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Original Article

Cloning and Expression of an Indigenous Mesophile Lipase and Evaluation of *Bacillus* Codon Translation in *Pichia pastoris* under Control of Two Different Promoters

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Abstract

Lipases are versatile biocatalysts with a wide range of application in food, dairy, leather, paper, pharmaceutical, and detergent industries. In this study, mesophilic lipase gene from an indigenous *Bacillus pumilus* F3, which its gene had been sequenced and identified previously, was cloned and expressed in methylotrophic yeast *Pichia pastoris* and codon translation of lipase gene was evaluated in *P. pastoris* under the control of two different promoters (alcohol oxidase (AOX1) and glyceraldehid phosphate dehydrogenase(GAPDH) promoters). In addition, the expression conditions of recombinant lipase F3 was optimized in *P. pastoris* expression system using BMMY medium at pH 3, 26ºC, and in 0.75% methanol. The 648 bp lipase gene with natural signal peptid sequence from *B. pumilus* F3 and the codon optimized lipase gene were cloned and expressed in methylotrophic yeast *P. pastoris*. The lipase gene was excised from the recombinant plasmid with *BamHI, EcoRI* enzymes and ligated into linearized pPIC9 and pGAP9 with the same enzymes. The recombinant plasmids were confirmed by the PCR and restriction enzyme digestion. The *Bgl II* linearized Ppic9 and pGAP9 recombinant plasmids were introduced into the yeast *P. pastoris* GS115 genome by electroporation and confirmed by PCR. Lipase expressing yeast was cultivated in a 250-ml shaking flask containing expression medium. Expression of lipase gene was confirmed using p-nitrophenyl palmitate test and SDS-PAGE. Codon optimized lipase was being expressed as well as native gene and the expression level was low in both cases. Also, these results suggest that protein structure is more important than codon preference in the expression of proteins such as lipases.

Keywords: Lipase, *Pichia pastoris*, *Bacillus pumilus F3*, Codon Translation, Expression, Optimization

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Introduction

Lipases (Triacylglycerol acylhydrolases; EC 3.1.1.3) catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids at the interface between water and oil and have been utilized widely in food, dairy, leather, paper, pharmaceutical and detergent industries [1-4]. Bacterial lipases are categorized into eight families and lipases from *Bacilli* belong to I.4 and I.5 subfamilies [5]. Lipases from *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus licheniformis*, belonging to subfamily I.4, are smaller than other subfamilies and lack the lid structure [5-6].

Recently, many lipases have been over expressed as an extracellular protein in *Pichia pastoris* [7-10]. The *P. pastoris* expression system has several advantages, such as easy purification, easy library screening, ability to perform eukaryotic protein modifications, and high levels of protein expression at the intra or extracellular level. Furthermore, in *P. pastoris* an alcohol oxidase 1 (AOX1) gene promoter is tightly regulated by methanol [11-13] and glyceraldehyde 3-phosphate dehydrogenase (GAP) gene promoter provides strong constitutive expression on glucose at a level comparable to that seen with the AOX1 promoter [14]. Thus in this study, mesophilic lipase gene from an indigenous *Bacillus pumilus F3* was cloned and expressed in methylotrophic yeast *P. pastoris* under control of two different promoters (AOX1 and GAPDH promoters). Expression of lipase in this system was approximately low. Low expression of the recombinant lipase was attributed to codon usage differences in the host yeast. Thus, lipase gene nucleotide sequence was compared in terms of codon usage with yeast *P. pasturis* host and codon optimized lipase gene cloned and expressed in methylotrophic *P. pastoris*. Since this bacterial lipase enzyme belongs to subfamily I.4 with lack of the lid structure [15], we suggest that lacking the lid structure of this lipase leads to cells death by hydrolysis of cell fatty acids.

Materials and Methods

Strains, Plasmids, and Media

The mesophilic lipase gene from an indigenous *B. pumilus F3* was isolated from the plasmid pym122 that was described by Heravi *et al*., [15]. The plasmid pPIC9 (Fig. 1a) and yeast strain *P. pastoris* GS115 were provided from Invitrogen and the plasmid pGAP9 (Fig. 1b) was constructed in this study. *E. coli DH5a F/ gyrA96 (Nalr) re-*

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cA1 relA1 endA1 thi-1 hsdR17(rk-mk+)glnV44 deoR Δ(lacZYA-argF)U169 [φ80dΔ(lacZ)M15]) was used for subcloning. The para nitrophenyl palmitate was supplied by Sigma (Deisenhofen, Germany). Yeast nitrogen base was purchased from invitrogen (Ausburg, Germany). Lauria Bertani (LB) medium was used for growing *E. coli* strains at 37℃, LB agar containing 100 mg/ml Ampicilin, 0.5 mM IPTG and 20 µg/µl X-Gal used to screen the recombinant bacterial colons. Yeast extract peptone dextrose (YPD), MM, MD, buffered glycerol complex media (BMGY), buffered methanol-complex medium (BMMY) media were prepared for growing and screen of recombinant yeasts according to invitrogen protocol.

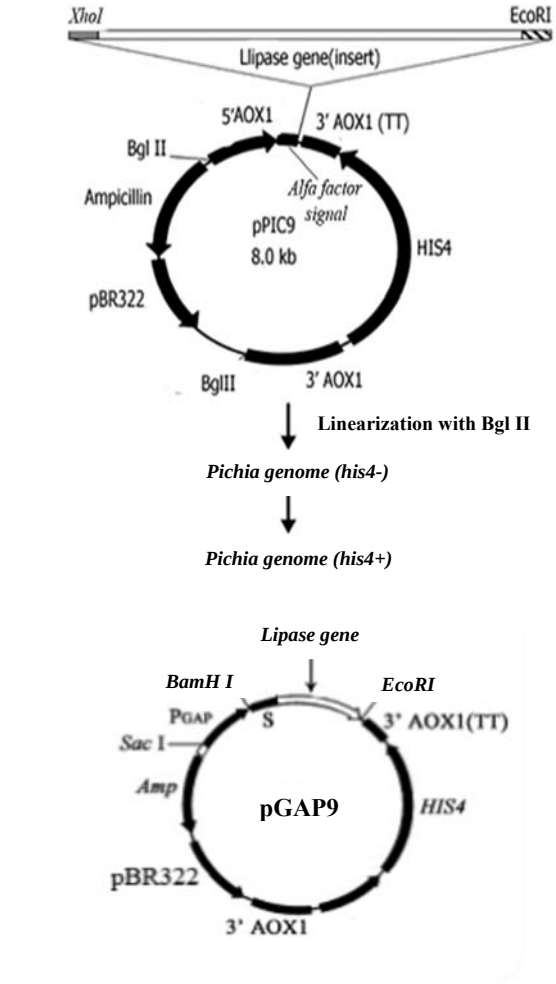

Figure 1. Expression vectors pPIC9 (a) and GAP9 (b) in *P. pastoris*.

Plasmid construction, subcloning, and transformation

The lipase 648 bp gene with natural signal peptid sequence from *B. pumilus F3* was amplified by PCR with two primers as forward (F) and reverse (R):

5′AGGGATCCCAACGATGAAGGTTATCAGATTCAA GAAAAGG3′ (F)

5′GAATTCTTATTAATTAGTATTTTGACCAA3′ (R)

The amplicon was inserted into pTZ57R/T vector. The lipase gene was excised from the recombinant plasmid with *BamHI*, *EcoRI* enzymes and ligated to the pPIC9, pGAP9 linearized with the same enzymes. The recombinant plasmids were confirmed by PCR, DNA sequencing and restriction enzyme digestion. The *BglII* linearized recombinant plasmids were introduced into the yeast *P. pastoris* GS115 by electroporation according to manufacturer's instructions. The insertion of lipase gene to genome of yeast was confirmed by PCR.

Expression of lipase F3 Gene

The yeast recombinant strains were cultivated in 250 ml BMGY medium [1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 5% biotin, and 1% glycerol] for 72 at 30℃ and agitated at 200 rpm. After 36 h of cultivation, the culture was centrifuged and the cell pellet was transferred into the BMMY medium, which contains the same compounds as the BMGY medium except for 0.5-1 % methanol instead of 1% glycerol. The culture was cultivated at 30℃, 200 rpm. As much as 0.5%-1 methanol was added to the culture every 24 h and 1mL of the culture was taken to determine the cell growth and the lipase activity. RT-PCR technique was used to detect mRNA expression levels. Total cellular RNA was isolated from the cells using the RNA isolation kit.

Lipase assay

Lipolytic activity was determined spectrophotometrically using 1.5 mM p-nitrophenyl palmitate (pNPP) as the substrate. One unit of lipase was defined as the amount of enzyme which cleaved 1 µmol of p-nitrophenol per minute [16].

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a 13% polyacrylamide gel on a vertical mini gel apparatus (paya pazhuhesh) at 150 V for 1 h. Molecular weight marker was provided from Fermentase. Gels were stained for protein detection by Coomassie Brilliant Blue R-250.

Optimization of expression conditions

Positive recombinant clones of lipase were selected for optimum expression. In order to enhancing the expression level of recombinant proteins in *P. pastoris*, the growth conditions such as type of medium, methanol concentration (0.25, 0.5, 1, 1.5, and 2%), pH (3, 4, 5, 6 and 7), temperature (25ºC-30ºC) and induction time were optimized. A general protocol was used for all experiments except of the parameter that was tested. A single recombinant *P. pastoris* colony was cultivated in 250 ml of buffered complex medium containing glycerol. The culture was grown at selected temperature for 48 hours. Cells were centrifuged and resuspended in 200 ml of buffered complex medium containing methanol, then the type of condition was tested. Para-nitrophenyl palmitate (pNPP) test was used to determine enzyme unit and lipolytic activity.

Evaluation of codon usage lipase gene in p. pastoris and optimization of Bacillus codons

The codon translation of the lipase was evaluated in *P. pastoris* using online softwares (Encore Biotechnology Inc and GenScript's OptimumGene^{TM} codon optimization

tool) based on yeast host codon usage. According to GenScript's OptimumGene™ codon optimization tool, possibility of high protein expression level is correlated to the value of Codon Adaptation Index (CAI). CAI of 1.0 is considered to be ideal, while a CAI of >0.8 is rated as good for expression in the desired expression system. This index for native lipase gene was 0.65 (Fig. 2), and for optimized lipase gene was 0.97 (Figure 2). Also Codon Frequency Distribution (CFD) was calculated, the value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. Codons with values lower than 30 are likely to hamper the expression efficiency. In this study, native lipase gene has 26 codons with values lower than 50 (Figure 3) and optimized lipase gene has any codons with values lower than 80 (Figure 4). Thus the codon optimized lipase gene was synthesized, then syntethic codon optimized lipase gene was cloned and expressed in *P. pastoris* under control of two different promoters (alcohol oxidase and glyceraldehid phosphate dehydrogenase promoters).

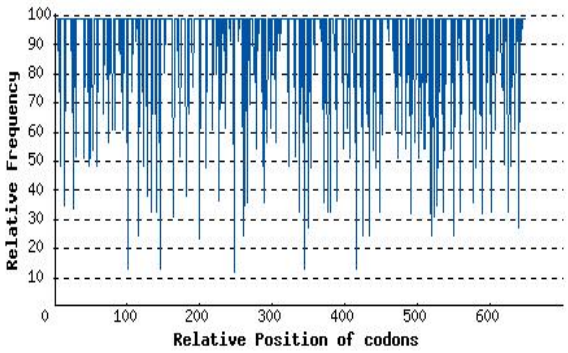

Figure 1. The distribution of codon usage frequency along the length of native lipase F3 gene to be expressed in *P. pastoris* host. Codon Adaptation Index (CAI) = 0.65.

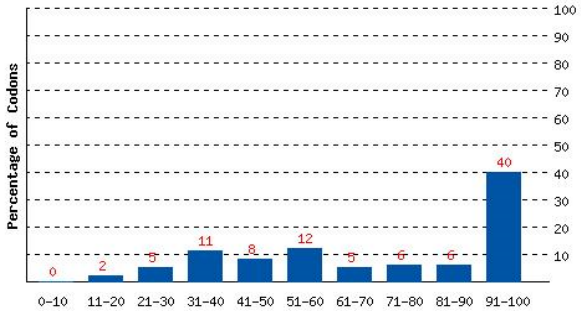

Figure 2. Codon Frequency Distribution (CFD), the percentage distribution of codons in computed codon quality groups for native lipase F3 gene.

Study of the secondary structures of lipase mRNA

Lipase mRNA sequence was applied to study its secondary structure and was predicted using web softwares (GeneBee).

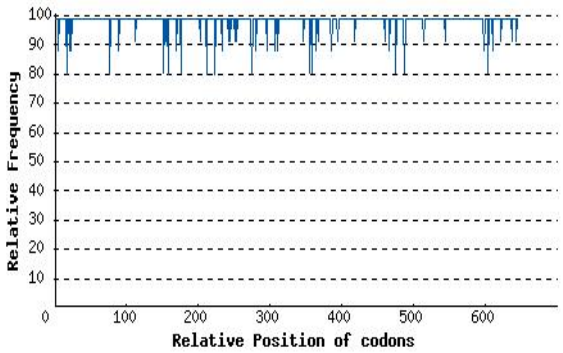

Figure 3. The distribution of codon usage frequency along the length of codon optimized lipase F3 gene to be expressed in *P. pastoris* host. Codon Adaptation Index (CAI) = 0.97.

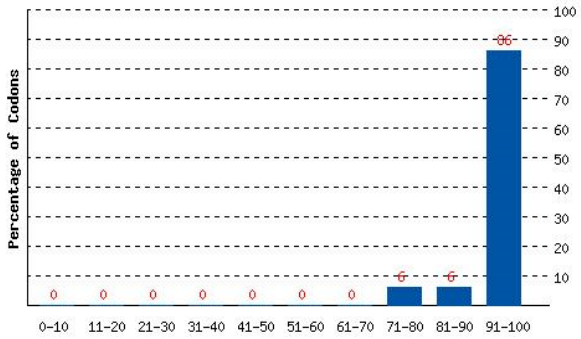

Figure 4. Codon Frequency Distribution (CFD), the percentage distribution of codons in computed codon quality groups for codon optimized lipase F3 gene.

Results

Plasmids construction

The lipase gene was amplified from the plasmid (pYM122) [15] by PCR using two primers complementary to the 5′ end and 3′ end of the lipase gene and containing *BamHI*, *EcoRI* restriction sites. The fragment was cut out of the gel, purified and cloned into pTZ57R/T vector followed by transformation of *E. coli DH5α*. After subcloning in *E. coli DH5a* and confirming by the PCR and restriction enzyme digestion, the lipase gene was excised from the recombinant plasmid with *BamHI* and *EcoRI* enzymes and ligated into the pPIC9 and pGAP9 with the same enzymes, resulting in the new recombinant plasmids (Fig. 1). After verification of the presence of lipase gene by the PCR and restriction enzyme digestion, the recombinant plasmids were sequenced using universal forward primer AOX1. The sequencing results revealed a 648 bp ORF encoding the lipase precursor of *Bacillus pumilus F3* which was cloned in expression vectors correctly.

Electroporation

The Ppic9 and Pgap9 recombinant plasmids linearized by *BglII* were introduced into the yeast *P. pastoris* GS115 genome by electroporation. Electroporation product was cultivated on MD plat. After 3-4 days transformants harboring lipase gene as integrating in its genomic DNA was appeared on MD plat (Figure).

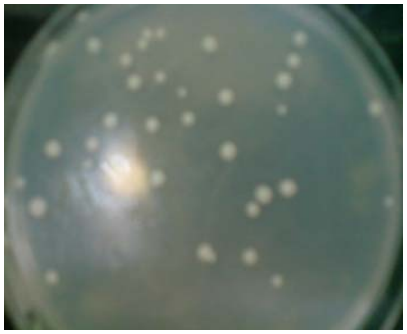

Figure 6. Recombinant yeast colonies grown on the MD plates after electroporation.

Lipase expression

P. pastoris GS115 transformants harboring lipase gene as integrating in its genomic DNA was cultivated in 1 litter shaking flask containing 200 ml BMMY medium. At different times, samples were taken from the culture and assayed for cell growth (OD_{600}) and for lipase activity by using p-nitrophenyl palmitateas substrate. The activity reached a maximum of 292 U/ml after 72 h of cultivation. Total protein extracted from recombinant *P. pastoris* cell showed the appearance of a novel 19 kDa protein at12 hours post induction, and its intensity peaked after 36 hours (Figure). The presence of the lipase F3 mRAN was confirmed by RT PCR technique.

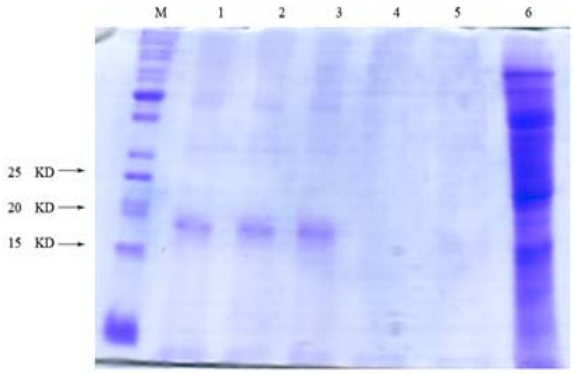

Figure 7. SDS–PAGE of the recombinant lipase F3 protein. Lane M: molecular mass standards indicated in kDa; Lane 1, 2, 3: sample of the supernatant after 48 h of cultivation; Lane 4, 5: negative control; Lane 6: intracellular protein sample.

The results of the optimization of expression conditions

The culture conditions were studied to improve the yield of lipase production. In this study, BMMY medium was found to be optimal for the expression of lipase (
Figure 5). To determine the optimal pH, expression was performed at pH values ranging from 3-7. The highest production was observed at pH 3 after 18 h of induction. A methanol feeding of 0.5 - 0.75 % was optimal for expression of lipase. When methanol feeding below 0.5% or above 2% was used, the expression was reduced (Figure). Optimal temperature for lipase production was 27ºC, because decrease in temperature, generally, reduces cell death, which prevents the release of proteases (
Figure 11).

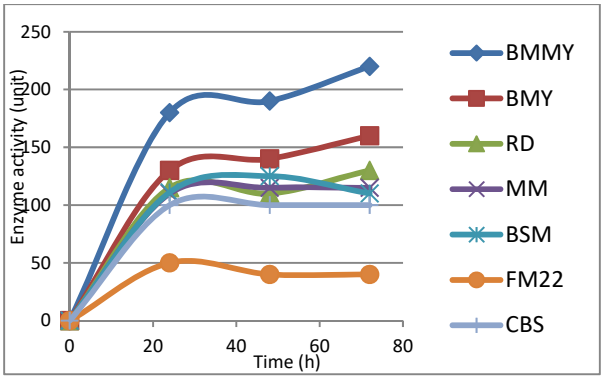

Figure 5. Effects of different media on the expression of recombinant lipase F3**.** The horizontal axis represents the time (hour) and the vertical axis represents the enzyme activity.

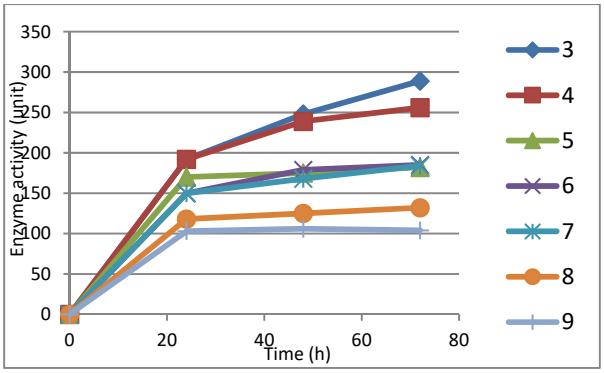

Figure 9. Effects of different pH on the expression of recombinant lipase F3. The horizontal axis represents the time (hour) and the vertical axis represents the enzyme activity**.**

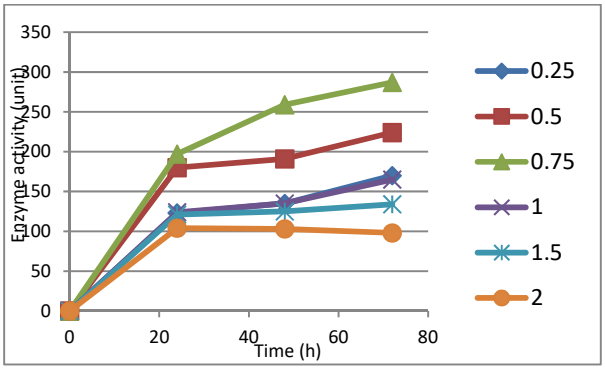

Figure 10. Effects of different concentrations of methanol (%) on the expression of recombinant lipase F3. The horizontal axis represents the time (hour) and the vertical axis represents the enzyme activity**.**

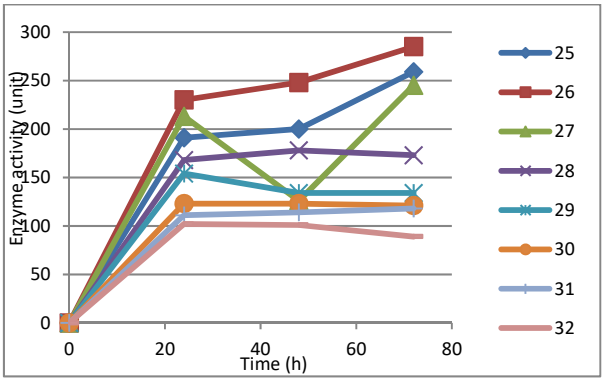

Figure 11. Effects of different temperatures on the expression of recombinant lipase F3. The horizontal axis represents the time (hour) and the vertical axis represents the enzyme activity

Optimization of lipase F3 codons and expression optimized gene in p. pastoris

Using web softwares, the codon translation of the lipase was evaluated in *P. pastoris*. Lipase codons were optimized according to the yeast host. The codon optimized lipase gene containing *BamHI* and *EcoRI* restriction sites was synthesized. This syntethic lipase gene was excised from the syntethic plasmid with *BamHI* and *EcoRI* enzymes and ligated into the linearized pPIC9 and pGAP9 with the same enzymes, resulting in the plasmids which cloned and expressed in *P. pastoris* under control of two different promoters (alcohol oxidase and glyceraldehid phosphate dehydrogenase promoters). Moreover, using web software, secondary structures of lipase mRNA with the sequence of its ribosome binding site was predicted. Results showed that the structures are appropriate for expression in *p. pastoris*. Lipase was produced as well as native gene and lipase expression was equal in both cases (Figure).

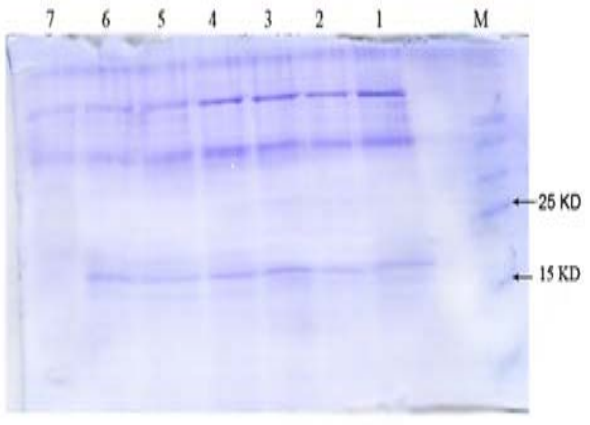

Figure 12. SDS–PAGE of the recombinant codon optimized lipase F3 protein. Lane M: molecular mass standards indicated in kDa; Lane 1 to 3: sample of the supernatant of codon optimized lipase under AOX1 promoter; Lane 3 to 6: sample of the supernatant of codon optimized lipase under GAP promoter; Lane 7: negative control.

Discussion

Already, this lipase gene has been cloned in *E. coli* under control of the strong T7 promoter, but the F3 lipase was not secreted by its native signal peptide from the cytoplasm of *E.coli* cells [15]. Thus, a lot of purification steps have to be done to obtain the purified enzyme. Using *P. pastoris* to produce the F3 lipase, the protein was secreted into the culture medium. The protein was relatively pure in the culture medium without need for any purification steps with a molecular weight of around 19 kDa on SDS gel, corresponding to the molecular weight of the mature lipase deduced from sequence calculation.

Among the media, highest yield for lipase production was obtained in BMMY medium. The components of BMMY had prominent effects on the growth and expression of recombinant proteins in *P. pastoris*. BMMY is a buffered medium that provides a stable pH condition, and its Yeast extract and peptone contain peptides, amino acids, vitamins, and trace elements, which can enhance the biomass and energy for protein synthesis during the expression. For the expression of protein using AOX1 promoter, high expression of foreign protein expression depends on rigorous control of methanol level. Excess of methanol can be toxic to the cells [17]. The highest lipase expression observed at pH 3, although *P. pastoris* can grow in a wide ranges of pH (3 to 7), pH values of 5-6 has been the most routinely used for expression in the growth culture. High lipase expression at pH 3 might be due to a decreased production or inactivation of proteases of the *P. pastoris* cells [18]. In this study, optimal temperature for growth and protein production was 26ºC, although, the recommended optimal growth and production temperature of *P. pastoris* is is 30ºC (according to the instruction manual of *P. pastoris* expression system). Increase in the protein expression at lower temperatures is due to the more stability of the recombinant protein and reduction of proteolytic degradation of recombinant proteins and, also, increase of cell viability [19] (Fig. 8 to 11).

Codon optimization is an efficient procedure to improve the expression level of genes in hetrologos expression systems. Due to the differences in codon usage of the host and their original strains, the expression levels of these lipases hardly reach to their optima. Codon usages of *B. pumilus* and *P. pastoris* are distinctly different. Thus, the lipase F3 gene sequence was synthesized based on the result of optimization procedure (Fig. 2 to 5). Results of our codon optimization reveal that codon optimized lipase was produced as well as native gene and lipase expression was equal in both of them. Therefore, a question that still exists is why the expression level remained unaffected? There are a few reports on the expression of recombinant Bacterial lipase enzyme without the lid structure, in a heterologos host. On the other hand, the lipases (subfamily I.4) containing artificial lid structures exhibited a lower activity than the wild type [20], in other words, insertion of the lid structures from structurally homologous enzymes

into a lipase that naturally does not possess such a lid structure, caused a reduction in the enzyme activity [21]. Thus, we suggest that the lipase without the lid structure, due to their high activity, kills cells with hydrolysis of host cell fatty acids. The invariant in expression of the codon optimized lipase F3 compared to native lipase F3, can be described by the fact that the protein structures are not adapted to the yeast host system and the protein structure is more important than codon preference in the expression of proteins such as lipases.

Conclusion

In summary, the methylotrophic yeast *P. pastoris* constitutes an efficient expression system for the production of recombinant proteins. Our next step will be the expression of the recombinant lipase F3 in *bacillus* strains and the evaluation of the effect of lipase lid structure in its expression and optimization of lipase expression in mesophilic indigenous *Bacillus pumilus* F3 by metabolic engineering.

Acknowledgments

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