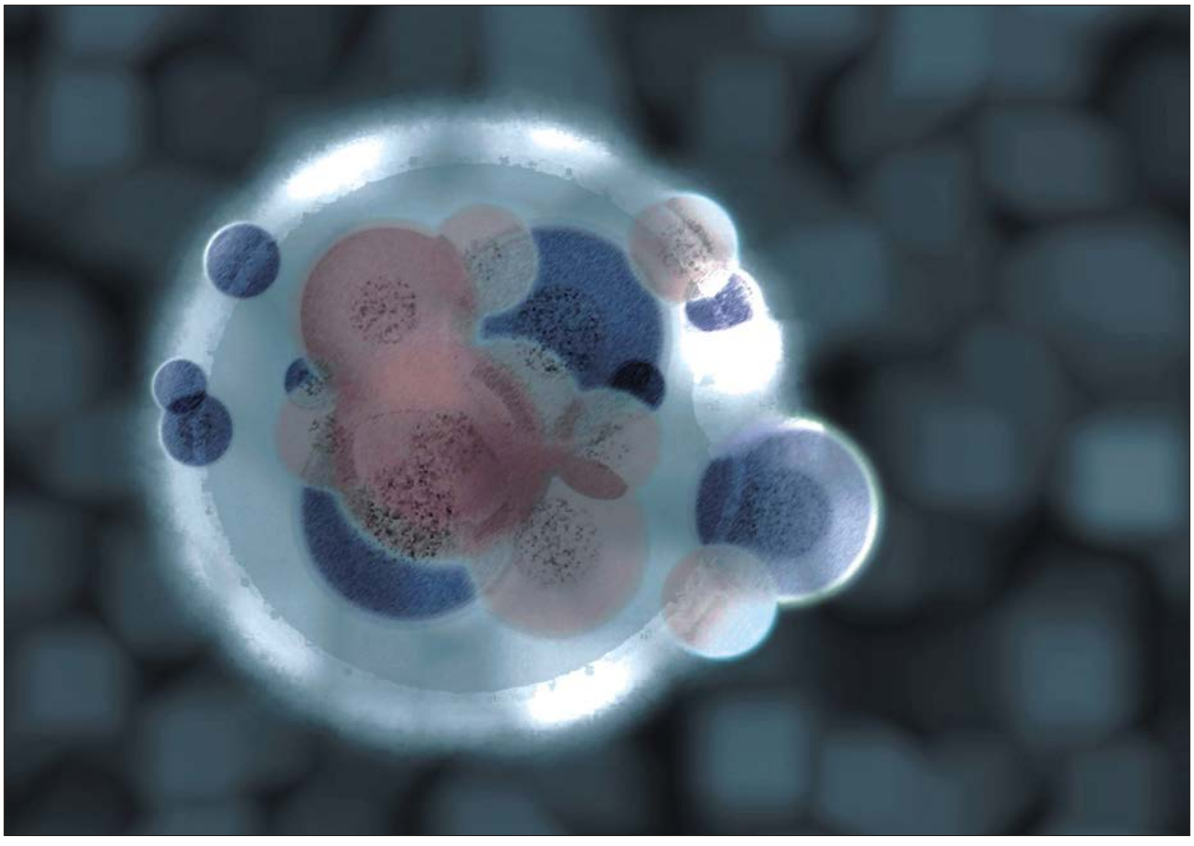

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Investigation of Isolated Lipase Producing Bacteria from Oil-contaminated Soil with Proteomic Analysis of its Proteins Responsive to Lipase Inducer

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Abstract: Forty bacterial strains isolated from oil-contaminated soil were screened for lipase production on selective medium using neutral red as an indicator. Nine lipase producing bacterial isolates indicated by the formation of red halo were found. The lipolytic activities produced by these bacteria were then compared. The highest enzyme activity of 163.51 U mL^{-1} was shown by the CFS14 isolate. Species identification of the CFS14 isolate was then performed by 16S rRNA gene sequencing. The DNA sequence showed the maximal similarity to *Pseudomonas xinjiangensis* with 99.6%. Lipase productions by *P. xinjiangensis* strain CFS14 were investigated in different cultured conditions. The bacteria produced the highest lipase activity when cultured in the medium of pH 8.0 supplemented with 0.5% $MgCl_2$ at 37°C for 36 h. Proteins related to the lipase induction by 0.5% $MgCl_2$ were examined by proteomic analysis. The protein patterns of *P. xinjiangensis* CFS14 cultured in the medium supplemented with 0.5% $MgCl_2$ were compared with those of controls. Fifteen spots (6 increasing, 1 decreasing, 8 supplementary) from treated bacteria were different in protein abundance from controls. Five chosen protein spots were identified by MALDI-TOF mass spectrometry combined with bioinformatic methods. Only 2 protein spots could be identified which were likely to be lipoprotein and hypothetical protein cdivTM_18888.

Key words: *Pseudomonas xinjiangensis*, proteomic, lipase, magnesium, oil-contaminated soil

INTRODUCTION

Lipases are important enzymes used in many industrial applications such as pharmaceuticals, food, detergents, paper and pulp, agrochemicals, biosurfactants and bioremediation (Pogori *et al*., 2007; Sharma *et al*., 2011). The main function of these enzymes is to hydrolyse triglycerides to glycerol and free fatty acids at an oil-water interface (Kulkarni and Gadre, 2002; Pahoja and Sethar, 2002). Lipases can be produced from various sources e.g., animals, plants and microorganisms. However, for industrial applications, lipases from microorganisms are more interesting because (1) they can be produced in the high yields (2) there are many varieties of catalytic activities which can be used in many applications and (3) the genetic manipulation is available (Hasan *et al*., 2006).

Lipase producing bacteria can be successfully isolated from oil-contaminated sites such as waste water (Mohan *et al*., 2008) and soil (Dahiya and Purkayastha, 2011; Heravi *et al*., 2008). To increase the production of lipase, many studies have worked on the optimization of culture condition. The lipase production of solvent tolerant *Pseudomonas fluorescens* P21 was highest in the cultured medium containing olive oil and peptone (Cadirci and Yasa, 2009). In *Bacillus pumilus*, yeast extract at 10% concentration contributed to the highest growth and lipase production (Heravi *et al*., 2008). *Bacillus stearothermophilus* AB-1 produced maximum yield of lipase at 35°C and pH 7.5 cultured conditions (El-moniem-Abada, 2008).

Several studies reported the elevation of bacterial lipase production by adding some metal ions such as Na^+, Ca^{2+}, Mg^{2+} in the cultured medium (Joseph *et al*., 2006; Kader *et al*., 2007; Sharma *et al*., 2009). Amanda *et al*. (2001) explained that Ca^{2+} may help the structure formation of active enzyme leading to higher enzyme activity. However, the mechanisms of metal ions as a lipase enhancer are still not clearly understood. To understand the effect of these metal ions towards the bacterial lipase production, investigation of proteins responding to metal ion enhancers by proteomic analysis is challenged. Proteomic technique is a powerful tool to analyze the

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global responses of proteins in cells or certain tissues. This technique comprises of two-dimensional gel electrophoresis (2DE) where proteins are firstly separated in two dimensions and mass spectra of interesting proteins are later identified.

In this study, the lipase producing bacteria were isolated from oil-contaminated soil. Isolate showing the highest lipase activity was identified and its culture condition for maximal lipase activity was investigated. Proteomic analysis of bacterial proteins responsive to lipase inducer was also examined.

MATERIALS AND METHODS

Screening for lipase producing bacteria: Ten grams of palm oil contaminated soil sample were cultured in 100 mL of NB (nutrient broth) containing 1% palm oil at 30°C for 24 h in rotary shaker. The isolation process was performed by serial dilution of samples on NA (nutrient agar). Grown colonies were picked and screened for lipase production using selective agar containing olive oil and neutral red. Colonies showing the red halo were picked out, purified on NA and transferred to agar slants.

Assay of lipase activity: The identified lipase producing bacteria were cultivated in NB for 24 h at 37°C with shaking as preculture. One millilitre of preculture was transferred to 99 mL NB supplemented with 1% olive oil and incubated at 37°C under the shaking condition for 48 h. The culture was then centrifuged at 10,000 rpm 4°C for 10 min. The supernatant was used as a crude enzyme for lipase activity measurement. The reaction mixture consisting of 880 μL reaction buffer (50 mM tris-HCl, 0.1% gum Arabic and 0.2% deoxycholic acid), 20 μL crude enzyme and 100 μL *p*-nitrophenyl laurate (*p*-NPL) substrate was incubated at 37°C for 2 min (Sangthong, 2001). The reaction was then immediately stopped by adding 500 μL 3 M HCl. After centrifugation at 12,000 rpm for 10 min, the released *p*-nitrophenol was measured at 405 nm. One unit of lipase activity was defined as the amount of enzyme that released 1 μmole of *p*-nitrophenol per min under assay condition. It should be noted that only extracellular lipase was measured in this report, even though both extra and intracellular lipases could be found (Jatta *et al*., 2009).

Identification of bacteria: PCR amplification of 16S RNA gene was performed. A genomic DNA was isolated from the highest lipase producer designated as the CFS14 strain and used as a template for PCR reaction in a reaction mixture containing 15-20 ng of template DNA, 2.0 μmole of primer 20F (5'-GTTACCTTGTTACGACTT-3'), 2.0 μmole of primer 1500R (5'-GTTACCTTGTTACGACTT-3'), 2.5 units of Taq polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 10 μL of 10×Taq buffer and H_2O up to 100 μL. The mixture was subjected to an initial 94°C denaturation step for 3 min, followed by an amplification program consisting of 25 cycling periods of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. The purified PCR products were sequenced using an ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.0, Applied Biosystems, Foster City, California, USA). The DNA sequencing was performed on an ABI Prism® 3730xl DNA Sequence (Applied Biosystem, Foster City, Califonia, USA). The nucleotide sequences were assembled using cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Homology search was performed using the standard nucleotide BLAST (BLASTn) from the NCBI web server http://blast.ncbi.nlm.nih.gov/Blast.cgi against previously reported sequences at the GenBank/EMBL/DDB database for determination of the nearest sequences.

Optimization of lipase production: The effects of various conditions including incubation temperature (30-50°C); pH of the medium (5-9) and supplementation of medium with various cations ($CaCl_2$, $MgCl_2$, $MnCl_2$, $BaCl_2$) at 0.1% w/v and different concentrations of $MgCl_2$ (0.2-0.7%) on the production of lipase by the CFS14 strain were investigated. All experiments were carried out in 3 replicates. In addition, for the study of inducer supplementation, the measurement of bacterial growth at 620 nm was determined to assure similar growth in media containing different cations.

Two-dimensional gel electrophoresis (2DE) of cellular proteins: The bacterial strain CFS14 was separately cultured in two medium conditions, with or without 0.5% $MgCl_2$ for 36 h at 37°C. Cells were harvested by centrifugation at 12,000 rpm for 10 min at 4°C and then resuspended with 200 μL lysis buffer (8 M Urea, 4% w/v CHAPS, 2% v/v IPG buffer 3-10 NL, 40 mM DTT) containing a protease inhibitor. The cell suspensions were placed on ice for 30 min and subsequently subjected to four freeze-thaw cycles. After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatants were cleaned using the 2D Cleanup kit (GE Healthcare Bio-Sciences). The protein concentrations of the supernatants were then determined using the 2D protein quantification kit (GE Healthcare BioSciences). Protein samples were stored as aliquots at -80°C. Two-dimensional gel electrophoresis was performed as described previously (Lomthaisong *et al*., 2008). Briefly, 15 μg of each protein extract was loaded by

in-gel rehydration 7 cm immobilized pH gradient (IPG) strips with pH range 3-10. Rehydration buffer consisted of 8 M urea, 2% w/v CHAPS, 0.002% w/v Bromophenol blue and 20 mM DTT. Isoelectric focusing was conducted using an Ettan IPGphor II (GE Healthcare BioSciences) at 20°C. Proteins were focused for a total of 8 kVh. Second-dimension electrophoresis was carried out on 12.5% polyacrylamide gel using a MiniVE vertical electrophoresis system (GE Healthcare BioSciences) with 10 mA per gel for 15 min then 20 mA per gel for 1.5 h. Proteins were detected by Coomassie staining. The experiment was repeated three times providing three replicated protein gels for each condition.

Proteomic analysis: The gels were digitalized using a UMAX UTA-1120 image scanner (GE Healthcare BioSciences). Six 2DE gels from three replicates of each condition were analyzed with IMAGEMASTER 2D PLATINUM version 5.0 (GE Healthcare BioSciences). The signal intensity of each spot was averaged over the replicates. The signal intensities of protein spots were then normalized. Protein spots presented in all replicates were added to the averaged gel. Subsequently, the averaged proteome profiles obtained from each condition were quantitatively compared. The statistical analysis of relative volume of each matched spot was accomplished using a two-sample t test offered by ImageMaster software. The p value correlated with t test value was available from the Critical t values calculation website (http://www.psychstat.missouristate.edu/introbook/tdist.htm). The protein spots with $p<0.05$ were considered as differentially expressed protein spots. For protein identification, protein spots found only in bacterial proteome cultivated with 0.5% $MgCl_2$ or those with changes in protein abundance more or less than one fold were chosen. These protein spots were excised from the gel, digested with trypsin before sending to Department of Biotechnology, Faculty of Science, Mahidol University, Thailand, for MALDI-ToF mass spectrometry analysis. The PMF (peptide mass fingerprint) obtained from each protein spot was searched for its peptide sequence by Mascot program. The Mascot program was available at http://www.matrixscience.com/.NCBInr20100624database was used for peptide sequencing. The taxonomy for data search was "other bacteria". The mass values and mass tolerance were monoisotopic and ±500 ppm. Two of missed cleavages were allowed. The variable modifications of carbamidomethyl and oxidation were chosen for consideration. Mowse score cut-off greater than 73 was qualified as a statistical significance ($p<0.05$) for a protein identification.

RESULTS

Forty bacterial strains isolated from oil-contaminated soil were screened for lipase production on selective medium agar with neutral red as an indicator. Nine strains (TH12, TH15, CFS14, Thb3, Thb5, Thb10, Thb11, MT1B and MT2) showed the red halo around their colonies indicating as the lipase producers. The cell-free supernatant of these strains were then measured for lipase activity using *p*-NPL as substrate. The CFS14 strain had the highest lipase activity (Fig. 1). This bacterial strain was then identified by comparing its 16S RNA sequence with sequences in the database. The nucleotide sequence of 609 bp long showed 99.6% identity to the partial sequence of *Pseudomonas xinjiangensis* strain S3-3 16S ribosomal RNA gene (Accession No. EU286805.1) with 100% coverage (data not shown). Therefore, it could be concluded that the CFS14 strain was *Pseudomonas xinjiangensis*.

The optimal conditions for the lipase production in *P. xinjiangensis* CFS14 culture were also studied. The lipase activity was of the highest (69.37 U mL^{-1}) when the bacterium was cultured at 40°C, when compared with 30 and 50°C conditions (Fig. 2). However, such lipase activity was lower than the results measured earlier at 37°C (163.51 U mL^{-1}, Fig. 1). Therefore, 37°C was chosen for further study. The *P. xinjiangensis* CFS14 cultivated in the pH 8 medium showed the highest lipase activity indicating that pH 8 was the optimal pH cultured condition (Fig. 3). To induce the production of lipase, 0.1% (w/v) of different divalent cations ($CaCl_2$, $MgCl_2$, $MnCl_2$, $BaCl_2$) were separately added into the medium. The results elucidated that magnesium chloride was the best inducer (data not shown). The appropriate concentration of $MgCl_2$ for the lipase induction was subsequently determined. It was revealed that the highest lipase activity

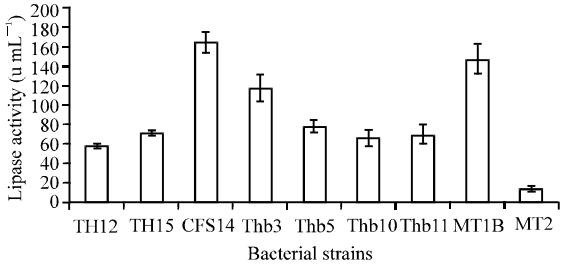

Fig. 1: Lipase activity from different strains of lipase producers. The lipolytic assay was carried out at 37°C for 2 min using p-NPL as a substrate. The experiments were performed in triplicates and bars represent the standard deviation

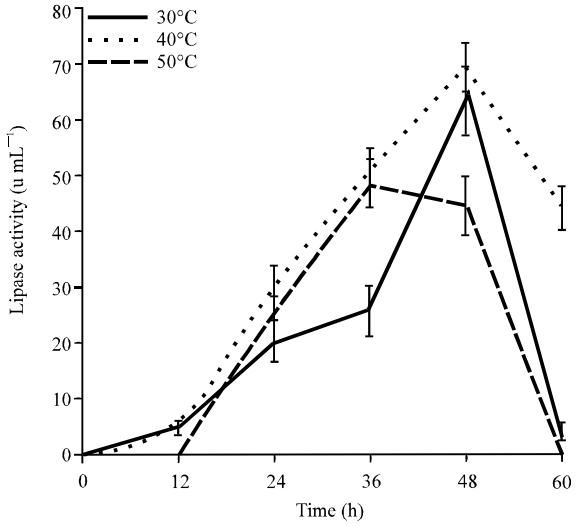

Fig. 2: Lipase activity in the medium of CFS14 strain cultured at different temperatures. The experiments were in triplicates and bars represent the standard deviation

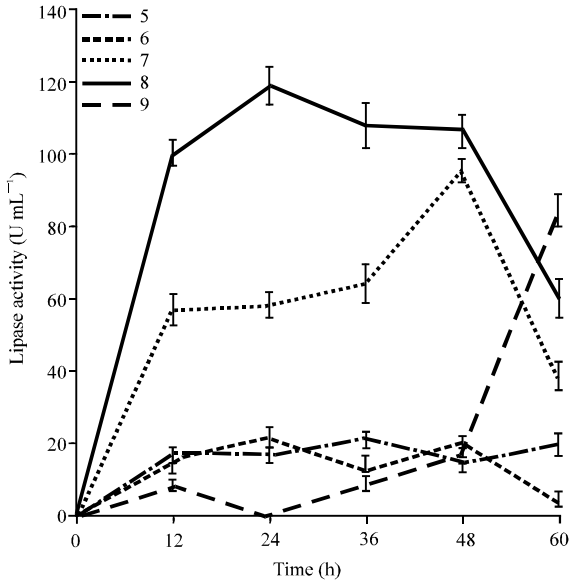

Fig. 3: Lipase activity in the medium of CFS14 strain cultured at different pH conditions. The experiments were in triplicates and bars represent the standard deviation

of 270.27 U mL^{-1} was observed at 36 h when incubated with 0.5% (w/v) $MgCl_2$ (Fig. 4). Therefore, 0.5% was shown to be the optimum concentration of $MgCl_2$ for lipase induction from *P. xinjiangensis* CFS 14. The effects of inducer on bacterial growth were also determined. No significant change in bacterial growth was observed

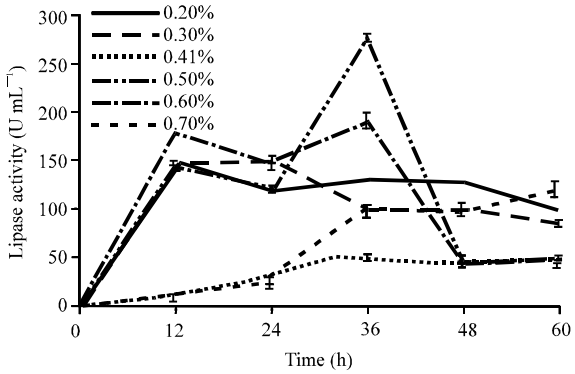

Fig. 4: Lipase activity in the CFS14 cultured with various concentrations of $MgCl_2$ supplementation. The experiments were in triplicates and bars represent the standard deviation

Table 1: Protein spots observed with the differential expression changes in *P. xinjiangensis* strain CFS14 which had been induced by 0.5% $MgCl_2$

Spot No.	pI	MW (kDa)	Quantity ratio[a] $MgCl_2$ treated/control
189	5.75	87.9	-[c]
203	5.65	80.6	1.21
219	5.18	68.4	1.84
228[b]	5.60	72.6	-
257[b]	5.55	66.2	1.18
262	5.72	64.8	-
391	5.02	47.1	1.11
502[b]	6.08	34.8	0.90
541	8.92	31.2	-
573[b]	8.84	29.6	-
603	5.91	26.4	-
617	8.88	25.6	-
635	8.96	23.5	-
636[b]	5.98	22.8	1.18
673	5.12	19.3	1.12

[a]Quantity ratio of each spot was derived from the normalization of average relative volume from their independent replicates. [b]Protein spots that were further analysed by MALDI-TOF MS. [c]Not found in control, *P. xinjiangensis* strain CFS14 cultured in medium with supplementation of $MgCl_2$

(data not shown). This result confirmed that the differences in lipase production resulted from inducer itself not the total number of bacteria in the medium.

Proteins involving in the $MgCl_2$ induction in *P. xinjiangensis* CFS14 were investigated. The 2DE profiles of the bacterium cultured in the medium supplemented with 0.5% $MgCl_2$ were compared with those of no addition. About 379 protein spots with pI 4.5-9.0 and MW 14-97 kDa were detected in both conditions. However, 15 protein spots were quantitatively different in protein abundances with 6 increasing, 1 decreasing and 8 newly induced (Fig. 5, Table 1). Five protein spots were chosen for protein identification by MALDI-ToF MS analysis including 2 increasing (spot no. 257 and 636), 1

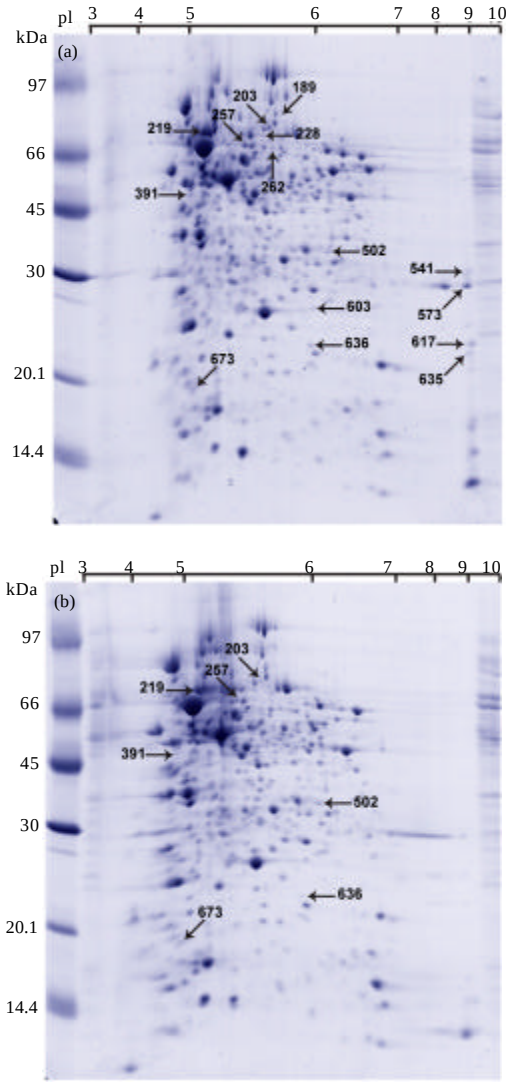

Fig. 5: 2DE gels of proteins extracted from *P. xinjiangensis* strain CFS 14 cultured in lipase production medium (A) with 0.5% $MgCl_2$ and (B) without $MgCl_2$. Protein spots that showed the expression-level changes are indicated by arrows and their pI and MW are listed in Table 1

decreasing (No. 502) and 2 newly induced (No. 228 and 573) protein spots. From 5 protein spots that were chosen for MS analysis, only 2 spots (spot no. 573 and 636) showed good PMF results. These PMFs were searched against the NCBI database with Mascot program. The results showed that protein spots no 573 and 636 were identified as putative lipoprotein and hypothetical protein, respectively (Table 2).

DISCUSSION

In this study, lipase producing bacteria isolated from oil contaminated soil were screened on selective agar containing olive oil and neutral red. Nine out of forty isolates were able to produce lypolytic enzymes. The activities of lipases produced were then quantified. The highest lipase producing isolate was identified to be *Pseudomonas xinjiangensis* strain CFS14. This bacterial strain was first described by Liu *et al.* (2009) in the study of their isolation of novel bacterial strain from Xinjiang, in north-west China. They found that the optimal cultured condition of this bacterium was at 37°C and pH 8. At this condition, the bacterium had also showed the positive lipase reaction. In agreement with their results, we found that the optimal condition for lipase production by *P. xinjiangensis* strain CFS14 culture was also at 37°C and pH 8.0.

The enhancement of lipase production by *P. xinjiangensis* strain CFS14 was then studied. The metal ion was chosen for this study as it had been reported as an enhancer for bacterial lipase production (Sharma *et al.*, 2009). Kulkarni and Gadre (2002) reported that the bacterial lipase production could be enhanced by Ca^{2+}. The mechanism of lipase stimulation by Ca^{2+} was suggested to attribute to the conformation of insoluble Ca-salt of fatty acids released in the hydrolysis preventing the product inhibition (Islam *et al.*, 2008). However, not every lipase producing bacteria could be effectively enhanced by Ca^{2+}. Sharon *et al.* (1998) studied the effects of various metal ions on the lipase activities produced by *P. aeruginosa* KKA-5. They found that Mg^{2+} stimulated the activity of lipase more efficiently than Ca^{2+} ion which was similar to our results.

Table 2: PMF analysis and identification of differentially expressed proteins in *P. xinjiangensis* strain CFS14 induced by 0.5% $MgCl_2$

Spot no.	Accession No.[a]	Protein identity	Species	%Sequence coverage[b]	Query matched[c]	pI/MW (kDa)[d] Theoretical	pI/MW (kDa)[d] Experimental
573	gi|124002018	Lipoprotein, putative	*Microscilla marina* ATCC 23134	29	5	10.04/23946	8.84/29647
636	gi|169839146 cdivTM_18888	Hypothetical protein	candidate division TM7 single-cell isolate TM7a	60	5	4.90/4729	5.98/22757

[a]Accession number of proteins derived from NCBI database using MASCOT search. [b]Percentage of sequence coverage calculated from sequence of matched amino acid/total amino acid x 100. [c]Number of searched peptides matched with peptide in database. [d]pI and MW (kDa) values calculated from amino acid sequence of protein in the database (theoretical) and the position of protein spots in 2DE gel using IMAGEMASTER 2D PLATINUM version 5.0 (experimental)

To understand the mechanism of Mg^{2+} ion as a lipase enhancer of *P. xinjiangensis* strain CFS14, proteins related to the $MgCl_2$ induction were examined by proteomic analysis. The protein patterns on 2DE gels of bacteria cultured in two different conditions (with or without 0.5% $MgCl_2$) were compared. Interestingly, most of newly detected protein spots and spots with increasing in protein abundance were found in bacteria cultured in the presence of $MgCl_2$. Similar evidence was observed in the study of rhamnolipid producing *P. aeruginosa* (Reis *et al*., 2010). The numbers of proteins were produced in the bacteria after fermentation in the condition that allowed rhamnolipid production. Therefore, this may explain that the addition of an appropriate enhancer could induce some protein expression and lead to more protein production including lipase.

Proteins related to $MgCl_2$ induction were then identified. The newly detected protein spot no.573 was similar to lipoprotein from *Microscilla marina* (ATCC 23134). Bacterial lipoproteins attach to outer membrane of gram-negative bacteria which are important for architecture of cell surface, the transport solutes and the maintenance of cell shape (Bos and Tommassen, 2004). Most of gram negative bacteria secrete extracellular enzymes via type II secretion pathway. The bacterial enzymes will secrete through the inner membrane via the Sec machinery. They were folded in the periplasm into enzymatically active conformation and translocated across the outer membrane as fully folded protein (Braun *et al*., 1999; Jaeger and Eggert, 2002). The relationship between lipoprotein and gram negative bacteria secretion has not been clearly explained. However, lipoprotein might involve in the protein secretion system of *P. xinjiangensis* CFS14.

Protein spot no. 636 showing the increasing protein abundance was similar to hypothetical protein cdivTM_18888 from candidate division TM7 single-cell isolate TM7a. Hypothetical protein is a protein with unknown function (Sivashankari and Shanmughavel, 2006). The candidate TM7 is one of newly described bacterial divisions characterized by environmental sequence data. TM7 was proposed based on partial 16S rRNA sequence which was not closely related to genes from any known Phyla. However, there were 135 numbers of TM7a genes with at least 30% sequence identity and 37 numbers of TM7a genes with at least 60% sequence identity to a member of class Gammaproteobacteria which was the class of *P. xinjiangensis* CFS14 (Marcy *et al*., 2007).

Although proteins related to lipase enhancer were not clearly shown in this experiment, this was the first study reporting proteins induced by $MgCl_2$ in *P. xinjiangensis* CFS14. It should be noted that protein extracts used in this proteomic study did not include secreted proteins. The presence of Mg^{2+} ion may involve in the folding steps to enhance lipase activity after its secretion. Therefore, the examination of extracellular proteins related to lipase enhancer may be useful.

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