Philippine Journal of Science
150 (2): 445-452, April 2021
ISSN 0031 - 7683
Date Received: 05 Aug 2020

Recombinant Expression of Alkane-1-Monooxygenase (AlkM) and Catechol 1,2-Dioxygenase (CatA) from Environmental Strains of *Acinetobacter baumannii*

Adrian Paul S. Tan, Vermando M. Aquino, and Cynthia T. Hedreyda*

National Institute of Molecular Biology and Biotechnology, College of Science
University of the Philippines, Diliman, Quezon City 1101 Philippines

Genes for the hydrocarbon-degrading enzymes – alkane 1-monooxygenase (AlkM) and catechol 1,2-dioxygenase (CatA) – were amplified from three environmental strains of *Acinetobacter baumannii* from Pasig River and oil sludge sample. Each gene has been previously cloned into a pBAD/Thio-TOPO plasmid expression vector and the recombinant pBAD/Thio-TOPO plasmids were introduced into *E. coli* TOP10 cells *via* transformation. This study was conducted to determine if AlkM and CatA proteins will be expressed by the recombinant *E. coli* TOP10 cells, to determine the optimized conditions for protein expression, and to perform purification of expressed AlkM and CatA fusion proteins. The pilot expression resulted in the production of the putative 62 kDa AlkM-thioredoxin and the 50 kDa CatA-thioredoxin fusion proteins, and the identity of the expressed proteins was verified to be AlkM and CatA using western blotting. The study showed that best AlkM and CatA expression was observed when recombinant cells were grown in Super broth using 6-h post-induction time with at least 0.2% arabinose. Purified AlkM and CatA fusion proteins were obtained using affinity chromatography and subsequent size exclusion chromatography with Sephadex G-75 column. The preliminary activity of the purified CatA fusion enzyme observed at 1.128 units/mg was much lower than activities reported for other species, while an AlkM activity assay could not be performed because AlkM fusion proteins from recombinant *E. coli* did not solubilize after purification. These suggest the need to perform an extensive study to address AlkM solubility problem and optimize parameters for enzyme activity of expressed CatA and AlkM. Successful expression of AlkM and Cat A genes from a human bacterial pathogen in non-pathogenic *E. coli* host cells is an initial step to the possible use of recombinant cells in producing AlkM and CatA enzymes from a pathogenic bacterial species in addressing problems of oil contamination.

Keywords: *Acinetobacter baumannii*, alkane 1-monooxygenase, catechol 1,2-dioxygenase, protein expression

INTRODUCTION

Hydrocarbons in oil are persistent environmental pollutants due to their hydrophobicity and stability. (Leahy and Colwell 1990). Species under the genus *Acinetobacter* produce two enzymes that are involved in the metabolism of hydrocarbons, AlkM and CatA. Alkane monooxygenase is responsible for oxidizing the terminal carbon in alkanes, converting them into alcohols (Rojo 2009). These alcohols can then be further oxidized into fatty acids, which are metabolized through β-oxidation. CatA is responsible for the opening of the aromatic ring in catechol and its derivatives, converting them into *cis,cis*-muconic acid,

*Corresponding Author: chedreyda@mbb.upd.edu.ph

which is further metabolized into succinyl-CoA and acetyl-CoA – both components of the citric acid cycle (Prince and Walters 2016). Compared to AlkM, the CatA of *Acinetobacter* is well-studied and the 3D structure for the *Acinetobacter calcoaceticus* CatA has been reported.

Previous studies detected genes encoding enzymes AlkM and CatA from an oil sludge strain (OS1) and two Pasig river strains (S341 and S354) of *Acinetobacter baumannii* that exhibited oil degradation in minimal broth and minimal agar plate with bunker oil as sole carbon source (Hedreyda and Sarmago 2014; Sarmago and Hedreyda 2014; Tan and Hedreyda 2020). Because this bacterial species is a harmful human pathogen, the three environmental strains could not be directly used for pollutant oil degradation studies. The *alkM* and *catA* genes from each of these environmental strains of *A. baumannii* were amplified and subjected to gene and corresponding amino acid sequence analyses. The complete *alkM* and *catA* genes from these strains were also cloned into pBAD/Thio-TOPO plasmids, and the recombinant pBAD/Thio-TOPO plasmids with either *alkM* or *catA* gene were used to transform *E. coli* TOP10 cells.

The main objective of this present study is to test the expression of the fusion proteins with either AlkM or CatA from environmental strains of the pathogenic *Acinetobacter baumannii* in non-pathogenic recombinant *E. coli* TOP10 cells. Experiments were conducted to determine the optimized expression parameters –including the type of culture media for growing the cells, arabinose concentration for induction, and an optimized length of time for arabinose induction. The study also included the conduct of procedures to achieve the purification of expressed AlkM and CatA fusion proteins for use in future enzyme activity assays. Results of this study to express AlkM and CatA from *A. baumannii* environmental strains OS1, S341, and S354 will pave the way for additional future studies to assess the possibility of using enzymes from pathogens like *A. baumannii* expressed in non-pathogenic bacterial host cells, to address problems of oil-contaminated environments.

MATERIALS AND METHODS

Pilot Expression of the *alkM* and *catA* Genes

E. coli TOP10 cells were previously transformed with pBAD Thio-TOPO plasmids containing either the *alkM* or the *catA* gene from *Acinetobacter baumannii* strains OS1, S341 or S354 (Tan and Hedreyda 2020). The transformants with *alkM* or *catA* gene from *Acinetobacter baumannii* strain 341 were grown in LB broth with ampicillin (100 µg/mL) for a pilot protein expression experiment. Protein expression experiments were conducted using 0 (no induction control), 0.002, 0.02, 0.2, 2, and 20% wt/vol arabinose. Cell lysates were subjected to SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) after 4 h of incubation with arabinose. Expression products were visualized in western blots using antibodies against the polyhistidine tag.

SDS-PAGE and western blot were performed using the Mini-Protean® Electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Cell lysates were boiled in SDS-PAGE treatment buffer and ran in Any kD™ Mini-Protean® TGX™ precast gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 100 V for 60 m. SDS-PAGE protein profiles were visualized after Coomassie Blue staining and proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 100 V for 60 min using the Mini-Protean® Mini-Trans-Blot® Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PVDF membrane was incubated in a skim milk solution overnight for blocking. Membranes were probed with a Mouse anti-His primary antibody (Invitrogen, Carlsbad, CA, USA) and further treated with Rabbit anti-Mouse secondary antibody conjugated to alkaline phosphatase (Invitrogen, Carlsbad, CA, USA). Proteins were visualized using the 1-step™ NBT-BCIP Substrate solution (Thermo Fisher Scientific, Waltham, MA, USA).

AlkM and CatA Protein Expression in Different Culture Broths and with Varying Lengths of Induction Time with Arabinose

AlkM and CatA expression studies in transformed *E. coli* TOP10 cells using different culture broths and different hours of arabinose induction were conducted. Transformed cells containing *alkM* gene from *A. baumannii* strains S341, S354, and OS1 and transformed cells containing *catA* gene from strain S341 were used. AlkM and CatA protein expression were assessed using arabinose induction time of 2, 4, and 6 h for each type of culture broth, the Luria broth (Condalab, Spain), Terrific broth (20 g tryptone, 24 g yeast extract, 4 mL glycerol per L), 2xYT broth (16 g tryptone, 10 g yeast extract, 5 g NaCl/ L), and Super broth (32 g tryptone, 20 g yeast extract, 5 g NaCl/ L).

Purification of Recombinant Enzyme

Prior to protein purification, recombinant cells producing AlkM and CatA proteins were lysed by performing three rounds of freezing at –80 ˚C for 10 min, followed by heating at 50 ˚C for 3 min. The soluble supernatant and the insoluble precipitate of recombinant cell lysates were subjected to SDS-PAGE after centrifugation at 14,000 x g for 5 min to determine which fraction of the lysate contains the AlkM or the CatA proteins.

Protein purification was performed starting with growing recombinant cells containing the *alkM* or *catA* gene in Super broth with 0.2% arabinose induction for 6 h. The cells that express AlkM were resuspended in denaturing buffer with 6 M GuHCl, 20 mM NaH_2PO_4 (pH 7.8), and 500 mM NaCl, while cells expressing CatA were resuspended in native buffer with 50 mM NaH_2PO_4 (pH 7.8) and 500 mM NaCl. Cell lysis was performed by sonication with 15–20-s pulses and the recombinant enzymes were purified from cell lysates through affinity chromatography using the HisTrap™ HP column (GE Healthcare UK Limited, United Kingdom), following the manufacturer's protocol. CatA enzymes were further purified through size exclusion chromatography using a Sephadex G-75 column (Sigma-Aldrich, St. Louis, MO, USA). Final enzyme concentrations were measured using Bradford reagent (Amresco, Radnor, PA, USA).

CatA Activity Assay

CatA activity was determined using a modified assay measuring the production rate of cis,cis-muconic acid (Nakazawa and Nakazawa 1970). Catechol solution (0.2 mL of 1 mM) was added to 5 mL of 0.1M K-Na phosphate buffer (pH 7.5), and this mixture was subsequently added to enzyme solution (0.2 mL). The mixture was incubated at room temperature and a 0.9-mL aliquot was taken from the mixture every minute after the addition of the enzyme. The reaction for each sampling was stopped by adding 0.2 mL of 1M HCl, and the absorbance of the mixture was measured at 260 nm. A calibration curve was generated using varying concentrations of *cis,cis*-muconic acid. The computed concentration of muconic acid was graphed against time and the slope of the graph is taken as the rate of muconic acid formation.

RESULTS

Pilot Expression of AlkM and CatA Proteins

The total protein gel profiles in the lanes loaded with lysates from transformed *E. coli* TOP10 cells containing *alkM* of *A. baumannii* strain S341 are shown in Figure 1A. Distinct protein bands (arrows) migrating in a position expected for a protein of about 50–75 kDa are present only in the lanes loaded with samples induced with 0.2, 2.0, and 20% arabinose. These protein bands are the putative AlkM thioredoxin fusion proteins that are approximately 62.71kDa. The lanes loaded with samples that were not induced with arabinose, as well as the samples that were induced with less than 0.2% arabinose, did not exhibit the same size protein band. The lanes loaded with lysates from recombinant bacteria that contain plasmid vector with no *alkM* gene did not exhibit the putative AlkM fusion protein.

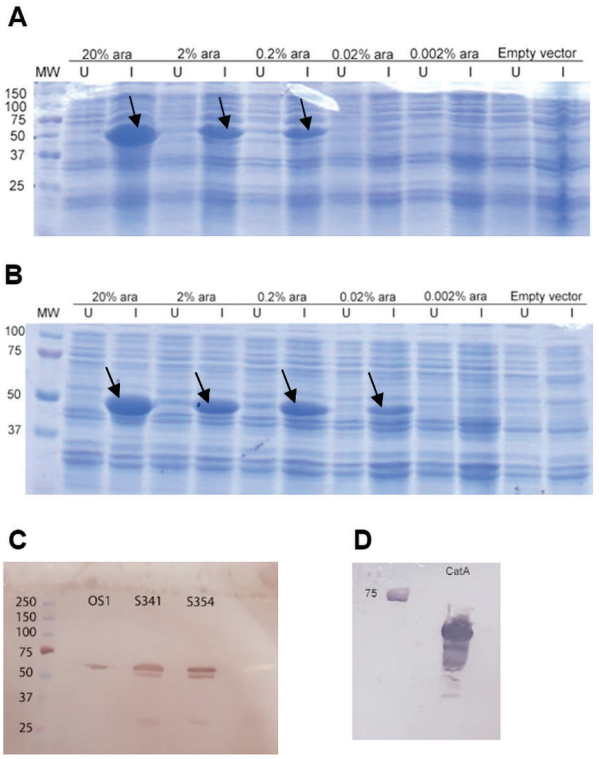

Figure 1. Expression of AlkM and CatA proteins in recombinant *E. coli* TOP10 cells containing pBAD Thio-TOPO plasmids with *alkM* or *catA*. A) AlkM proteins (arrows) in SDS-PAGE of lysates from cells with pBAD Thio-TOPO plasmids containing *alkM* from strain S341; B) CatA protein (arrows) in SDS-PAGE of lysates from cells with pBAD Thio-TOPO plasmids containing *catA* from strain S341; C) western blot of lysates from cells with pBAD Thio-TOPO plasmids containing *alkM* from strains OS1, S341, and S354; D) western blot of lysates from cells with pBAD Thio-TOPO plasmids containing *catA* from strain S341. For A and B, U is for no induction control and I is with arabinose induction. For C and D antibodies targeting poly-His was used to blot AlkM or CatA. The last two lanes in A and B are protein profiles of transformants containing only the vector pBAD Thio-TOPO plasmid.

Distinct protein bands that migrated in the position expected for a fusion protein of about 50 kDa (arrows) are present only in lanes loaded with cell lysates induced with 0.02–20% arabinose from transformed *E. coli* TOP10 cells containing *catA* (Figure 1B). These bands were observed in the position expected for a 49.5 kDa thioredoxin-CatA fusion protein. Lanes with lysates from transformants containing the plasmid vector without *catA* gene did not exhibit the correct size putative CatA protein.

The western blots (Figures 1C and D) revealed that the putative AlkM and CatA fusion proteins were expressed, resulting in the positive signal that indicates the interaction of the antibodies that target the His-tag in the fusion proteins.

AlkM and CatA Expression in Different Types of Culture Broths and Using Different Arabinose Induction Time

When the recombinant *E. coli* TOP10 cells containing the *alkM* from strain S341 gene were grown in Terrific and Super broths (with arabinose induction), more intense bands of the putative AlkM proteins of about 60 kDa (Figure 2A) were observed compared to the lanes loaded with samples grown in LB and 2xYT broth. Intense bands of the putative CatA proteins were also observed from cells grown in all types of culture media (Figure 2B, arrow), with the highest level of expression of the 50-kDa size CatA protein for cells grown in Super broth.

Total protein profiles observed from lysates of recombinant transformants (containing *alkM* from three strains of *A. baumannii*) after incubation with arabinose in Super broth exhibited the putative 60-kDa AlkM proteins (Figures 3A and B). Visually more intense bands were observed in lanes loaded with lysates from transformant cells in Super broth induced with arabinose for 6 h. Total protein profiles in lanes loaded with lysates from the transformants containing the *catA* gene from strain OS1 (Figure 3B) exhibited highly expressed putative 50kDa size CatA protein. Visually higher expression of the putative CatA protein in the lane loaded with the lysates from cells induced for 6 h with arabinose was observed.

Purification of Recombinant AlkM and CatA

SDS-PAGE exhibited the expected size of the AlkM and CatA fusion proteins (Figure 4A, bands with arrows). The gels revealed that the AlkM fusion proteins were more

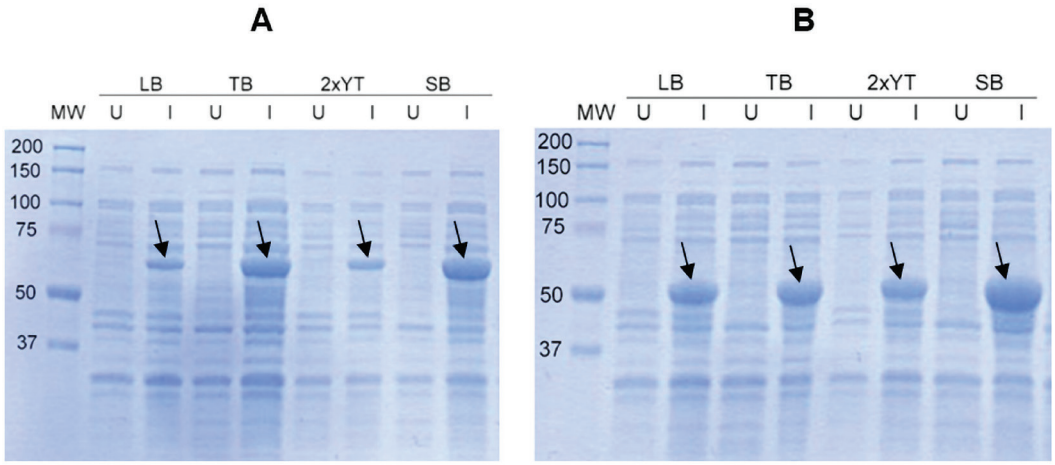

Figure 2. Total protein profiles of recombinant *E. coli* TOP10 cells with *alkM* (A) and *catA* (B) genes from *A. baumannii* strain S341 after incubation in different culture broths with (I) or without (U) 0.2% arabinose induction. A) AlkM proteins(arrows) expressed after incubation of *alkM*-containing transformed cells in different culture media; B) CatA proteins(arrows) expressed with 0.2% arabinose induction (I) after incubation of *catA*-containing transformed cells in different culture media. LB – Luria broth; TB – Terrific broth; 2xYT – 2xYT broth; SB – Super broth.

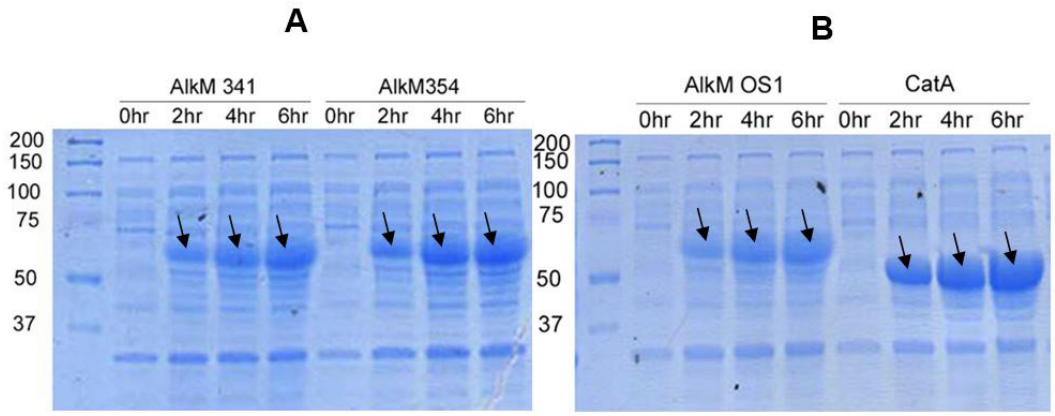

Figure 3. Total protein profiles of recombinant *E. coli* TOP10 cells with *alkM* gene (from *A.baumannii* strains S341, 354, and OS1) and *catA* gene (from *A.baumannii* strain S341) after 0–6 h induction with 0.2% arabinose in Super broth.

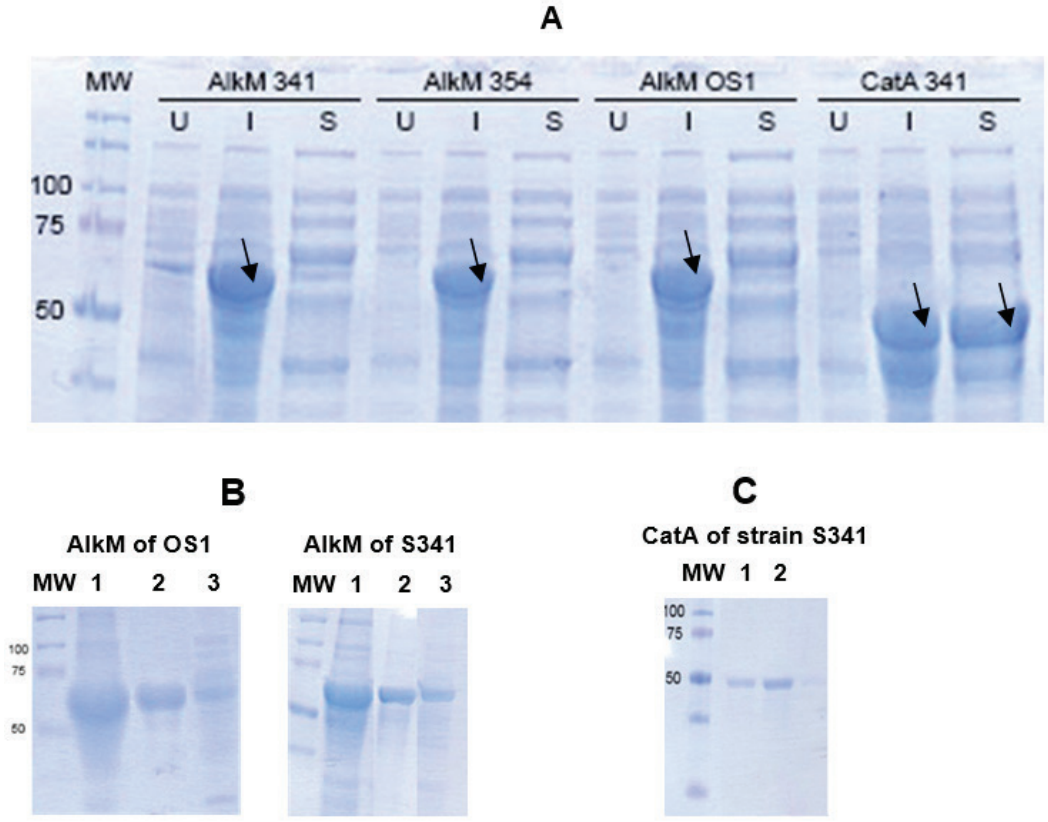

Figure 4. SDS-PAGE of AlkM and CatA after different steps of purification. A) Insoluble (I) and soluble (S) fractions of cell lysates from recombinant cells expressing AlkM and CatA and U are lysates from cells grown with no arabinose induction; B) cell lysate fractions from recombinant bacteria expressing AlkM after purification using a His-Trap-column where Lane 1 is for cell lysate or total cell protein prior to purification, Lane 2 is for the eluate or the eluted fraction containing the His-tagged recombinant proteins, and Lane 3 is for the flowthrough fraction prior to elution of the column; C) Lanes 1 and 2 are eluate fractions from recombinant bacteria expressing CatA after purification using a His-Trap-column followed by purification using a Sephadex G-75 column. Arrows in A point to recombinant AlkM or CatA fusion proteins.

abundant in the insoluble phase, while the CatA fusion protein was equally present in the soluble and insoluble phases. Purification of all AlkM samples was achieved, as indicated by the absence of bands in the SDS-PAGE profiles of the eluates from His-tag chromatography (Figure 4B). After purification, protein refolding was attempted by removing urea through dialysis. This resulted in the precipitation of recombinant proteins, and there was no protein remaining in the solvent post-dialysis.

After His-tag chromatography, partial purification of the CatA protein (data not shown) was observed. Faint bands of about 100 kDa were present in lanes loaded with an eluted fraction containing the His-tagged recombinant proteins. The faint bands were successfully removed resulting in bands of 50 kDA CatA protein after size exclusion chromatography (Figure 4C, Lanes 1 and 2). The protein concentration of the purified enzyme was computed to be 0.1284 mg enzyme/ mL.

Preliminary CatA Activity Assay

Because sequence analysis of the complete *catA* gene in *A. baumannii* strains S341, S354, and OS1 showed that the predicted CatA protein sequences from all these strains are identical, only the strain S341 CatA activity assay was performed. The CatA assay shows that the setup containing the enzyme had increasing *cis,cis*-muconic acid concentration over time (Figure 5), indicating purified CatA enzyme activity.

The slope of the trendline corresponds to the rate of catechol conversion into muconic acid in µM/min. This means that about 4.3803 µM of catechol is converted by CatA into muconic acid per minute. This value was used to compute for enzyme activity parameters – including enzyme units [4.818 X 10^{-3} U (µmol/min)], unit per mL (0.14455 U/mL), and specific enzyme unit (1.126 U/mg).

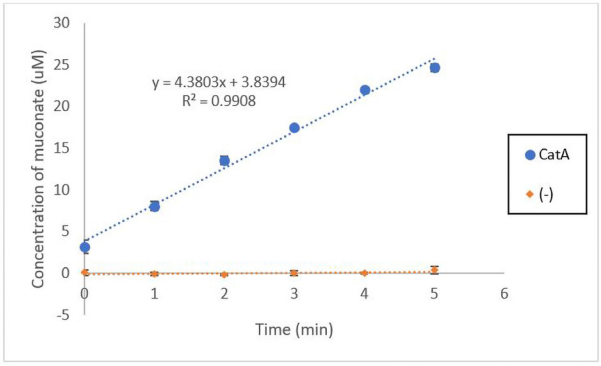

Figure 5. CatA enzyme activity measured as the rate of *cis,cis*-muconic acid formation. Blue dots represent cis,cis-muconic acid formation using purified S341 CatA. Orange diamonds represent the no-protein negative control. The equation of the trendline for the CatA setup is shown in the graph.

DISCUSSION

In previous studies that were conducted to isolate oil-degrading bacteria, isolates that exhibited bunker oil utilization were identified to belong to *Acinetobacter baumannii* (Hedreyda and Sarmago 2014; Tan and Hedreyda 2020). The *alkM* and *catA* genes coding for AlkM and CatA enzymes that are involved in hydrocarbon degradation were detected in this species. *Acinetobacter baumannii* is a human pathogen with wide spectrum of antimicrobial resistance that can cause serious complications such as pneumonia, nosocomial meningitis, and wound infections (Merriman 2014). Oil degrading strains of *A. baumannii*, therefore, could not be directly used for bioremediation. In this research, non-pathogenic recombinant *E. coli* TOP10 cells containing a complete *alkM* gene from strains of *A. baumannii* (oil sludge strain OS1, Pasig River strain S341 or Pasig River strain S354) or recombinant *E. coli* TOP10 cells with the complete *catA* gene from strain S341 were used to study AlkM and CatA protein expression. The possibility of using recombinant cells that AlkM or CatA enzymes for bioremediation studies requires knowledge of optimized parameters for enzyme expression and activity.

The plasmids used to clone the genes for AlkM and CatA in this study regulate expression using the P_{BAD} promoter. This promoter is induced by arabinose (Lee *et al.* 1987) and is repressed by glucose (Miyada *et al.* 1984). Results show that AlkM and CatA expression levels increase with arabinose induction, with the highest expression attained when using 20% arabinose. High AlkM and CatA expression did not result in cell decline, showing that AlkM and CatA are not toxic to the transformants. The four types of culture media used in the expression experiments have the same components but with different nutrient concentrations. The culture media did not contain glucose to prevent repression of the P_{BAD} promoter. Expression of AlkM and CatA was highest when using Super broth with the highest nutrient concentration among the culture media used. Expression of the alkaline phosphatase in previous studies using the same expression vector was threefold less efficient in rich media compared to minimal media (Guzman *et al.* 1995). It is suggested that expression using a minimal medium be included in future expression studies.

Previous studies report that the thioredoxin fusion tag increases protein yield and may reduce the inclusion body formation of proteins (LaVallie *et al.* 1993). The thioredoxin fusion tag was used in this study to improve the expression of active membrane proteins (McNiff *et al.* 2016). Because AlkM is an integral membrane protein (Ratajczak *et al.* 1998a) and is expected to be insoluble, the pBAD Thio-TOPO plasmid was used to express the recombinant protein with thioredoxin. Results show, however, that the AlkM fusion protein remained in the insoluble fraction. Attempts to refold AlkM after denaturation resulted in protein precipitation, which indicates that AlkM is insoluble even with the thioredoxin tag. Therefore, the AlkM enzyme activity experiment could not be performed. An additional study to address the AlkM enzyme insolubility needs to be pursued. The solubility problem of a related protein AlkB in *Pseudomonas oleovorans* (Peters and Witholt 1994) was addressed by testing various detergents that may solve the insolubility problem without affecting enzyme activity.

Purification of the recombinant CatA was achieved and a preliminary CatA activity assay suggested that conversion of catechol into *cis,cis*-muconic acid. Moreover, the results show that the thioredoxin tag does not eliminate the enzyme activity. CatA specific enzyme activity, which was calculated to be 1.126 units per mg, is relatively low compared to the specific enzyme activity reported for CatA of *Acinetobacter calcoaecticus* ADP-96, with a specific activity of 20 units/mg after six purification steps (Patel *et al.* 1976). Another study reported that *Acinetobacter radioresistens* CatA with a specific activity of 24.5 after two purification steps (Briganti *et al.* 1997). Sequence and structure differences of CatA from different species need to be analyzed to gain insight into the possible role of protein structure in enzyme activity. More studies to determine optimal parameters for improved specific enzyme activity of expressed CatA is highly recommended. The effect of CatA recombinant protein thioredoxin tag on enzyme activity also needs to be studied.

Bioremediation can be achieved through bioaugmentation, which is the introduction of microbes that are capable of degrading the pollutant to supplement biodegradation (Tyagi *et al.* 2011). The *Acinetobacter baumannii*, however,

is a pathogen that cannot be used for bioaugmentation. This limitation may be addressed by using recombinant bacteria that express the *A. baumannii* AlkM and CatA enzymes exhibiting sufficient activity. The result of this study is an initial step towards using recombinant non-pathogenic host cells transformed with genes for enzymes important in oil pollutant degradation. There is a need, however, to address concerns of releasing recombinant bacteria into the environment.

This study showed that the expression of the AlkM and CatA enzymes requires induction by arabinose to allow the binding of the AraC transcription factor to the P_{BAD} promoter upstream of the inserted gene. If arabinose is not present in the environment, the enzyme may not be expressed by the transformants. This problem can be addressed by replacing the expression system with one that responds to hydrocarbons (Yuste *et al.* 1998) like the studies on AlkS of *Pseudomonas* (Smits *et al.* 2001) and AlkR in *Acinetobacter* (Ratajczak *et al.* 1998b). Recombinant bacteria have been used to express catechol 2,3-dioxygenase (XylE) in the presence of catechol and has been used to express other enzymes involved in hydrocarbon biodegradation such as alkane monooxygenase (AlkB) and cytochrome P450 (CYP153) (Koch *et al*. 2009). Transformants with plasmids containing *alkB* of *Pseudomonas putida* GPo1 were successfully used to improve the degradation of diesel by a bacterial consortium in a laboratory setting (Luo *et al.* 2015). Furthermore, the release of recombinant bacteria with plasmids containing antibiotic resistance genes is undesirable as it may lead to the spread of antibiotic resistance (Tiedje *et al.* 1989). Alternative methods for bacterial selection such as resistance to herbicide or heavy metals (Ramos *et al.* 1994) have been reported. These issues must be addressed. It is also possible to introduce cell-free enzymes to the environment to catalyze the degradation of pollutants. This may also be achieved by introducing crude or purified enzymes expressed by recombinant bacteria into the environment.

In this study, genes for enzymes like AlkM and CatA was cloned into plasmid expression vectors, and the recombinant plasmids were introduced by transformation to non-pathogenic *E. coli* host cells. The protein gene products were expressed and parameters for significant expression were successfully obtained. Successful expression of the enzyme by the recombinant host cells, however, must be coupled with high enzyme activity comparable if not better than expression levels previously reported for the enzyme. An extensive study that will focus on obtaining optimized parameters for enzyme activity of both AlkM and CatA expressed in non-pathogenic host cells is deemed necessary before the recombinant cells can be used in addressing problems of oil contamination.

REFERENCES

BRIGANTI F, PESSIONE E, GIUNTA C, SCOZZAFAVA A. 1997. Purification, Biochemical Properties and Substrate Specificity of a Catechol 1,2-Dioxygenase from a Phenol Degrading *Acinetobacter radioresistens*. FEBS Letters 416(1): 61–64.

GUZMAN LM, BELIN D, CARSON MJ, BECKWITH J. 1995. Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose PBAD Promoter. Journal of Bacteriology 177(14): 4121–4130.

HEDREYDA CT, SARMAGO AMG. 2014. Detection and Sequence Analysis of the AlkM Gene in *Acinetobacter baumannii* Strain OS1 from Oil Sludge Sample. Philippine Science Letters 7(1): 1–6.

KOCH DJ, CHEN MM, VAN BEILEN JB, ARNOLD FH. 2009. *In Vivo* Evolution of Butane Oxidation by Terminal Alkane Hydroxylases AlkB and CYP153A6. Applied and Environmental Microbiology 75(2): 337–344.

LAVALLIE ER, DIBLASIO EA, KOVACIC S, GRANT KL, SCHENDEL PF, MCCOY JM. 1993. A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the *E. coli* Cytoplasm. Bio/Technology 11(2): 187–193.

LEAHY JG, COLWELL RR. 1990. Microbial Degradation of Hydrocarbons in the Environment. Microbiological Reviews 54(3): 305–315.

LEE N, FRANCKLYN C, HAMILTON EP. 1987. Arabinose-Induced Binding of AraC Protein to AraI2 Activates the AraBAD Operon Promoter. Proceedings of the National Academy of Sciences of the United States of America 84(24): 8814–8818.

LUO Q, HE Y, HOU D-Y, ZHANG J-G, SHEN X-R. 2015. GPo1 AlkB Gene Expression for Improvement of the Degradation of Diesel Oil by a Bacterial Consortium. Brazilian Journal of Microbiology 46: 649–657.

MERRIMAN H. 2014. Infectious Diseases. In: Acute Care Handbook for Physical Therapists, 4th Edition. Paz JC, West MP eds. Elsevier. p. 313–334.

MCNIFF ML, HAYNES EP, DIXIT N, GAO FP, LAURENCE JS. 2016. Thioredoxin Fusion Construct Enables High-Yield Production of Soluble, Active Matrix Metalloproteinase-8 (MMP-8) in *Escherichia coli*. Protein Expression and Purification 122: 64–71.

MIYADA CG, STOLTZFUS L, WILCOX G. 1984. Regulation of the AraC Gene of *Escherichia coli*: Catabolite Repression, Autoregulation, and Effect on AraBAD Expression. Proceedings of the National Academy of Sciences 81(13): 4120– 4124.

NAKAZAWA T, NAKAZAWA A. 1970. Pyrocatechase (*Pseudomonas*). Methods in Enzymology 17(A): 518–522.

PATEL RN, HOU CT, FELIX A, LILLARD MO. 1976. Catechol 1,2-Dioxygenase from *Acinetobacter calcoaceticus*: Purification and Properties. Journal of Bacteriology 127(1): 536–544.

PETERS J, WITHOLT B. 1994. Solubilization of the Overexpressed Integral Membrane Protein Alkane Monooxygenase of the Recombinant *Escherichia coli* W3110[PGEc47]. Biochimica et Biophysica Acta 1196: 145–153.

PRINCE RC, WALTERS CC. 2016. Biodegradation of Oil Hydrocarbons and Its Implications for Source Identification. In: Standard Handbook Oil Spill Environmental Forensics. Elsevier. p. 869–916.

RAMOS JL, DÍAZ E, DOWLING D, DE LORENZO V, MOLIN S, O'GARA F, RAMOS C, TIMMIS KN. 1994. The Behavior of Bacteria Designed for Biodegradation. Bio/Technology 12(12): 1349–1356.

RATAJCZAK A, GEISSDÖRFER W, HILLEN W. 1998a. Alkane Hydroxylase from *Acinetobacter* sp. Strain ADP1 Is Encoded by AlkM and Belongs to a New Family of Bacterial Integral-Membrane Hydrocarbon Hydroxylases. Applied and Environmental Microbiology 64(4): 1175–1179.

RATAJCZAK A, GEISSDÖRFER W, HILLEN W. 1998b. Expression of Alkane Hydroxylase from *Acinetobacter* sp. Strain ADP1 Is Induced by a Broad Range of n-Alkanes and Requires the Transcriptional Activator AlkR. Journal of Bacteriology 180(22): 5822–5827.

ROJO F. 2009. Degradation of Alkanes by Bacteria. Environmental Microbiology 11(10): 2477–2490.

SARMAGO AMG, HEDREYDA CT. 2014. Sequence Analysis of the Complete Catechol 1,2-Dioxygenase Gene in *Acinetobacter baumannii* Strain OS1. Philippine Science Letters 7(2): 406–412.

SMITS THM, SEEGER MA, WITHOLT B, VAN BEILEN JB. 2001. New Alkane-Responsive Expression Vectors for *Escherichia coli* and *Pseudomonas*. Plasmid 46(1): 16–24.

TAN APS, HEDREYDA CT. 2020. Sequence Analysis and Cloning of Alkane 1-Monooxygenase and Catechol 1,2-Dioxygenase Genes from *Acinetobacter baumannii* Isolated from the Philippine Pasig River. Philipp J Sci 149: 159–168.

TIEDJE JM, COLWELL RK, GROSSMAN YL, HODSON RE, LENSKI RE, MACK RN, REGAL PJ. 1989. The Planned Introduction of Genetically Engineered Organisms: Ecological Considerations and Recommendations. Ecology 70(2): 298–315.

TYAGI MM, DA FONSECA MR, DE CARVALHO CCCR. 2011. Bioaugmentation and Biostimulation Strategies to Improve the Effectiveness of Bioremediation Processes. Biodegradation 22(2): 231–241.

YUSTE L, CANOSA I, ROJO F. 1998. Carbon-Source-Dependent Expression of the PalkB Promoter from the *Pseudomonas oleovorans* Alkane Degradation Pathway. Journal of Bacteriology 180(19): 5218–5226.