Recombinant Production and One-Pot Purification for Enhancing Activity of Haloacid Dehalogenase from Bacillus cereus IndB1

Enny Ratnaningsih*1, Sulistiya Nirta Sunaryo1, Idris Idris2, and Rindia M. Putri1

1)Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Indonesia
2)Mycrobiology Division, Research Center for Biology, Indonesian Institute of Sciences, Indonesia

*Corresponding author: enny@chem.itb.ac.id

(Received: June 05, 2021; Accepted: June 28, 2021)

Abstract

In recent years we have witnessed the emergence of organohalogen utilization in various chemical-based industries, particularly polymer-based, agricultural, and pharmaceutical sectors. Despite this, organohalogen compounds are actually very dangerous to the environment, as they are difficult to be naturally degraded and generally toxic to organisms. A green and biocompatible method to overcome this issue is by employing enzymes that could convert organohalogens into non-toxic compounds, such as the class of enzymes known as haloacid dehalogenases. To enhance the activity of haloacid dehalogenase isolated from local strains of Bacillus cereus IndB1, we have developed a recombinant expression system using pET-bcfd1 plasmid in E. coli BL21 (DE3) host cells. Following enzyme production, we also demonstrated a one-pot purification system for the expressed dehalogenase, harnessing the presence of His-tag in the recombinant clones. Purification was carried out using Ni-NTA affinity column chromatography, using imidazole eluent with a concentration gradient of 10 mM to 500 mM. The enzyme activity was tested against the monochloroacetic acid (MCA) substrate according to the Bergmann and Sanik method, and the protein content in the solution was measured using the Bradford method. The purity of the enzyme after one-pot purification was confirmed by SDS-PAGE analyses, showing a single band of 40 kDa in size. Remarkably, the purified haloacid dehalogenase specific activity was increased by 12-fold compared to its crude enzyme extract. Therefore, the expression and purification system developed in this study allow further exploration of dehalogenases from local strains as an efficient catalyst for MCA biodegradation.

Keywords: recombinant expression, haloacid dehalogenase, monochloroacetic acid, enzyme purification

How to Cite This Article: Ratnaningsih, E., Sunaryo, S. N., Idris, I., and Putri, R. M., (2021), Recombinant Production and One-Pot Purification for Enhancing Activity of Haloacid Dehalogenase from Bacillus cereus IndB1, Reaktor, 21(2), 59-64, https://doi.org/10.14710/reaktor.21.2.59-64

INTRODUCTION

Organohalogens are a group of organic compounds containing one or more halogen atoms, which are widely used in polymer industry, agriculture, and pharmaceutics (Fetzner and Lingens 1994). Despite their benefit, exposure to organohalogen compounds can cause great damage to organisms and ecosystem, as they are neurotoxic,
immunotoxic, and possibly carcinogenic (Rose et al., 2009). As organohalogen compounds are difficult to be naturally degraded in the environment, the risk of exposure to these compounds is relatively high (Rose et al., 2009). Monochloroacetic acid (MCA) is an example of organohalogen that would survive in the environment for a long time (European Commision, 2005). The chemical hydrolysis of MCA is slow and photolysis of MCA in air or in water does not occur because this compound does not absorb UV rays. Due to its persistence in the environment and its use in industries, the risk of exposure to MCA is significantly high (European Commision, 2005).

To address this environmental issue, accelerating MCA degradation by employing microbes and green plants is an attractive option, owing to their green and biocompatible nature (Megharaj et al., 2011). Specifically, a group of enzymes obtained from microorganisms can be employed for this purpose, as they are capable of altering the chemical structures of organohalogen pollutants into non-toxic compounds. This class of enzymes is known as the haloacid dehalogenases that catalyze the carbon-halogen bond-breaking reaction in halogenated aliphatic acid compounds (Fetzner and Lingens, 1994). We have previously reported a local strain of Bacillus cereus IndB1 that expressed a haloacid dehalogenase (Ratnaningsih and Idris, 2018). However, as the yield of the enzyme obtained from Bacillus cereus IndB1 wild type was relatively low, it was not quite sufficient for an efficient MCA biodegradation. A recombinant expression system is generally very effective approach to improve enzyme production (Ratnaningsih and Idris, 2018). Furthermore, in order to enhance the activity of such enzyme, a purification method of the recombinant expressed enzyme is generally required.

In this study, we aimed to produce the recombinant haloacid dehalogenase from Bacillus cereus IndB1 local strains in its recombinant pET-bcfd1 clone using E. coli BL21 (DE3) as host cells and improve the activity of the expressed enzyme by means of purification. Efficiency of the purification was expected to be high since the recombinant clone contains the His-tag. Therefore, a one-pot purification system for this dehalogenase utilizing Ni-NTA affinity chromatography, could be developed (Bornhorst and Falke, 2000; Mahmoodi et al., 2019). The enzyme purity was evaluated using SDS-PAGE. To further confirm that the purification would result in the expected enhancement of specific activity, we compared the pure enzyme specific activity to the crude enzyme extract expressed under identical environments.

**MATERIALS AND METHODS**

**Source of the recombinant microorganism**

The E. coli BL21 (DE3) harboring pET-bcfd1 recombinant clone carrying the haloacid dehalogenase gene from Bacillus cereus IndB1 local strain was obtained from our previous research (Ratnaningsih and Idris, 2018). The haloacid dehalogenase gene, namely bcfd1, has been sequenced and the sequence has already been deposited in GenBank (accession number KU49803). This gene was initially cloned into pGEM-T for analysis and subsequently sub-cloned into pET-30(a) expression vector under the control of the T7 promoter. The clone has been stored as a stock culture at −80°C in 20% (v/v) glycerol-LB medium.

**Refreshing the E. coli BL21 (DE3)/pET-bcfd1 culture**

To refresh the E. coli BL21 (DE3) carrying pET-bcfd1 recombinant plasmid, the culture from glycerol stock was inoculated into selective LB broth medium without NaCl (contains 5 g/L yeast extract and 10 g/L tryptone, and 50 μg/mL kanamycin). The culture was shaken overnight at 37°C rotary shaker. Single colony for further experiments was obtained by streaking this fresh culture onto a solid LB medium containing 50 μg/mL kanamycin, followed by overnight incubation at 37°C. Confirmation of the recombinant plasmid was conducted by restriction analysis as well as by PCR employing the primers used for initial gene isolation.

**Recombinant expression of haloacid dehalogenase from E. coli BL21 (DE3)/pET-bcfd1**

The crude enzyme was prepared by inoculating 500 μL of overnight fresh culture of E.coli BL21 (DE3)/pET-bcfd1 into 100 mL LB broth medium without NaCl, containing 50 μg/mL kanamycin. The culture was incubated at 37°C with shaking until the OD<sub>600</sub> reached 0.6. Then, IPTG was added into the culture with 10 μM final concentration. The culture was further incubated at 30°C for 2 hours with shaking, moved into ice for 10 minutes, and the cells were harvested by centrifugation. The supernatant was collected, and the cells pellet was re-suspended into 10 mL of 50 mM Tris-acetate buffer pH 7.5. The cells were lysed by intermittent ultrasonication for 20 minutes, 30 seconds on and 30 seconds off with 40% amplifier level at 4°C. The lysate was then centrifuged at 4°C and the supernatant (which is the crude enzyme extract) was transferred into a new tube and store at 4°C for further analysis.

**Enzyme purification**

Purification of the crude enzyme was carried out using batch method on Ni-NTA affinity column, chromatography employing the His-tag in the recombinant enzyme (Bornhorst and Falke, 2000; Mahmoodi et al., 2019). One milliliter of Ni-NTA resin suspension was transferred into a 15 mL centrifuge tube. The alcohol in the resin mixture was decanted and the resin was washed several times with MilliQ water until the alcohol odor disappears. Into the tube, 2 mL of phosphate buffer binding solution was added, and the mixture was vigorously shaken at 10°C for about 10 minutes. The supernatant was then discharged by means of centrifugation. Then, 3 mL of cold crude enzyme extract was added into the resin in the tube, and the mixture was incubated overnight on rotary shaker at 10°C. The supernatant was collected...
RESULTS AND DISCUSSION

Culture of E. coli BL21 (DE3)/pET-bcfd1

The gene encoding for haloacid dehalogenase from Bacillus cereus IndB1 local strain was referred as bcfd1 gene. This gene has been inserted to a pET-30a(+) expression vector, resulting in pET-bcfd1 recombinant clone. The E. coli BL21 (DE3) harboring pET-bcfd1 recombinant clone carrying the haloacid dehalogenase gene from Bacillus cereus IndB1 was named as E. coli BL21 (DE3)/pET-bcfd1, which was obtained from our previous research (Ratnaningsih and Idris, 2018). In this study, these recombinant clones were cultured in a selective LB medium containing 50 g/mL kanamycin. In this medium, only the bacteria that carrying the recombinant plasmid would grow as the kanR gene responsible for resistance to kanamycin was carried by the recombinant pET-bcfd1 plasmid. Using serial streaking technique on selective agar plates, followed by overnight incubation at 37°C, we obtained a single colony of E. coli BL21 (DE3)/pET-bcfd1 for further experiments (Figure 1). A single colony that healthily grew on the medium and well separated from other colonies was assumed to be derived from a single cell. Such a single colony was required as to ensure that the cultures were genetically homogeneous.

Figure 1. Culture of recombinant E. coli BL21(DE3)/pET-bcfd1 on selective solid LB medium

Isolation and analyses of pET-bcfd1 recombinant plasmid

To confirm that E. coli BL21 (DE3) was carrying the correct pET-bcfd1 recombinant plasmid, we isolated the plasmid from the single colony (Figure 1) derived culture by alkaline lysis method (Birnboim and Doly, 1979). The isolated recombinant plasmid was then subjected to agarose gel electrophoresis using 1% (w/v) agarose gel in TA buffer and run at 55 V for 60 min. The electrophoregram revealed that the size of pET-bcfd1 from the single colony (line S) was larger compared to the empty pET-30a(+) clone (line E) (Figure 2a). The larger size of the recombinant plasmid was indicated by its smaller migration distance in comparison to empty pET-30a(+). Such an increase in size confirmed that the single colony

into a new tube as flowthrough and stored at −20°C for further analysis. The resin was subsequently washed 5 times with 2 mL each of washing buffer 1 (10 mM imidazole), and the supernatants were pooled into a new sterile tube. The washing process was repeated using washing buffer 2 (50 mM imidazole) under identical procedure.

After completing the washing process, 600 μL of elution buffer 1 (100 mM imidazole) was used to take up the enzyme from the resin, which were carried out 3 times, and each resulted eluates were placed in separate new sterile tube. The elution process was repeated using elution buffer 2 (250 mM imidazole) and elution buffer 3 (500 mM imidazole) with the same procedure. All imidazole solution was prepared in 50 mM Tris-Cl pH 7.5. Overall, this elution process resulted in 9 fractions, namely fraction 1 of eluate 1, fraction 2 of eluate 1, fraction 3 of eluate 1, fraction 1 of eluate 2, fraction 2 of eluate 2, fraction 3 of eluate 2, fraction 1 of eluate 3, fraction 2 of eluate 3, and fraction 3 of eluate 3. Enzyme purity of each fraction was evaluated by SDS-PAGE and its protein content was quantified by Bradford method (Bradford, 1976; Kruger, 2009; Aminian et al., 2013), using BSA standard curve. All fractions were subsequently subjected to haloacid dehalogenase activity assay, where fractions from the same eluates were pooled.

Haloacid dehalogenase activity assay

The enzymatic reactions were performed in 1.0 mL solutions containing 50 μL of enzyme crude extract or wash buffer, or 5 μL of enzyme fractions from Ni-NTA column (diluted to 50 μL with buffer), 850 μL of 50 mM glycine-NaOH buffer pH 10, and 100 μL of 5 mM MCA to make 0.5 mM final concentration. The control was prepared with the same composition except that the enzyme was replaced with buffer solution. The reaction mixture was homogenized by inverting the tube several times, followed by incubation at 37°C water bath for 10 minutes. The reaction was stopped by placing the tube on ice for about 5 minutes and the amount of chloride ion released into the medium was quantified by spectrophotometer according to Bergmann and Sanik method (Bergmann and Sanik, 1957).

Chloride ion determination

Bergmann and Sanik method (Bergmann and Sanik, 1957) for chloride ion determination was performed as follows. One milliliter of the enzymatic reaction sample was mixed with 100 μL of 0.1% (w/v) Hg(SCN)\textsubscript{2}, mixed well by inverting the tube several times, and incubated for 5 min at room temperature. Then, 100 μL of 0.25 M FeNH4(SO\textsubscript{4})\textsubscript{2} in 9 M HNO\textsubscript{3} was added into the mixture, mixed well by inverting the tube, and further incubated for 5 min at room temperature. Absorbance of the resulted color solution was measured at 460 nm, and the chloride ion concentration was determined using NaCl standard curve.
cultivated in this study indeed contained a recombinant plasmid.

To ensure that the inserted gene in the plasmid was indeed the bcfd1 gene, restriction analysis was performed using the EcoRI and HindIII restriction enzymes (Ratnaningsih and Idris, 2018). Following double-digestion with these restriction enzymes, we analyzed the samples by agarose gel electrophoresis using 1% (w/v) agarose gel in TA buffer and run at 55 V for 60 minutes. The electrophoreogram is presented in Figure 2(b), revealed that the double-digested pET-bcfd1 (line S) produced two DNA fragments with the size of ~5500 bp and ~850 bp. The heavier fragment was indicated to be the pET-30a(+) whose original size was 5422 bp. On the other hand, the lighter fragment of ~850 bp confirmed the presence of the bcfd1 gene as this particular size is in agreement with the estimated 852 bp of bcfd1 gene suggested by previous in silico analyses (Ratnaningsih and Idris, 2018). Further confirmation was performed by amplifying the bcfd1 gene using pair of primers utilized in initial gene isolation, which also resulting the same size of strong single band amplicon (Figure 2(c)).

Expression and one-pot purification of haloacid dehalogenase

The recombinant expression of the bcfd1 gene in E. coli BL21 (DE3) hosts occurred upon the addition of the inducer to the growth medium (Rosano and Ceccarelli, 2014; Rosano, et al., 2019). In this study, we employed isopropyl-β-D-thiogalactopyranoside (IPTG) as the inducer, which is an analogue compound of lactose (Ma et al., 2020; Schein, et al., 1988; Winograd, et al., 1993). Following after induction incubation at 30°C for 2 h with shaking, the resulting cultures were harvested by centrifugation and the cells were lysed by intermittent ultrasonication.

Upon centrifugation, the lysate was transferred into a new sterile tube and used as the crude enzyme extract.

To improve the purity of the enzyme, and thus enhancing its specific activity, we set to purify the enzyme using a Ni-NTA affinity column. The recombinant haloacid dehalogenase gene in E.coli BL21 (DE3)/pET-bcfd1 contains 6 x His-tag in its N-terminal sequence, which facilitated the separation of this enzyme from other cellular proteins using this column (Hengen, 1995). At certain pH, histidine residue in His-tag would specifically bind to nickel ions immobilized with nitrilo triacetic acid in the resin matrices (Ni-NTA) (Hengen, 1995). Once all other proteins were washed off from the resin, the target protein could be eluted with improved gradient concentration of imidazole. This approach allowed a one-pot purification system for the expressed enzyme.

All protein fractions from Ni-NTA column were subjected to SDS-PAGE and the observed electrophoreogram is presented in Figure 3. In our one-pot purification system, the resin was initially washed 5 times with 2 mL each of washing buffer 1 (10 mM imidazole) and subsequently with washing buffer 2 (50 mM imidazole). After the completion of resin washing, elution buffer 1 (100 mM imidazole) was used to elute the enzyme out of the resin. The elution process was repeated using higher concentrations of imidazole in elution buffer 2 (250 mM imidazole) and then elution buffer 3 (500 mM imidazole). Particularly at the highest imidazole concentration (elution buffer 3), all fractions from imidazole elution gave only a single band at around 40 kDa, indicating that the recombinant haloacid dehalogenase was successfully purified using this method. Our previous research had identified the size of haloacid dehalogenase from Bacillus cereus IndB1 as 37 kDa (Ratnaningsih and Idris, 2018).

Enhanced activity of haloacid dehalogenase upon one-pot purification

The activity of haloacid dehalogenase bcfd1 was quantitatively analyzed by measuring the amount of
free chloride ions into the medium as a result of MCA degradation by haloacid dehalogenase (Adamu, et al., 2020; Torz and Beschkov, 2005; Ang, et al., 2018). The measurement of chloride ions in solution was carried out using the Bergmann and Sanik method (Bergmann and Sanik, 1957). In this method, the chloride ions from MCA degradation would bind the Hg²⁺ ions from Hg(SCN)₂, releasing thiocyanate ion (SCN⁻) into the solution. As iron(III) ions were also present in the solution, the colored complex of Fe(SCN)₃⁺was accordingly formed and monitored at the wavelength of 460 nm (A₄₆₀). In this method, the concentration of the colored complex was directly proportional to the chloride ions released by MCA degradation (Bergmann and Sanik, 1957). To obtain the extinction coefficient required for spectrophotometric concentration determination, we prepared standard solutions of NaCl and separately carried out the Bergmann and Sanik reactions under identical procedure.

Specific activities of all fractions obtained from this purification are presented in Table 1. Fractions of the same elutes were pooled, because they all contained the same single band in SDS-PAGE. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of chloride ion into the medium in a 1-minute enzymatic reaction using 0.5 mM of MCA as the substrate. Accordingly, unit of specific activity was concurrently defined as unit activity per microgram protein in the sample in a 1-minute enzymatic reaction.

Table 1. Specific activity of haloacid dehalogenase in each fraction from purification steps

| Sample          | [Cl⁻ (mM)] | Activity (U/mL) | [Total protein] (µg/ml) | Specific activity (U/µg protein) |
|-----------------|------------|-----------------|------------------------|---------------------------------|
| Medium          | 0.065      | 0.130           | 11.11                  | 0.011                           |
| Crude extract   | 0.329      | 0.658           | 95.21                  | 0.007                           |
| Wash 1          | 0.238      | 0.476           | 15.38                  | 0.030                           |
| Wash 2          | 0.197      | 0.394           | 6.20                   | 0.064                           |
| Elute 1         | 0.155      | 0.100           | 8.98                   | 0.345                           |
| Elute 2         | 0.179      | 3.580           | 11.11                  | 0.322                           |
| Elute 3         | 0.249      | 4.980           | 5.94                   | 0.838                           |

Notes: Unit activity was calculated from observed chloride ion concentration divided by reaction time (10 minutes) and enzyme volume used in the reaction. The volumes of medium, crude extract, wash 1, and wash 2 solutions were 50 µL each in 1 mL of total reaction volume, whereas the volume of elution 1, elution 2, and elution 3 were 5 µL each, in 1 mL of total reaction volume.

Table 1 clearly indicated that the specific activity of the enzyme was significantly increased with the purification steps. Best specific activity was obtained in eluates 3, where 500 mM imidazole was used as an eluent. Specific activity in this fraction was 0.838 U/µg protein, which was 12-fold higher compared to the crude enzyme extract (0.007 U/µg protein). Gradual increase of imidazole concentration in the eluent apparently did not give any significant effect on this haloacid dehalogenase purification.

CONCLUSIONS

Through genetic engineering, we have produced haloacid dehalogenase from local strain as an intracellularly expressed active enzyme for MCA degradation, employing E. coli BL21 (DE3) as the host cells. Double-digestion and gel analyses of the recombinant pET-bcfd1 isolated from E. coli BL21 (DE3) single colony confirmed that the gene inserted to the pET-30a(+) was indeed the bcfd1 from Bacillus cereus IndB1 encoding for haloacid dehalogenase. Moreover, IPTG-induced expression of the enzyme resulted in a ~40 kDa protein on SDS-PAGE analyses, which was in agreement with the predicted size of haloacid dehalogenase from Bacillus cereus IndB1. Importantly, a one-pot purification set-up using Ni-NTA affinity column chromatography had successfully increased the specific activity of the purified enzyme by 12-fold compared to its crude enzyme extract. Such an increase in activity was obtained particularly at higher concentration of imidazole as the eluent. The reported findings are useful to further utilize the dehalogenase from local strain as an efficient catalyst for MCA biodegradation as well as for fine chemical synthesis.

ACKNOWLEDGMENT

The authors would like to thank Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Indonesia in facilitating this research.

REFERENCES

Fetzner, S., and Lingens, F. (1994), Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications, Microbiological Reviews, 58(4), pp. 641–685.

Roze, E., Meijer, L., Bakker, A., Van Braeckel, K. N. J. A., Sauer, P. J. J, and Bos, A. F., (2009), Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age, Environmental Health Perspectives, 117(12), pp. 1953–1958.

European Commision (2005), Monochloroacetic acid: Summary risk assessment report, Institute for Health and Consumer Protection, The Netherlands.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., and Naidu, R. (2011),
Recombinant Production and One-Pot Purification ...

Bioremediation approaches for organic pollutants: A critical perspective, *Environment International*, 37(8), pp. 1362–1375.

Ratnaningsih, E. and Idris, I., (2018), Cloning and expression of haloacid dehalogenase gene from *Bacillus cereus* IndB1, *Indonesian Journal of Biotechnology*, 22(2), pp. 55–60.

Bornhorst, J. A., and Falke, J., (2000), Purification of protein using polyhistidine affinity tags, *Methods Enzymology*, 324, pp. 245–254.

Mahmoodi, S., Pourhassan-Moghaddam, M., Wood, D. W., Majdi, H., and Zarghami, N., (2019), Current affinity approaches for purification of recombinant proteins, *Cogent Biology*, 5(1), 1665406.

Bradford, M. M., (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, 72, pp. 248–254.

Kruger, N. J., (2009), The Bradford method for protein quantitation, In: Walker, J.M. (eds) The Protein Protocols Handbook, Springer Protocols Handbook, Humana Press, Totowa, N.J., pp. 17–24.

Aminian, M., Nabatchian, F., Vaisi-Raygani, A., and Torabi, M., (2013), Mechanism of Coomassie Brilliant Blue G-250 binding to cetyltrimethylammonium bromide: An interference with the Bradford assay, *Analytical Biochemistry*, 434(2), pp. 287–91.

Bergmann, J. G., and Sanik, J., (1957), Determination of trace amounts of chlorine in naphtha, *Analytical Chemistry*, 29, pp. 241–243.

Birnboim, H. C., and Doly, J. (1979): A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA, *Nucleic Acids Research*, 7(6), pp. 1513–1523.

Rosano, G. L., and Ceccarelli, E. A., (2014), Recombinant protein expression in *Escherichia coli*: Advances and challenges, *Frontiers in Microbiology*, 5(172), pp. 1-17.

Rosano G. L., Morales E. S., Ceccarelli, E. A., (2019), New tools for recombinant protein production in *Escherichia coli*: A 5-year update, *Protein Science*, 28(8), pp. 1412-1422.

Ma, Y., Lee, C. J., and Park, J. S., (2020), Strategies for optimizing the production of proteins and peptides with multiple disulfide bonds, *Antibiotics*, 9, pp. 541.

Schein, C. H., and Noteborn, M. H. M., (1988), Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature, *Biotechnology*, 6, pp. 291–294.

Winograd, E., Pulido, M.A., and Wasserman, M., (1993), Production of DNA-recombinant polypeptides by Tac-inducible vectors using micromolar concentrations of IPTG, *Biotechniques*, 14, pp. 886–887.

Hengen, P., (1995), Purification of His-tag fusion proteins from *Escherichia coli*, *Trends in Biochemical Sciences*, 20(7), pp. 285–286.

Adamu, A., Wahab, R. A., Aliyu, F., Aminu, A. H., Hamza, M. M., and Huyop, F., (2020), Haloacid dehalogenases of *Rhizobium* sp. and related enzymes: Catalytic properties and mechanistic analysis, *Process Biochemistry*, 92, pp. 437–446.

Torz, M., and Beschkov, V., (2005), Biodegradation of monochloroacetic acid used as a sole carbon and energy source by *Xanthobacter autotrophicus* GJ10 strain in batch and continuous culture, *Biodegradation*, 16(5), pp. 423–33.

Ang, T. F., Maiangwa, J., Salleh, A.B., Normi, Y. M., and Leow, T.C., (2018), Dehalogenases: from improved performance to potential microbial dehalogenation applications, *Molecules*, 23, pp. 1100.