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# Dehalogenation of Dichlorobenzoates by *Acidovorax* sp. KKS102's beta class Glutathione S-transferase and its Mutants

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***Abstract***

*Glutathione s-transferases (GSTs) are ubiquitous family of enzymes well known for their detoxification function. Several different classes of the enzyme exist with beta class being the one specific to bacteria. Recently, the enzymes were found to exhibit other functions, in particular dehalogenation of some organic compounds. This property could be extremely useful especially in the bioremediation of some organochlorine pollutants. A beta class GST from Acidovorax sp. KKS102 designated as KKS-BphK was previously cloned and characterized. In this research, molecular docking study was first employed to investigate the possibility of binding of the protein to dichlorobenzoates; byproducts of polychlorobiphenyl degradation. The wild type enzyme together with other mutants were expressed using E. coli BL21 (DE3) cells and purified. The dehalogenation function of the enzymes against dichlorobenzoate derivatives was also investigated through chloride ion detection assay. The results of the molecular docking study indicated the possibility of binding of KKS-BphK to these substrates. Both the wild type and the mutants showed dehalogenation function against the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). Furthermore, the enzymes also showed dehalogenation function against 2,4-dichlorobenzoate derivatives. However, in testing the activity of the enzymes toward 2,5-dichlorobenoate and 2,6-dichlorobenzoate, only K107T and A180P mutants showed some activity while the wild type and C10F mutant showed zero activity. The research indicates the usefulness of beta class GST in the dehalogenation of dichlorobenzoates in addition to their known function of dehalogenating monochlorobenzoates.*

Keywords: Glutathione s-transferase, Mutants, Beta class, dehalogenation, dichlorobenzoates.

## INTRODUCTION

Glutathione S-transferases (GSTs) constitute several different classes of enzymes which deals with detoxification, and, in some instances, activation of different classes of substrate through catalytic conjugation with the tripeptide glutathione (GSH) and thereby increasing their solubility and subsequent excretion from the body (Shehu & Alias, 2018). GSTs are classified based on location as cytosolic, mitochondrial, microsomal and bacterial specific fosfomycin resistant proteins. GSTs have the ability to recognize wide repertoire of substrates including drugs, pesticides, herbicides, environmental toxicants/carcinogens and other endogenously produced molecules (Shehu *et al.*, 2019). In bacteria, GSTs also played a role in the detoxification of several types of pollutants and are therefore employed for bioremediation purposes (Shehu *et al.*, 2019).

*Acidovorax* sp. KKS102 formerly known as *Pseudomonas* sp. KKS102 is a biphenyl/polychlorinated biphenyl (PCB) degrading organism isolated from a soil sample containing biphenyl/PCB hydrocarbons in Japan (Ohtsubo*et al.*, 2012). PCBs are synthetic and very stable organic molecules that carry multiple chlorine atoms on a biphenyl carbon skeleton. They have excellent properties and as such they are used industrially for various applications. However, PCB's are among the persistent pollutants that can remain in soil and water bodies for decades (Ponce *et al.*, 2011). Their persistent in the environment necessitated the inclusion of PCBs among the list of chemicals to be eliminated by 2025 in the Stockholm convention (Xu *et al.*, 2013).

Recently, we cloned and characterized a Beta class GST from *Acidovorax* sp. KKS102 which showed a dechlorination function against monochloro benzoates (metabolites generated during PCBs biodegradation) (Shehu & Alias, 2019).

The number of chlorine atoms attached to the biphenyl carbon skeleton determines how difficult a given PCB can be degraded by microbial consortia. Generally, the higher the number of chlorine atom attached to the biphenyl carbon skeleton the more toxic is the PCB congener (Harmon, 2015). Most of the chorine substituent are found at the *para* and *meta* positions and are considered to be less toxic while those substituted at *ortho* positions are considered to be more toxic. In addition to the monochloro benzoates, the dichloro benzoates also appeared to be among the metabolites generated during PCB biodegradation (Adebusoye *et al.*, 2017). Considering the dehalogenation function exhibited by the BphK-KKS against monochloro benzoates, we aimed to investigate the possibility of binding interaction and dechlorination reaction between some dichloro benzoates derivatives and the BphK-KKS by molecular docking studies and chloride ion detection assay.

## MATERIALS AND METHODS

### Materials

**Organism (*Acidovorax sp. KKS102*)**

*Acidovorax* sp. KKS102 (JCM 17234) was supplied by Japan's collection of microorganisms and appropriate instructions were followed to revive the organism.

**Chemicals**

The substrates: 2,4-dichlorobenzoates 2,5-dichlorobenzoates and 2,6-dichlorobenzoates were purchased from Merck Millipore Germany. Molecular biology reagents were purchased from Invitrogen, USA. Other chemicals were purchased from Sigma Aldrich, USA.

### Methods

**Cloning and site directed mutagenesis of BphK-KKS gene**

Cloning of BphK-KKS gene and site-directed mutagenesis of some amino acids was performed as previously reported by Shehu and Zazali, (2019). The pair of primers used to produce all mutant were as follows; for the C10F mutation, forward 5'-CCGGTGCCTT**T**CTCGCTCGCCGTCCACATTGCCTTG-3' and reverse 5'-GAGCGAG**A**AGGCACCGGGGGCGTAGTAGAGCTTC AT-3', for the K107T mutation; forward 5'-CTGCACA**C**GGGCTTCAGCCCCTGGCTGTGGCAC-3' and reverse 5'-GAAGCCC**G**TGTGCAGTTCGGTGCTGACGAAGGTG-3', for the A180P; forward 5'-ACCTGCAG**C**CCTGGATGGCACGCGTGGCGGCCCG CCC -3' and reverse 5'-CCATCCAGG**G**CTGCAGGTGCGGGTAGGCAGTGAG CGGG-3'. All mutations were confirmed by DNA sequencing (Shehu and Zazali, 2019).

**Protein expression and purification of wild type and mutants BphK-KKS**

Both the wild type and all mutant recombinant BphK-KKS were expressed and purified based on the protocol below. About 10ng of the plasmids (pET101 D-TOPO vector) containing the wild type and mutants BphK-KKS were used to transform *E. coli* BL21$^{star}$ (DE3) competent cells by heat shock at 42°C for exactly 30 seconds. Recombinant BphK-KKS was over expressed using BL21$^{star}$ (DE3) competent cells as follows. The transformed BL21$^{star}$ (DE3) competent cells was transferred into 10 mL Luria Bertani (LB) broth containing 100 µg/mL ampicillin and was allowed to grow overnight at 200 rpm and 37°C temperature. 500 mL of LB containing 100 µg/mL ampicillin was then inoculated with the entire 10 mL from the overnight culture. The culture was grown at 37°C with shaking (200rpm) until the optical density $(OD)_{600}$ reaches about 0.5, 1 mM Isopropyl ß-D-1-thiogalactopyranoside (IPTG) was then added to induce the protein expression and the cells were grown for further three hours. The cells were centrifuged at 6000rpm for 12 mins at 4°C to obtain the pellets. The cells were lysed by sonication and centrifuged at 8000 rpm for 60 mins. The supernatant was collected for GST purification.

Protein purification was carried out using Amersham Bioscience AKTA FPLC$^{TM}$ connected to a fraction collector. The purification was done using a GST-trap column. The column was first equilibrated with 25 mM sodium phosphate buffer pH 7.4 and 5 mL of crude lysate was then injected. The flow rate was adjusted to 0.5 mL/min and thereafter the bound protein was eluted using 50 mM GSH.

**Chloride ion detection assay**

Chloride ion detection assay was performed according to the method described by (McGuinness*et al*,. 2007) with some modifications. 900 µl of purified GST was incubated with 50 µl of 10 mM of GSH and 50 µl of 10 mM substrates (CDNB, 2,4-dichlorobenzoates, 2,5-dichlorobenzoates and2,6-dichlorobenzoates) overnight for 16 hours at 100 rpm and 28°C temperature. The reaction was terminated by the addition of 20 µl of 5 M $H_2SO_4$ and 200 µl of 13 mM Hg $(SCN)_2$ in 95% ethanol and 200 µl of 0.25 M $Fe(NH_4)(SO_4)_2$ × $12H_2O$ in 9 M $HNO_3$ were then added to 0.6 ml of the reaction mixture. The absorbance of Fe $(SCN)^{2+}$produced was measured after 5 min at 450nm. Blank containing all the above chemicals with the exception of the enzyme preparation was used to zero the instrument.

The concentrations of the chloride ions released were determined from the known concentration of sodium chloride in the standard curve.

**Protein concentration**

Bradford assay (Bradford, 1976), was used to determine the protein concentration using bovine serum albumin as a standard.

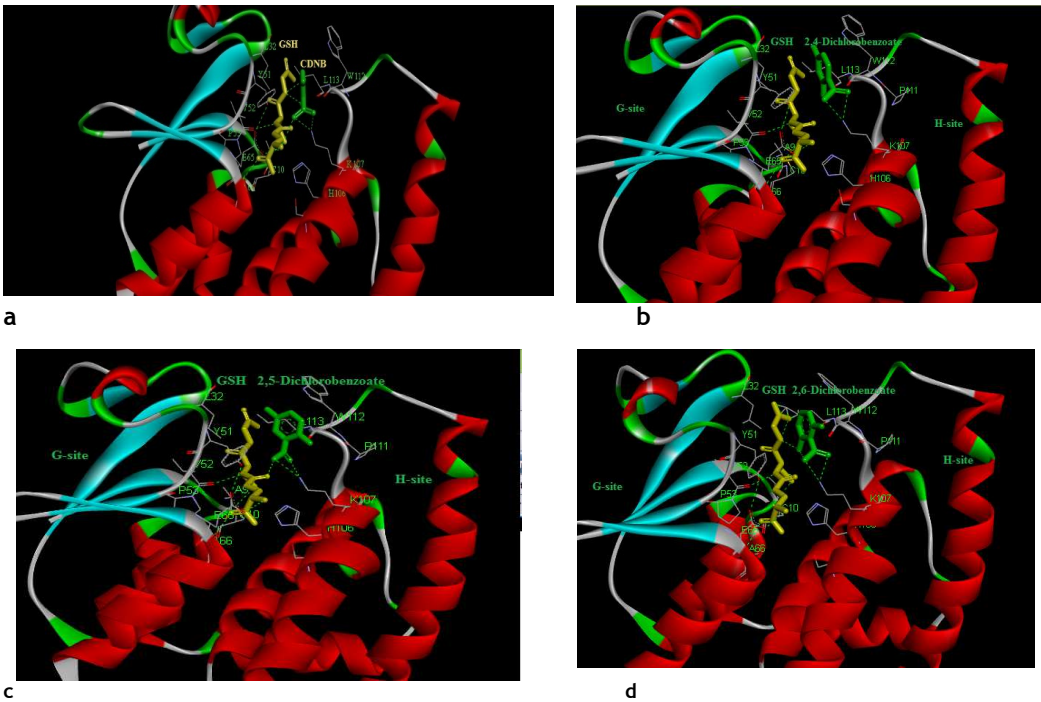
**Statistical analysis**

Analysis of variance (ANOVA) and student's t-test were used to analyze the data. 95% level of confidence level (significance p=<0.05) was use as criterion to determine whether the differences among variances of the data are statistically significant or not. All the data are reported as mean ± SD of three independent experiments using different enzyme preparations.

**Molecular docking studies**

Freely accessible software autodock 4.2 was used for the molecular docking studies (Goodsell*et al.*, 1996). The three-dimensional structure of KKS-BphK was built using SWISS MODEL (https://swissmodel.expasy.org/), because the crystal structure of the protein is not available (Waterhouse et al., 2018). The selected KKS-BphK model was built using a crystal structure of glutathione s-transferase from *Bulkhoderia xenovorans* strain LB400 known as BphK (PDB code: 2gdr.1). The protein shares 48% sequence similarity with KKS-BphK. The quality of the built model was assessed using global QMEAN scoring function (http://swissmodel.expasy.org/qmean/cgi/index.cgi) (Benkert *et al*, 2011). The structures of all ligands under study (mol2 file) were constructed and optimized using Chemsketch software. The mol2 files were then used as input for Open babel software to generate the protein data bank (PDB) file of all the ligands (O'Boyle et al., 2011). The PDF files of both the protein and ligands were used as input files in autodock tools (ADT). Polar hydrogen, Kollman charges and solvation parameters were added to the protein while rotatable bonds for the ligands were defined. The prepared files were saved in the form of pdbqt format. A blind docking analysis was set up using an autogrid size of 126,126 and 126 for the x,y and z-axis respectively. A total of 100 runs was made for each binding site using Lamarckian genetic algorithm as search engine. Finally, cluster analysis was performed on the docked result using a root mean square deviation (rmsd) of 2Å. The docked conformations were visualized using Discovery studio software.

## 4.0 RESULTS AND DISCUSSION




a b

c d

**Figure 1:** Predicted docking orientation of the lowest docking energy conformation of various ligands (a=CDNB b=2,4-dichlorobenzoate, c= 2,5-dichlorobenzoate, d=2,6-dichlorobenzoate) to the binding pocket of KKS-BphK. The co-substrate is shown in yellow ball and stick presentation while the green ones represent the various substrates. The green dash lines represent the hydrogen bondings.

**Table 1**: Summary of the docking results showing the minimum binding energy, amino acids involved, hydrogen bondings and run number.

| Ligands | Amino acids involved in binding | Minimum binding energy (kJ $mol^{-1}$) | Run | Number of Hydrogen bondings |
|---|---|---|---|---|
| CDNB | Ser102, His106, Lys107, Trp112, Leu113 | -6.63 | $52^{nd}$ | 4 |
| 2,4-dichlorobenzoate | Ser102, His106, Lys107, Pro111, Trp112, Leu113 | -6.25 | $71^{st}$ | 2 |
| 2,5-dichlorobenzoate | Ser102, His106, Lys107, Pro111, Trp112, Leu113 | -6.34 | $81^{st}$ | 2 |
| 2,6-dichlorobenzoate | Ser102, His106, Lys107, Pro111, Trp112, Leu113 | -5.87 | $96^{th}$ | 2 |

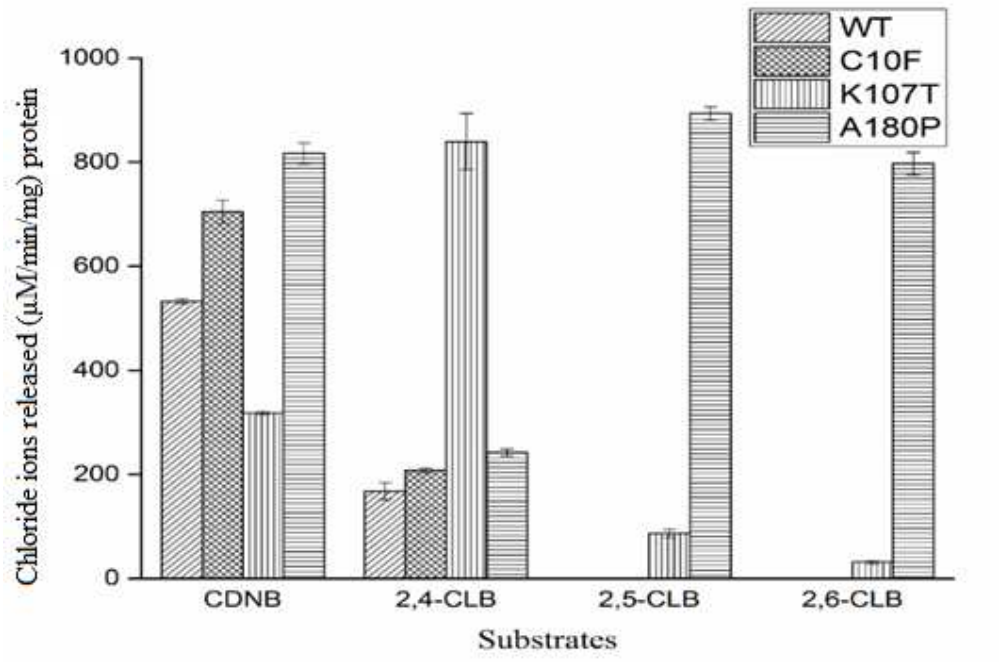



**Figure 2:** Chloride ion detection assay for the substrates CDNB, 2,4-dichlorobenzoate, 2,5-dichlorobenzoate, and 2,6-dichlorobenzoate. The results are mean ± S.D of three independent determinations.

## RESULTS AND DISCUSSION

Molecular docking study is an emerging field that plays a vital role in understanding the interaction between ligands and proteins. The field nowadays plays very important role in environmental sciences by determining the possibility of interaction between various detoxification enzymes and pollutants (Bhatt*et al*., 2020). In the present study, molecular docking was first employed in order to understand the possibility of interaction between some dichloro benzoates and KKS-BphK. All the ligands employed showed possibility of binding with the protein. The results were analyzed using root mean square deviation of 2Å. Most docking programs employ lowest docked binding affinities and top ranked pose as a standard for selecting and ranking of dockings. Cluster analysis of the dichloro benzoate derivatives as well as model substrate CDNB revealed A9, C10, L32, Y51, V52, P53, E65 and A66 as amino acids from KKS-BphK responsible for the binding of co-substrate GSH (Figure 1; a,b,c,d). S102, H106, K107, W112, and L113 were found to be responsible for binding the various ligands with different binding energies (Table 1). Based on the results obtained, CDNB showed the lowest minimum binding energy of -6.63 kJ $mol^{-1}$, which occurred in the $52^{nd}$ run of the cluster containing 11 members. This is followed by 2,5-Dichlorobenzoate with minimum binding energy obtained of -6.34 kJ/mol which occurred in the $81^{st}$ run of the second most populated cluster containing 44 members.

2,4-Dichlorobenzoate had a minimum binding energy of -6.25 kJ/mol, which occurred in the 71st run of the least populated cluster containing 12 members. 2,6-Dichlorobenzoates was found to have the highest minimum binding energy among all the ligands analyzed. The minimum binding energy obtained was -5.87 kJ $mol^{-1}$which occurred in the 96th run of the most populated cluster containing 70 members. In addition to the hydrophobic interactions, at least two hydrogen bonds were predicted between the oxygen atom of all the ligands and Lys107 of KKS-BphK with the exception of CDNB which showed four (4) hydrogen bondings. The molecular docking result showed the possibility of binding of these substrates to KKS-BphK. Based on the criteria of ranking, 2,5-dichlorobenzoate appeared to possess better binding affinity, followed by 2,4-dichlorobenzoate and 2,6-dichlorobenzoate has the least binding affinity. In addition to other hydrophobic interactions, the presence of hydrogen bonding between KKS-BphK and all the substrates further affirmed the possibility of interaction and possible dehalogenation of all the substrates by KKS-BphK.

Chloride ion detection assay was used to measure the activity of purified wild type and mutants (C10F and K107T, A180P) KKS-BphK towards dichlorobenzoate substrates. Moreover, the model substrate for GSTs; 1-chloro-2,4-dinitrobenzene (CDNB) was included for comparison purposes. In the most widely used assay for GSTs, CDNB conjugated with GSH after removal of the chloride ion is measured at 630nm. However, in this assay, we tried to quantify the amount of chloride ion released by the CDNB as well as other substrates instead of the CDNB-GSH conjugate. The activity of KKS-BphK towards CDNB as measured by chloride ion detection showed that the wild type had dehalogenating power of 532.5 ± 14.51 (µM/min/mg protein). A statistically significant increase of 1.32-fold in dehalogenating power was observed in the C10F mutant while for the K107T mutant a statistically significant 1.67-fold decrease was observed compared with wild type. For A180P mutant, a statistically significant 1.53-fold increase in the dehalogenating power against CDNB was observed. C10F showed 1.24-fold and K107T showed a statistically significant 5.0-fold increase in dehalogenating power when compared with wild type. Wild type KKS-BphK and C10F did not posses dehalogenation activity toward 2, 5- dichlorobenzoate and 2,6-dichlorobenzoate, however, K107T and A180P mutants were found to exhibit dehalogenation activity towards the substrates. The specific activities of A180P mutants were found to be 851.45 ± 31.27 µM/min/mg and 782.27 ± 21.23 µM/min/mg towards 2,5-dichlorobenzoate and 2,6-dichlorobenzoate respectively. K107T mutant was also found to display little activity against 2, 5 and 2,6-dichlorobenzoates. 2,5-dichlorobenzoate appeared to be a better substrate with specific activity of 45.11 ± 7.64 µM/min/mg as against 18.22 ± 3.21 µM/min/mg displayed by 2,6-dichlorobenzoate.

Dichlorobenzoates are relatively water-soluble compounds, however, they among the highly recalcitrant compounds because of the scarcity of gene for their biodegradation in microbial population (T'Syen et al., 2018). This is evident from the fact that investigation into the presence of genes for chlorobenzoic acid degraders relative to benzoic acid degraders revealed an astonishing one to a million (Adebusoye *et al.*, 2017; T'Syen *et al.*, 2018). This may not be unconnected with the presence of carbon-chlorine bond which is difficult to break and expose the carbon for further degradation by benzoic acid degraders. This also necessitated the use of microbial consortium in the biodegradation of some recalcitrant compounds. The present data demonstrated the ability of beta class GST to dechlorinate dichlorobenzoate derivative in addition to monochlorobenzoates previously studied (Shehu & Alias, 2019). However, the biodegradation of dichlorobenzoates was shown to be very low when compared with the monochlorobenzoates counterparts previously reported by Shehu and Alias (2019). Furthermore, the present data demonstrated the implication of mutating some amino acids in the binding pocket of KKS-BphK. While the wild type enzyme could not be able to dechlorinate 2,5- and 2,5-dichlorobenzoates, the mutants were able to display a very good dechlorination activity. In biodegradation of chlorobenzoate compounds, the number of chlorine atoms together with their positions are the two determining factors that facilitated their biodegradation (Adebusoye, 2017).

Biodegradation of monochlorobenzoates appeared to be facilitated by various organisms having the capability to break up the carbon chlorine bond and utilize the remining product to generate energy (Mahmoud*et al.*, 2019). While glutathione s transferases are mainly known to be part of the detoxification enzymes, recent studies have shown their usefulness also in the removal of some chlorinated pollutants (Shehu et al., 2019). This is clearly exemplified by the presence of beta class GST in the BphK pathway for the biodegradation of polychlorobiphenyls (PCBs).

The results presented above have shown that beta class GSTs have the potential also in the dehalogenation of dichlorobenzoates, though to some little extent when compared with monochlorobenzoates.

However, having known the facts that biodegradation of PCBs is always accompanied with the generation of dichlorobenzoates, the enzymes would clearly be useful in the complete biodegradation of polychlorobiphenyls and other chlorobenzoates derivatives.

## CONCLUSION

In conclusion, binding interaction between a beta class GST from *Acidovorax* sp. KKS102 and some dichlorobenzoates was investigated in order to ascertain the possibility of interaction between the protein and the ligands. The protein together with some generated mutants were also expressed, purified and their dehalogenation function against some dichlorobenzoate derivatives was determined. The binding interaction clearly showed the possibility of binding of dichlorobenzoates to the beta class GST which was confirmed using the chloride ion detection assay. Furthermore, some of the mutants especially K107T and A180P showed higher versatility in their dehalogenation when compared with the wild type and C10F mutants. The result showed that the enzyme could be useful in the bioremediation of dichlorobenzoate in addition to monochlorobenzoates.

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**Conflict of Interest**

The authors declare no conflict of interest.

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