CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This study is divided into several phases. The first phase was the establishment of single knockout mutants of two *Burkholderia* species, *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 using a suicide vector system. The second phase involved physiological and metabolic characterisation of the resulting mutants. The third phase involved investigation of dibenzofuran degradation by the two wild type strains and their single knockout mutants. The expression for *bphA* gene in the degradation of dibenzofuran through biphenyl degradation pathways was also investigated. The final phase involved the evaluation of the bioremediation on potential single knockout mutant (Figure 3.1).

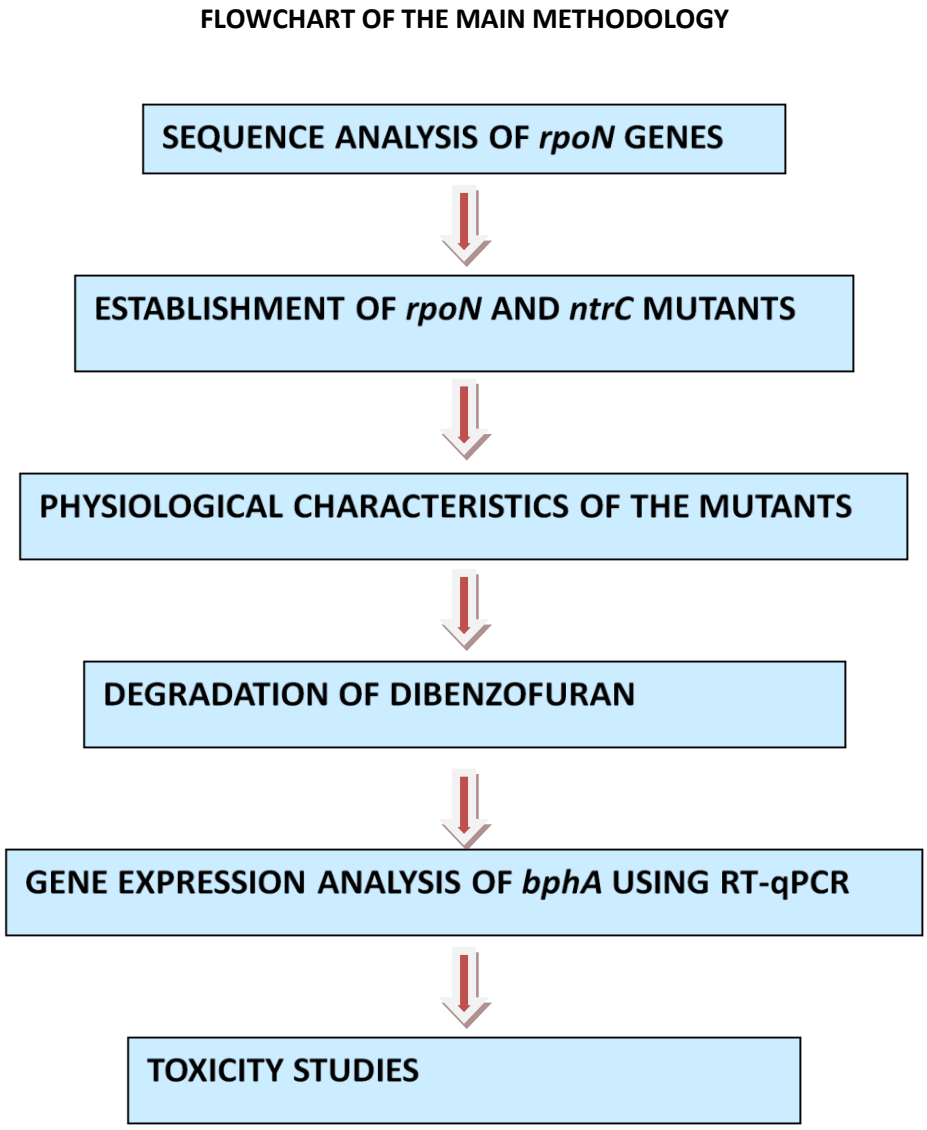

Figure 3.1: Flowchart of the main methodology used in this study

3.2 Bacterial strains.

Two species of *Burkholderia* have been used in developing *rpoN* knockout systems; *B. cenocepacia* strain J2315 and *B. xenovorans* LB400, which were originally isolated from a patient suffering with CF and a PCB-contaminated landfill soil respectively. Three *E. coli* strains were used for plasmid maintenance. *E. coli* strain JM109 was used for molecular cloning in the pGEM-T Easy plasmid (Promega) while *E. coli* BW19851 was specifically used to maintain plasmids with the R6K origin (*ori)* of replication (pKNOCK-cm and pKNOCK-tc; Markey *et al*., 2006). *E. coli* strain DH5α was used for molecular cloning in the pUCP28T plasmid for complementation studies. Table 3.1 and Table 3.2 show bacterial strains and plasmids used in this study and its description respectively.

Table 3.1: Bacterial strains used in this study

Bacterial strain	Description	Reference
Burkholderia xenovorans LB400	Environmental isolate from PCB-containing landfill	Goris *et al*., 2004
Burkholderia cenocepacia J2315	Clinical isolate from patient with cystic fibrosis	Govan *et al.* 1993
E.coli JM109	*recA1, endA1, gyrA96, thi, hsdR17* (rK–,mK+), *relA1, supE44*, Δ(*lac-proAB*), [F´, *traD36*, *proAB*, *lacIqZ*ΔM15]	Promega
E. coli BW19851	*RP4-2(Km::Tn7,Tc::Mu-1, ΔuidA3::pir*$^{+}$ *recA1 endA1 thi-1 hsdR1 creC5107*	CGSC, University of Yale. Metcalf *et al*., 1994
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Taylor *et al*., 1993

Table 3.2: Plasmids used in this study

Vector	Description	Reference
pGEM T Easy	Cloning vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase.	Promega
pKNOCK-cm	Suicide vector contains oriT (origin of transfer) of RP4 and gamma oriV (origin of replication) of R6K; polylinker of pBluescriptII, chloramphenicol resistance cassette	Alexeyev, 1999
pKNOCK-tc	Suicide vector contains oriT (origin of transfer) of RP4 and gamma oriV (origin of replication) of R6K; polylinker of pBluescriptII, tetracycline resistance	Alexeyev, 1999
pUCP28T	Cloning vector	West *et al.*, 1994
pGEM::rpoN$_{J2315}$	pGEM-T Easy vector containing 1500bp fragment of *rpoN* gene from *B. cenocepacia* J2315, Ampicillin resistance	This study
pGEM::rpoNS$_{J2315}$	pGEM-T Easy vector containing 800 bp partial fragment of *rpoN* gene from B. cenocepacia J2315, Ampicillin resistance	This study
pKNOCKcm::rpoNS$_{J2315}$	pKNOCKcm suicide vector containing 800bp partial fragment of *rpoN* gene from *B. cenocepacia* J2315, chlorampenicol resistance	This study
pKNOCKcm::rpoNS$_{LB400}$	pKNOCKcm suicide vector containing 600bp partial fragment of *rpoN* gene from *B. xenovorans* LB400, chlorampenicol resistance	This study

pKNOCKtc::rpoN2S$_{LB400}$	pKNOCKcm suicide vector containing 600bp partial fragment of *rpoN* gene from *B. xenovorans* LB400, tetracycline resistance	This study
pGEM::rpoN$_{LB400}$	pGEM-T Easy vector containing 1500bp fragment of *rpoN* gene from *B. xenovorans* LB400, Ampicillin resistance	This study
pGEM::rpoN2$_{LB400}$	pGEM-T Easy vector containing 1500bp fragment of *rpoN* gene from *B. xenovorans* LB400, Ampicillin resistance	This study
pGEM::rpoNS$_{LB400}$	pGEM-T Easy vector containing 600bp partial fragment of *rpoN* gene from *B. xenovorans* LB400, Ampicillin resistance	This study
pGEM::rpoN2S$_{LB400}$	pGEM-T Easy vector containing 600bp partial fragment of *rpoN* gene from *B. xenovorans* LB400, Ampicillin resistance	This study
pUCP28T::rpoNE$_{LB400}$	pUCP28T cloning vector containing *rpoN* gene of *B. xenovorans* LB400 from fragment with 20bp upstream and downstream of the gene	This study
pUCP28T::rpoN2E$_{LB400}$	pUCP28T cloning vector containing rpoN2 gene of *B. xenovorans* LB400 from fragment with 20bp upstream and downstream of the gene	This study
pUCP28T::rpoNE$_{J2315}$	pUCP28T cloning vector containing *rpoN* gene of *B. cenocepacia* J2315 from fragment with 20bp upstream and downstream of the gene	This study

3.3 Growth and maintenance media.

All *Burkholderia* strains were grown in Luria Bertani (LB) media at 30°C while *E. coli* JM109, BW19851 and DH5α were grown at 37°C in LB supplemented with ampicillin (100µg/ml), chloramphenicol (30µg/ml), kanamycin (30µg/ml), tetracycline (15µg/ml) and trimethroprim (15µg/ml) where appropriate.

Luria Bertani medium

LB media is a nutritionally rich media which can support the growth of most bacteria.

Ingredient	Amount
Tryptone	10g
Yeast extracts	5g
NaCl	5g
Distilled water	to 1000 ml

The ingredients were mixed and the pH was adjusted to pH 7.0-7.5. For solid media preparation, 1.5% of Bacto agar was added. The mixture was then autoclaved at 121°C for 20 min.

M9 minimal media

M9 minimal media was used in nitrogen utilisation assays. Different nitrogen sources were added to determine the utilisation of specific nitrogen by mutant bacterial stains.

5X M9 Salt media

Ingredient	Amount
$Na_2HPO4.7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
Distilled water	to 1000 ml

M9 minimal media

Ingredient	Amount
5X M9 salts media	200ml
1M $MgSO_4$	2ml
1M $CaCl_2$	0.1ml
Distilled water	to 1000 ml

The mixture was autoclaved at 121°C for 20 min. Filter-sterilised (0.22-µm pore size membranes; Millipore) 20% glucose (wt/vol) was added to the mixture as a carbon source and filter- sterilised solutions of different nitrogen sources at 20 mM concentration was added.

3.4 Bioinformatics analysis

The amino acid sequences of *rpoN* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 were obtained from GenBank database through the NCBI webpage. BLAST analysis was performed. The obtained sequences were then realigned using Multiple Sequence Alignment tool, ClustalX2. The alignment of amino acid sequences was followed by construction of phylogenetic tree for the *rpoN* genes of *Burkholderia* spp. using Neighbor-Joining method with Kimura-2 parameter. The distance matrix was also generated from the alignment. The generation of distance matrix and phylogenetic tree were performed using MEGA Version 5.05.

3.5 Establishmnet of the single gene knockout mutants

Single gene knockout mutants of *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 were established using suicide vector pKNOCK series (Alexeyev, 1998). The *rpoN* knock-out in the bacterial chromosome was achieved via homologous recombination and subsequently confirmed by negative and positive PCR screening.

3.5.1 Bacterial DNA extraction

Bacterial DNA from suspension cultures and plate cultures were extracted using QIAamp®DNA Mini kit (Qiagen). For the isolation of genomic DNA from bacterial plate cultures, 1 loopful of bacterial colony was suspended in 180µL of ATL by vigorous stirring while for suspension

cultures, 1mL of bacterial culture was pipetted into a 1.5mL microcentrifuge tube and centrifuged for 5 min at 7500 r.p.m. Subsequently, 180µL of Buffer ATL were then added to the bacterial pellet and suspended vigorously. The ATL mixed samples were then added with 20µL proteinase K and mixed thoroughly and subsequently incubated at 56ºC until the tissue completely lysed. Subsequently, 200µL of Buffer AL were added to the sample and mixed thoroughly to yield a homogeneous solution. The solution was then incubated at 70ºC for 10 minutes. After the incubation, 200µL ethanol (96-100%) were added to the sample and mixed thoroughly. The mixture was then transferred to QIAamp Mini spin column which was placed in 2mL collection tube. The spin column was centrifuged at 8000r.p.m. for 1 minute. The filtrate was discarded and the spin column was placed in a new collection tube followed by addition of 500µL buffer AW1 into the spin column and centrifuged at 8000r.p.m. for 1 minute. Subsequently 500µL buffer AW2 were then added to the spin column and centrifuged at 14,000 r.p.m. for 3 minutes. The QIAamp Mini spin column was placed in a clean 1.5mL microcentrifuge tube before 200µL buffer AE was added. The spin column was then incubated at room temperature for 1 minute followed by centrifugation at 8000 r.p.m. for 1 minute to elute the DNA sample. The DNA sample was stored in -20ºC for further analysis.

3.5.2 Polymerase chain reaction (PCR) amplification

Polymerase chain reaction (PCR) was performed to amplify the selected target fragments of *rpoN* and *ntrC* genes in *Burkholderia xenovoran*s LB400 and *Burkholderia cenocepacia* J2315. Table 3.3 shows the list of primers used in this study. PCR amplification was also utilised for confirmation of RpoN mutants using the combination of primers specially designed to detect the

insertion of suicide vector containing partial target DNA into bacterial chromosome. This will be discussed in later section (Section 3.5.13)

Table 3.3: Primers designed to amplify *rpoN* and *ntrC* genes from *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400.

Primer	Sequence(5-3')	Description
J*Rpo*N-FS J*Rpo*N-R1	5'-ATGAAAGCCAGCCTTCAACTCCGCCTG-3' 5'-TTAAAGAGACTTGCGCAGATTCACTGCCGG-3'	Amplification of *rpo*N gene of *B. cenocepacia* J2315
J*Rpo*N-F2 J*Rpo*N-R2	5'-GAGAACGACGACGAATGGATCGCGA-3' 5'-GGACGGATCGAGACGCAGCAGCT-3'	Amplification of partial *rpo*N gene of *B. cenocepacia* J2315
RpLBF RpLBR	5'-ATGAAAGCCAGCTCCAA-3' 5'-GGTTGACTGCCGGAATCT-3'	Amplification of *rpo*N gene of *B. xenovorans* LB400
RpLBIF	5'-CGAAGCGGACTATGTCGTG-3'	Amplification of partial *rpoN* gene of *B. xenovorans* LB400
Rp2LBF Rp2LBR	5'-ATGCCTTCAATTGAACTACGCACA-3' 5'-TCAAACCTGGCGGCGCAATTC-3'	Amplification of *rpo*N gene from chromosome 2 of *B. xenovorans* LB400
Rp2LBIF Rp2LBIR	5'-CGAATTGACCGAAGACGAGT-3' 5'-GGATCGTATCGAAGCGTTGT-3'	Amplification of partial *rpo*N gene from chromosome 2 of *B. xenovorans* LB400
*Ntr*C-U1F *Ntr*C-R1	5'-CTCACGCTCGCGCAGACGTTCGT-3' 5'-TCAGGGCTCCAGATGGAGCTCCTG-3'	Amplification of *ntr*C gene of *B. cenocepacia* J2315
*Kpn*I-*Ntr*C-F1 *Xba*I-*Ntr*C-R2	5'-GGTACCATTGAGCGGCGACA-3' 5'-TCTAGACTCGAGATTCTGGT-3'	Amplification of partial *ntr*C gene of *B. cenocepacia* J2315
NtLB178F NtLB1514R	5'-GGACTCGAATTGCTGCAAAC-3' 5'-GGCTCGAGATTCAATTCCTG-3'	Amplification of *ntr*C gene of *B. xenovorans* LB400
NtLB591F NtLB1134R	5'-TCCGAAGGATCTGTTGGAAT-3' 5'-CAGGTCCTTGATCTCGATGG-3'	Amplification of Partial *ntr*C gene of *B. xenovorans* LB400

Primer sets (forward and reverse sequences) designed from *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 genome sequences were used to amplify the *rpoN* and *ntrC* genes from both species.

Colony PCR amplification

Colony PCR amplification, using bacterial DNA obtained directly from bacterial colonies grown on agar plates, and PCR amplifications from bacterial DNA isolated by a phenol chloroform procedure were performed in the presence of dimethyl sulphoxide (DMSO) to enhance amplification from *Burkholderia* species DNA which is known to possess a high GC content.

Reaction contents	**Amount**
5X PCR Buffer	10µl
MgCl2	2µl
10mM dNTPs	1µl
Forward primer	4µl
Reverse primer	4µl
DMSO	2.5µl
Taq DNA polymerase	1µl
Sterile distilled water	to 50µl

PCR conditions

PCR amplification conditions were determined empirically for each primer pair and template. Melting temperatures for each primer pair were estimated using PCR primer design tools such as primer3 (Rozen and Skaletsky, 2000). Typically a range of annealing temperatures was chosen near and slightly above the calculated melting temperatures.

Initial denaturation	:	95°C, 5 min	
Denaturation	:	95°C, 1 min	30 cycles
Annealing	:	55°C, 1 min	
Extension	:	72°C, 1min	
Final extension	:	72°C, 5min	

For the confirmation of any particular mutant using colony PCR, the extension period was extended to 4 min. PCR amplicons were analysed by agarose gel electrophoresis.

3.5.3 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis in 1% agarose gels prepared in 1X Tris-Acetate-EDTA (TAE) buffer which was heated to dissolve solid agarose before addition of ethidium bromide (50µg/100ml) on cooling to 55°C. The gel was poured immediately after mixing onto a casting tray and left to set for 45 min. The DNA samples were mixed with 5X loading buffer (50% glycerol, 0.2% bromophenol blue in 1X TAE before being loaded into the wells. Electrophoresis was performed at 80 V in 1X TAE buffer for 2 h. The separated DNA fragments in the gels were then observed and photographed using a UV trans-illuminator and a Gel-Doc system (BioRad).

3.5.4 Recovery of DNA from agarose gels.

The QIAEX II kit (QIAGEN) was used to recover DNA from agarose gels. Following electrophoresis, the desired band was excised from the gel and 300µl of QXI was added for every 100 mg of excised gel prior to adding 10-15µl of DNA binding matrix (QIAEX II). The solution was then incubated at 50°C for 10 min with vortexing at 2 min intervals. The solution was then centrifuged for 30s at 14,000 rpm and 500µl of QXI was used to wash and resuspend the pellet. The suspension was then centrifuged for 30s at 14,000 rpm and 500 PE buffer was used to wash and resuspend the pellet by vortexing. The mixture was centrifuged for 30s at 14,000 rpm, the supernatant discarded and the pellet air-dried for 10-15 min. The pellet was re-suspended in 20µl of sterile-distilled water, re-centrifuged and the supernatant transferred to fresh tube.

3.5.5 Ligation of amplicons for transformation

Purified PCR products were ligated into the pGEM T Easy cloning vector (Promega).

Ligation reaction mixture	
2X rapid ligation buffer	5µl
pGEM T Easy plasmid	1µl
DNA template	3µl
T4 DNA ligase	1µl

Ligation reaction mixture was incubated at 4°C for a minimum of 16 h.

3.5.6 Preparation of competent cells

Overnight cultures of *E. coli* JM109, *E. coli* BW19851 or *E. coli* DH5α cells (1ml) were transferred to 100ml LB and incubated at 37°C until an OD_{600} 0.3 was achieved. Then 50ml of the culture was centrifuged at 10,000 rpm for 5 min at 4°C. The pellet was re-suspended in 25ml cold 30 mM $CaCl_2$ and centrifuged. The pellet was resuspended once more in 5ml cold 30 mM $CaCl_2$ and left on ice for 20 min. The suspension was then centrifuged for 5 min at 5,000 rpm at 4°C and the pellet re-suspended in 1ml cold 30 mM $CaCl_2$ plus 25% glycerol and 100µl aliquots stored at -70°C for later use.

3.5.7 Transformation of DNA into *E. coli* JM109

Competent *E. coli* JM109 cells were left to thaw on ice and 10µl of ligation mixture was added to the tube and the mixture left on ice for 20 min. The cells were then heat-shocked at 42°C for 1 min and placed on ice for a further 5 min prior to the addition of 900µl of LB and incubation at 37°C for 90 min. The culture was centrifuged at 14,000 rpm for 10s and 800µl of the supernatant discarded. The remaining pellet and supernatant were re-suspended and spread onto LB agar Petri plates containing 100µg/ml ampicillin/ 0.5mM isopropyl-ß-D-thio-galactopyranoside (IPTG)/ 80µg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) and were then incubated at 37°C overnight.

3.5.8 Isolation of plasmid DNA using the mini-prep kit

Recombinant white colonies were inoculated into 5ml LB broth containing 100µg/ml ampicillin and incubated overnight at 37°C with shaking at 200 rpm. Following incubation, the bacterial cultures were then centrifuged at 5,000 rpm for 10 min and the pelleted bacterial cells re-suspended in 250µl P1 buffer and transferred to a fresh microcentrifuge tube. The cells were lysed by the addition of 250µl P2 buffer and gentle inversion of the tube before addition of 350µl of neutralisation buffer N3 and mixing. The mixture was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to QIA prep spin column centrifuged at 13,000 rpm for 1 min. The flow through was discarded and the column was washed with 500µl of PB buffer by centrifugation at 13,000 rpm for 1 min. The column was then washed once with 750µl PE buffer

by centrifugation as above and then re-centrifuged for a further 1 min to remove all of the ethanolic solution. The DNA was eluted in 50µl of nuclease free water by centrifugation for 1 min.

3.5.9 Restriction enzyme digestion

The restriction enzyme *Eco*RI was used to digest the plasmid to confirm the presence of DNA insert in putative recombinants.

Reaction mixture	
Buffer H	1µl
*Eco*RI	0.5µl
DNA	2µl
Sterile distilled water	to 10µl

The mixture was incubated at 37°C for 1 h and the products analysed by agarose gel electrophoresis.

3.5.10 DNA Sequencing

The recombinant plasmid DNA was sent to commercial DNA sequencing service (1st BASE, Malaysia) to determine the DNA sequences. The sequence data were analysed, aligned and compared for similarity with global databases using the BLAST *via* the NCBI web server (Altschul *et al.*, 1990), LALIGN programme (Lipman and Pearson, 1985) of the FASTA package from eh.EMBnet.org and MULTALIN (Corpett, 1988).

3.5.11 Ligation of DNA amplicons to the suicide vector pKNOCK

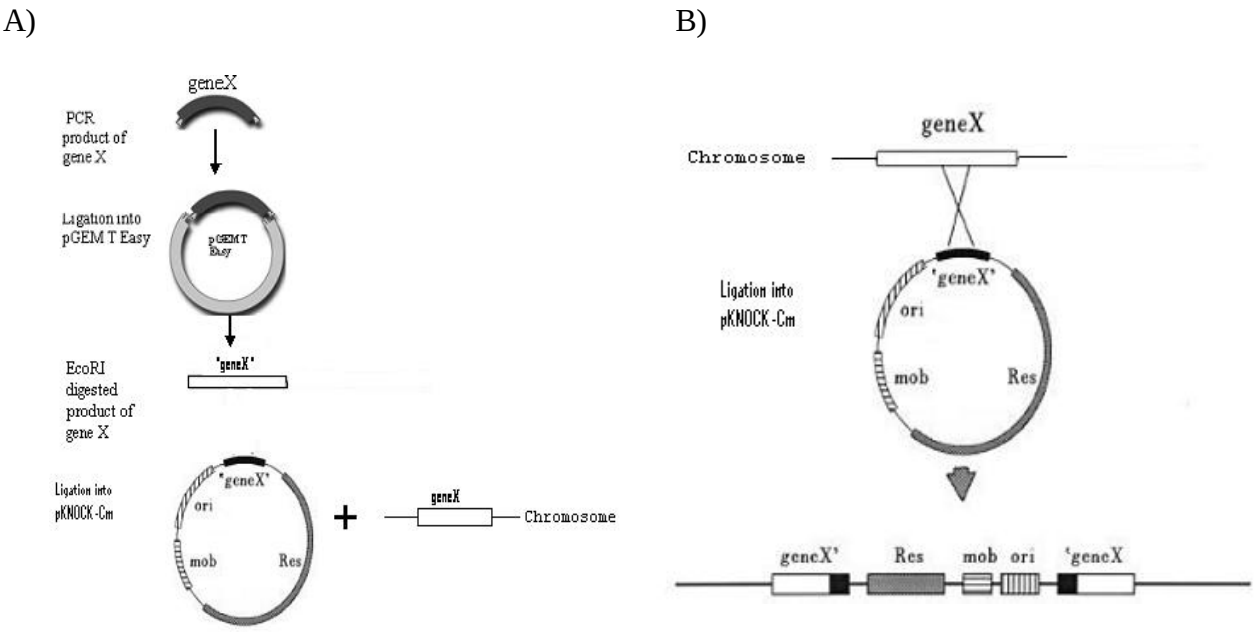

Figure 3.2: General scheme for insertional mutagenesis using the pKNOCK vector. A) DNA fragment is inserted into pKNOCK vector. B) Insertion of recombinant plasmid into *E. coli* BW19851 by transformation.

The suicide vector pKNOCK-Cm or pKNOCK-Tc (Alexeyev, 1999) were used to knock-out selected genes on the *Burkholderia sp* p. chromosome by homologous recombination. Figure 2.1 illustrates the general scheme for insertional mutagenesis using the pKNOCK vector. The pKNOCK-Cm plasmid, which carries a chloramphenicol resistance gene or pKNOCK-Tc plasmid, which carries a tetracycline resistance gene was linearised by *Eco*RI digestion at 37°C for 6 h and the restricted product phosphorylated with 1µl of shrimp alkaline phosphate (SAP) following further incubation for 1 h. The mixture was then incubated at 55°C for 10 min to denature all enzymes and the linear pKNOCK-cm or pKNOCK-Tc was used for ligation to amplicons of interest.

Ligation reaction mixture	
5X ligation Buffer	3µl
PCR generated DNA containing EcoR1 site	9µl
*Eco*R1 restricted and dephosphorylated pKNOCK	2µl
T4 DNA ligase (Promega)	1µl

The ligation mixture was then incubated at room temperature for 1 h and transformed into competent *E. coli* BW19851 cells. Following overnight incubation on LB agar plates supplemented with 30µg/ml chloramphenicol or 25µg/ml tetracycline, transformants were selected and used to produce further overnight liquid cultures as before. Plasmid DNA was then extracted using the mini-prep method and the presence of inserted DNA confirmed following agarose gel analysis electrophoresis of *Eco*RI digestion products.

3.5.12 Biparental conjugation

E. coli BW19851 recombinants (carrying pKNOCK with a DNA insert) and wild type *Burkholderia* species were cultured overnight in LB broth and LB broth supplemented with chloramphenicol or tetracycline respectively. Then, 1ml of each overnight culture was then centrifuged at 14,000 rpm for 1 min and the pellets washed with 200µl fresh LB broth. Equal amount of re-suspended *E. coli* BW19851 and wild type *Burkholderia sp* p. cells were mixed and subsequently 20µl of the mixture plated on LB agar and incubated overnight at 37°C. The mixed culture was then re-suspended in 200µl LB broth and spread on LB agar containing 100µg/ml

chloramphenicol or 50µg/ml tetracycline and 50µg/ml kanamycin. Any colonies growing on these plates were assumed to be mutants who were then confirmed as such following colony PCR.

3.5.13 Screening for single gene knockout mutants

Candidate mutants were screened by negative PCR amplification using primers designed to amplify the full length of the gene. Candidate mutants were also screened using PCR amplification with primers designed from the internal sequence of the pKNOCK plasmid for positive identification (Table 3.4). Combination primers of CmF/CmR and OriF/OriR were used in the PCR amplification reaction for the potential mutants which were knocked out using suicide vector pKNOCK-Cm whereas only combination primers of OriF/OriR were used in PCR amplification reaction for the potential mutant with pKNOCK-Tc. The strategy used in screening of candidate mutants is illustrated in Figure 3.3.

Table 3.4: Primers designed to amplify internal region of suicide vector pKNOCK

Primer	Sequence(5-3')	Description
CmF	5'-TGTCGGCAGAATGCTTAATG-3'	Internal primer within chloramphenicol cassette of pKNOCK-Cm
CmR	5'-CCAGCTCACCGTCTTTCATT-3'	
OriF	5'-TACTTCACCTATCCTGCCCG-3'	Internal primer within oriT- oriV of pKNOCK vector
OriR	5'-CGTTACATCCCTGGCTTGTT-3'	

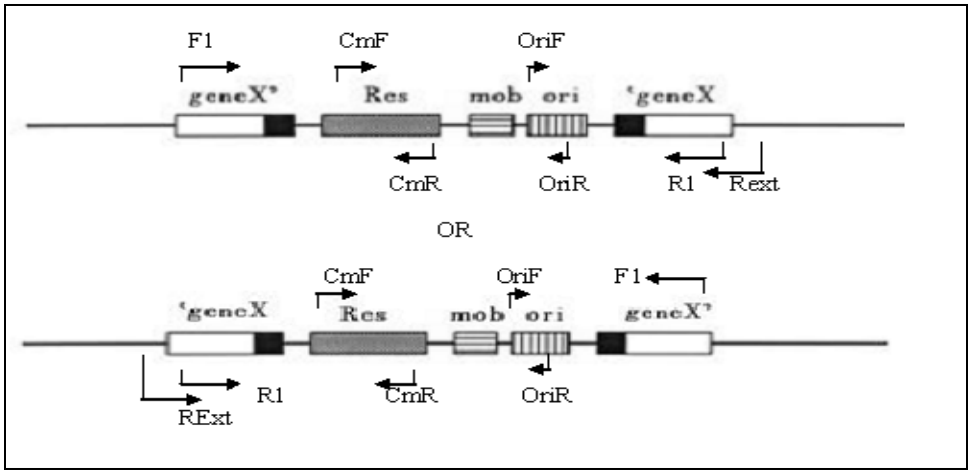

Figure 3.3: Combination of primers used for the confirmation of potential mutants.

3.5.14 Construction of *rpoN*-complementing plasmid

The complete *rpoN* genes and 20bp upstream and downstream of the sequences were PCR amplified from *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 chromosomal DNA with primer pairs R1LxF/R, R2LxF/R and RpoN-F3/RpoNR, respectively. The PCR amplicon were cloned in pGEM T Easy, digested with *Eco*RI and then inserted into the plasmid pUCP28T at a unique *Eco*RI site to produce plasmid p28TRLB (pUCP28T::rpoNE$_{LB400}$), p28TR2LB (pUCP28T::rpoN2E$_{LB400}$), p28TFR3 (pUCP28T::rpoNE$_{J2315}$). The sequence of the insert DNA in p28TRLB, p28TR2LB and p28TFR3 were verified prior to co-transformation with helper plasmid pUCP28T (Schwiezer *et al*., 1996) into *rpoN1*LB400, *rpoN2*LB400 and *rpoN*J2315 respectively by electroporation (MicroPulser, BioRad) at 1.8kV in electroporation buffer (1mM MgCL2, 1mM HEPES, pH 7.0) (Yu *et al*., 2007). Transformants were selected on solid LB plates containing chloramphenicol (300µg/mL) or tetracycline (50µg/mL) and trimethoprim (500µg/mL) and maintained in liquid cultures with both antibiotics at a final concentration of 200µg/mL.

3.6.1 Nitrogen utilisation assay

Wild-type *Burkholderia* species and mutants were grown in LB broth at 37°C to an OD_{600} of 0.4-0.6 was reached. The cells were washed twice with M9 minimal medium and the cell densities equalised to OD_{600nm} 0.005. The cells were then sub-cultured into fresh M9 medium containing glucose and thiamine supplemented with different nitrogen sources (20 mM) which included asparagine, histidine, alanine and glutamine, ammonia in the form of ammonium chloride and nitrate in the form of sodium nitrate. Cultures were incubated at 37°C with shaking at 250 rpm until an OD_{600} of 1 or saturation was achieved. Growth rate during the exponential phase was calculated based on equation:-

$$\text{(Growth rate) } \mu = \frac{\ln O.D_2 - \ln O.D_1}{t_2 - t_1}$$

3.6.2 Biofilm formation assay

Glass tubes (16mm X 100mm) containing 500µl LB medium were inoculated in triplicate with the individual strains suspended at an OD_{600} of 0.005 and incubated at 37°C under static conditions for 24h. Planktonic bacteria were discarded, and the tubes were gently rinsed with 1 ml of water. Crystal violet was added (800µl of a 1% [wt/vol] solution), and the mixture incubated at room temperature for 1 min the tubes rinsed three times with water and dried. The dye was then dissolved with 1 ml of 100% methanol. The absorbance of the solubilised crystal violet was determined at 540 nM with spectrophotometer (Saldias *et al.* 2008).

3.6.3 Motility assay

Bacterial motility was analysed with motility agar plates (1% [wt/vol] LB, 0.3% [wt/vol] agar) inoculated with 2 µl of culture at an OD_{600} of 2 and incubated at 37°C. The diameter of the motility zone was measured every 24 h for 48 h (Saldias *et al.* 2008).

3.7 Biodegradation studies

Degradation of furan was conducted using the simplest member of furans group, non-chlorinated furan (dibenzofuran) CxHxOx. Optimisation of growth parameters such as concentration of dibenzofuran, incubation temperature and pH of the media were conducted in 250mL baffled flask.

3.7.1 Utilisation of dibenzofuran

Burkholderia cenocepacia J2315 and *Burkholderia xenovorans* LB400 were grown in LB broth at 37°C to an OD_{600} of 0.4-0.6. The cells were washed twice with minimal media (M9) and the cell densities equalised. The washed cells were then subcultured into 150ml fresh M9 media containing dibenzofuran as sole carbon source. Cultures were incubated at 37°C with shaking at 250 rpm until an OD_{600} of 1 or saturation was achieved. Several concentration of dibenzofuran was used for optimization of its utilisation (1µg/mL, 5µg/mL and 10µg/mL). Furthermore, the optimal temperature was also determined using baffled flask. *Burkholderia* isolates were also cultured in 150mL M9 media with 5µg/mL dibenzofuran at temperatures of 30ºC, 37ºC and

42ºC. Different pH level at pH 5.0, pH 7.0 and pH 9.0 were used to optimise the optimal growth of bacterial cell both for wild type and mutants. Different intervals at 5, 17, 24, 48, 72, 96, 120, 144 hours were measured.

3.7.2 Extraction of dibenzofuran

Reverse phase solid phase extraction (SPE) is considered as simplest alternative to conventional methods such as Soxhlet and liquid-liquid extraction to separate analytes of interest from aqueous matrix interferences. The extraction of dioxin was conducted using SPE ENVI-18 (Supelco), a silica-based hydrophobic octadecyl bonded (C18) sorbent which is a valuable tool for extraction of dioxin-like compounds and other POPs. SPE ENVI-18 was conditioned using 2mL Acetonitrile followed by 2mL distilled water with flow rate of 20mL/min. the sample was then added to the SPE column and passed through the column at 10mL/min. Subsequently, the column was washed with 3mL distilled water twice before adding 2mL of methanol to help the column to dry faster. The column was left to dry under vacuum for 1 hour. After drying, the glass tube was placed in the vacuum chamber for sample collection. The sample retain within the silica in SPE column was eluted using 6mL hexane. The sample was then concentrated to 2mL using gentle blow of nitrogen gas. The concentrated sample was ready to be quantified using GC/MS.

3.7.3 Standard curve

Standard curve was generated using external standard with different concentration of dibenzofuran; 5µg/mL, 10µg/mL, 15µg/mL and 20µg/mL in hexane. R^2 was obtained for the linear graph using Agilent MassHunter Quantitative analysis software.

3.7.4 Qualitative and Quantification of dibenzofuran by Gas Chromatography-Mass Spectrometry (GC/MS)

Dibenzofuran was quantitated by Agilent 7000B Triple Quadrupple GC/MS/MS system. A fused-silica capillary column, HP-5MS of 60m x 0.25mm i.d. 0.25µm film thickness (Agilent technologies) was used for ion separation. The GC oven temperature used for the column was programme from 40ºC (1 min hold) to 250ºC at 4ºC/min and held for 15 min. The injection port and transfer line temperatures were at 250ºC, and the helium carrier gas was at 30cm/s liner velocity in a split injection mode (1:40). The qualitative and quantitative analyses were conducted using MassHunter Workstation application (Agilent Technologies).

3.8 Expression analysis of *bphA* gene of *Burkholderia xenovorans* LB400

The transcription of *bphA* gene that involves in degradation of dibenzofuran was demonstrated to be initiated by alternative sigma-54 (encoded by *rpoN* gene). Furthermore, the level of alternative sigma-54 (σ^{54}) might also affect the expression of σ^{54}-dependent *bphA* gene.

3.8.1 Extraction of total RNA

Bacterial cultures with or without dibenzofuran as supplement in Minimal media M9 were centrifuged at 14,000 rpm for 10 seconds. The extraction of total RNA was perfomed using Nucleospin®RNA II kit (Macheney-Nagel). The pellets were resuspended in 100µL TE buffer containing 1mg/mL lysozyme by vigorous vortexing and incubated at 37ºC for 10 minutes. To lyse the cells, 350µL Buffer RA1 and 3.5µL β-mercaptoethanol were added to the suspension and vortexed vigorously. The mixture was then applied into NucleoSpin filter which was placed in 2mL collection tube and centrifuged for 1minute at 11,000xg to reduce viscosity and turbidity of the solution. Subsequently, the RNA binding conditions was adjusted by addition of 350µL 70% ethanol to the lysate. The NucleoSpin Column was placed in a collection tube. The lysate was pipetted 2-3 times up and down and subsequently loaded into the column. The lysate was then centrifuged for 30 seconds at 11,000 x g. The NucleoSpin column was placed in new collection tube and 350µL MDB (membrane Desalting Buffer) was added and centrifuged at 11,000 x g for 1 minute to dry the membrane. After the membrane dry, 95µL DNase reaction micture was directly applied onto the centre of the silica membrane of the column and incubated at room temperature for 15 minutes. Three washing steps were performed to wash and dry the silica membrane. First wash was done using 200µL Buffer RA2 which was added to the NucleoSpin RNA II column and centrifuged for 30 seconds at 11,000 xg. For the second wash, 600µL Buffer RA3 was added to the column and centrifuged for 30 seconds at 11,000 x g. The flow-through was discarded and the column was placed into the new collection tube. The third step of washing was performed by adding 250µL Buffer RA3 and centrifuged for 2 minutes at 11,000 x g to dry the membrane completely. The column was then placed into a nuclease-free

collection tube. The RNA was then eluted with 40µL RNase-free water and centrifuged at 11,000 x g for 1 minute.

3.8.2 Synthesis of cDNA

Synthesis of cDNA from total RNA was conducted using iScriptTM cDNA synthesis kit (BioRad). iScript synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. It can produce excellent results in both real time and conventional RT-PCR. The mixture was prepared as recommended by manufacturer.

Component	Volume per Reaction
5X iScript reaction mix	4µL
iScript reverse transcriptase	1µL
Nuclease-free water	10µL
RNA template	5µL
Total volume	20µL

Reaction protocol

The mixture was incubated as follows:-

25°C for 5 minutes

42°C for 30 minutes

85°C for 5 minutes

Hold at 4°C before storage.

3.8.3 Real Time PCR for gene expression analysis

The degradation of xenobiotic compounds by microorganisms is from the reaction of specific enzymes excreted during the process. The production of the enzyme is a result of expression of the gene that involved in the degradation pathway. This study aimed to determine if the *bphA* gene that responsible for dibenzofuran degradation can be expressed when RpoN was inactivated. Quantitative PCR is able to measure the expression of these genes based on its expression from mRNA. Quantitative PCR was conducted using CFX96 Real-Time PCR Machine (BioRad). The primers and probes used in this study were listed in Table 3.5.

Table 3.5: Primers and probes used in gene expression analysis using RT-qPCR.

Primer	Sequence
bphAF	5'-TGCAACTGGAAGTTTGCCGCCG-3'
bphAR	5-'TGATTGCCCTTGGTGGGTATCTGCG-3'
gyrBF	5'-TGCTTCACGAACAACATTCCGCAGC-3'
gyrBR	5-'TTGTTCAACACGCGCGTCATCGC-5'
Probe	Sequence
bphA Cy5 probe	5'-/5Cy5/TCCTGGCGGGCATTCCGCCGGAAAT/3IAbRQSp/-3'
gyrB FAM probe	5'-/56-FAM/TGGGACGCACCTGACCGGATTGCGT/3IABkFQ/-3'

The PCR mixture was prepared using SsoFastTM Probes Supermix. SsoFast Probes Supermix is a 2x concentrated ready-to-use cocktail for probe based real time quantitative PCR. The Reaction mixture was prepared as follows:-

Component	Volume per reaction (µL)
SsoFast probes supermix	10
Forward primer	1
Reverse primer	1
Fluorogenic probe	1
RNase/DNase-free water	2
DNA template	5
Total	20

Cycle condition for qPCR for BioRad CFX96 Real Time PCR System

Cycling Steps	Temperature	Time	Cycle Number
Enzyme activation	95°C	30 sec	1
Denaturation	95°C	5 sec	30-40
Annealing/Extension	60°C	1-10 sec	

3.9 Phytotoxicity studies for evaluation of bioremediation potential of the single gene knockout mutants

Dioxins and furans which include dibenzofuran were known for their toxicity towards human, environment, plants and animals. The toxicity test of the dibenzofuran was performed using Phytotestkit (MicroBioTest Inc.). In this study, seeds of three terrestrial plants, *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba* were exposed to dibenzofuran and the end-product from biodegradation of dibenzofuran by *Burkholderia xenovorans* LB400 and its *rpoN* mutants (NRPLB and NRP2LB). The seeds were washed with 10% sodium hypochlorite solution for 10 minutes (USEPA, 1996) and dried prior to be used in the toxicity test in order to ensure sterility. The seeds were then hydrated with water for 60 minutes to prepare the seed for germination.

For optimisation purpose, the seeds were exposed to water and dibenzofuran for 30 minutes, 90 minutes, 3 hours and 24 hours. The test plate was placed with foam pad and white filter paper and subsequently filled thoroughly with 10 mL water. The black paper filter was then placed on the top of hydrated white filter paper and 5 ml water was thoroughly added on top of the black paper. The seeds were then placed on top of the hydrated black filter paper in one row at equal distance of each other. The seeds were placed near the top of the filter paper, at about 1cm of the middle ridge of the test plate.

For the toxicity test, the seeds were exposed to 5 different treatments:-

1. 60 minutes of hydration+ 24 hours of exposure to water (negative control).
2. 60 minutes ofhydration + 24 hours exposure to of 5µg/mL dibenzofuran in M9 minimal medium
3. 60 minutes of hydration + 24 hours exposure to of product/metabolite from dibenzofuran degradation by wildtype *Burkholderia xenovorans* LB400.
4. 60 minutes of hydration+ 24 hours exposure to of product/metabolite from dibenzofuran degradation by NRPLB (*rpoN1* mutant)
5. 60 minutes of hydration + 24 hours exposure to of product/metabolite from dibenzofuran degradation by NRP2LB (*rpoN2* mutant)

The cover of the test plate was carefully placed starting from the middle of the test plate to avoid the seeds to move from its original position. The test plate was then placed in vertical position in the cardboard holder. The test plate was then incubated at 25°C for 3 days in the dark.

3.9.1 Image recording and growth measurement.

The pictures of the test plates were captured at the end of the incubation period using digital camera. The test plate was placed horizontally on a flat surface directly under the camera where the camera was mounted on a table top tripod stand. The image was then processed using image tools software for the length measurement. Calculation of the percentage effect of the chemical on seed germination and root and shoot growth was calculated as following:-

$$([A - B]/ A) \times 100\%$$

: A= average number of germinated seeds/ average root or shoot length in the control.

B= average number of germinated seeds/ average root or shoot length in the treatment.

3.10 Statistical analysis

The data were statistically analysed for their significant differences using one way analysis of variance (ANOVA) at $p = 0.05$. All statistical analyses were performed with Statistical Package for the Social Sciences program (SPSS 20.0.0).