CHAPTER 8

CONCLUDING REMARKS

8.1 Introduction

The main focus of this research is to gain insights into the role of alternative sigma factor-54 (RpoN) of *Burkholderia xenovorans* LB400 in degradation of dibenzofuran via biphenyl degradation pathway by utilising the biphenyl dioxygenase (BPDO) encoded by *bph* genes.

Sequence analysis of the *rpoN* genes using bioinformatics tools revealed that *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 is not closely related (58.2%) therefore both genes may evolved differently and possibly transcribed independently. This study had also showed that the single copy of the *rpoN* gene of *Burkholderia cenocepacia* J2315 is closely related (90%) to *rpoN1* gene of *Burkholderia xenovorans* LB400. However, the *rpoN* gene of *Burkholderia cenocepaci*a J2315 is located in different cluster to *rpoN1* of *Burkholderia xenovorans* LB400 (*rpoN* gene of *Burkholderia cenocepacia* J2315 was clustered in the Bcc group while *rpoN1* gene of *Burkholderia xenovorans* LB400 was clustered in soil and rhizospheric group). This clearly demonstrates the difference of environmental isolated *Burkholderia* from pathogenic *Burkholderia* species.

Single-gene knockout mutants of the *rpoN* gene were established using pKNOCK suicide vector series resulting in two *rpoN* mutants of *Burkholderia xenovorans* LB400 [NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant)] and one *rpoN* mutant of *Burkholderia cenocepacia* [NRPJ (*rpoN* mutant)]. In addition, the gene encodes for NtrC which involved in nitrogen assimilation in both *Burkholderia* species were also obtained namely NTLB (*ntrC* mutant of

Burkholderia xenovorans LB400) and NTJ (*ntrC* mutant of *Burkholderia cenocepacia* J2315). The mutants were successfully screened using negative and positive PCR amplification and DNA sequencing.

In order to determine the differences between the wildtype strains and their mutants, physiological and metabolic response analyses were conducted. Two physiological analyses were conducted (biofilm formation and motility test) on all the mutants. Physiological responses revealed that *rpoN1, rpoN2* and *ntrC* genes of *Burkholderia xenovorans* LB400 were not involved in biofilm formation. Inactivation of *rpoN1* gene reduced the motility of *Burkholderia xenovorans* LB400 suggesting the involvement of *rpoN1* gene in motility. In *Burkholderia cenocepacia* J2315, inactivation of *rpoN* and *ntrC* genes reduced the biofilm formation but not motility suggesting the involvement of *rpoN* gene in motility but not in biofilm formation. However the reduced formation of biofilm and motility in NNTJ (*ntrC* mutant of *Burkholderia cenocepacia* J2315) was unexpected as NtrC was only known for its involvement in nitrogen assimilation but not in biofilm and motility of the bacteria.

Metabolic response analysis revealed that utilisation of nitrogenous compounds was affected with inactivation of *rpoN* and *ntrC* genes of *Burkholderia xenovorans* LB400 indicating their involvement in nitrogen utilisation. However the growth of *Burkholderia xenovorans* LB400 and its mutants were not observed when supplemented with nitrate as sole nitrogen source. This might due to the difficulties of the *Burkholderia xenovorans* LB400 in utilising inorganic nitrogenous compounds such as nitrate. For *Burkholderia cenocepacia* J2315, both *rpoN* and *ntrC* genes were observed to play important role in nitrogen utilisation. The altered physiological

and metabolic responses shown by the mutants indicate the successfulness of the procedure for the establishment of the *rpoN* and *ntrC* mutants.

Biodegradation analysis revealed that *Burkholderia xenovorans* LB400 is a potential dibenzofuran degrader. Wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were able to utilised dibenzofuran as sole carbon and energy source. However, better growth and degradation was observed in NRP2LB (*rpoN2* mutant) compared to wildtype *Burkholderia xenovorans* LB400. This result indicates the involvement of *rpoN1* gene in expression of BphA thus enhanced the degradation of dibenzofuran. Furthermore the gene expression study also showed that *bphA* gene was overexpressed in NRP2LB (*rpoN2* mutant) compared to moderate expression by wildtype *Burkholderia xenovorans* LB400.

For bioremediation to be successful and beneficial, the byproducts of biodegradation have to be safe or harmless towards, human and environment. In order to evaluate the bioremediation potential of the single-gene knockout mutants, simple phytotoxicity test using three selected terrestrial plants was conducted. The result showed that all byproducts from biodegradation of dibenzofuran were less toxic compared to the original compound (dibenzofuran). However byproducts from NRP2LB (*rpoN2* mutants) was able to enhance the growth of *Sorghum saccharatum* suggesting that the transformation of dibenzofuran is not toxic and possibly produced a specialised byproducts that could enhanced growth of *Sorghum saccharatum*.

8.2 Possible roles of *rpoN1* and *rpoN2* genes in degradation of dibenzofuran

In this study, the *rpoN1* gene was observed to play an important role in degradation of dibenzofuran as demonstrated in previous chapter. The overexpression of *bphA* gene that involved in first step of breaking the aromatic ring of dibenzofuran was regulated by *rpoN1* gene. Even though the expression of *bphA* gene in wildtype *Burkholderia xenovorans* LB400 during degradation of dibenzofuran was also detected, the expression was moderate. This result suggesting that the regulation of *bphA* gene expression was controlled by the presence of both *rpoN* genes in the wildtype strains. However this phenomenon was not observed when r*poN2* gene was inactivated suggesting the negative autoregulation by RpoN2 towards *rpoN1* gene. As illustrated by Michiels *et al.* (1998), negative autoregulation by RpoN2 can happen when the active site for *rpoN1* gene to initiate the transcription process was occupied by RpoN2 thus inhibit the expression of related genes (e.g. *bphA* gene). This result suggested that by knocking out the *rpoN2* gene in *Burkholderia xenovorans* LB400, the expression of *bphA* gene can be enhanced thus increase the degradation of dibenzofuran. However, further experiments are needed before the solid conclusion can be made for utilisation of NRP2LB in degradation of dibenzofuran.

8.3 Future Directions

The results presented in the thesis indicated the potential of *Burkholderia xenovorans* single-gene knockout mutant NRP2LB (*rpoN2* mutant) in degradation of dibenzofuran due to its high growth rate and degradation capability towards dibenzofuran. Furthermore, the byproducts from

degradation of dibenzofuran by NRP2LB (*rpoN2* mutant) wass less toxic and possibly consist of specialised byproducts that could enhance the growth of *Sorghum saccharatum*.

However, this study is only considered the establishment of single knockout of *rpoN* gene. As the *rpoN1* and *rpoN2* genes showed the autoregulation mechanisms in their functions, further investigation on the relationship between these genes should be conducted. The double-knockout of both *rpoN1* and *rpoN2* genes in *Burkholderia xenovorans* LB400 is needed in order to inactivate both *rpoN* genes. The effects of double-knockout in the mutant towards metabolic and physiological responses need to be investigated.

Following the successful in biodegradation of dibenzofuran which was evaluated by the reduction amount of dibenzofuran, the identification of its degradation byproducts will be very valuable information. The extraction and purification procedures for degradation byproducts need to be established to obtain the pure metabolites and subsequently the identification of the purified metabolites can be conducted using analytical instruments such as Nuclear Magnetic Resonance (NMR) or Gas Chromatography/ (Mass Spectrometry)^2 GC-MS/MS).

The degradation byproducts of dibenzofuran not only can affect the plants but also other living organisms. In order to complement the results obtained from the phytotoxicity test, the toxicity of degradation byproducts from wildtype *Burkholderia xenovorans* LB400 and its single-gene knockout mutants can be further evaluated using different test system such as nematodes, earthworms, cladora and daphnia.