

CHAPTER 6

ANALYSIS OF DIBENZOFURAN DEGRADATION IN WILDTYPE *Burkholderia xenovorans* LB400, *Burkholderia cenocepacia* J2315 AND THE SINGLE-GENE KNOCKOUT STRAINS

6.0 Introduction

Microorganisms are known for their ability to degrade many recalcitrant pollutants and xenobiotic compounds. Dibenzofuran is a member of the Dioxin family that also include dibenzo-p-dioxin and ortho-substituted polychlorinated biphenyls (ortho-PCBs). As dibenzofuran has similar basic structure as PCBs, therefore it can be degraded using the same pathways as PCBs. *Burkholderia xenovorans* LB400 is a well known PCBs degrader utilising biphenyl degradation pathways initiated by biphenyl dioxygenase (BPDO). It was previously described that the biphenyl pathway is not always directly involved in the degradation of biphenyl and PCBs by *Burkholderia xenovorans* LB400 (Parnell *et al.*, 2006). The pathway can also be used to degrade other dioxin-like compounds. Biphenyl dioxygenase, a key enzyme in degradation of dibenzofuran via biphenyl dioxygenase pathway not only catalyses the first enzymatic step of biphenyl catabolic pathway but also oxygenates a range of biphenyl analogs, including PCBs, dibenzofuran and dibenzo-p-dioxin (Mohammadi and Sylvestre, 2006). Using biphenyl dioxygenase of *Burkholderia xenovorans* LB400, dibenzofuran can be transformed via lateral oxygenation resulting in formation of dihydro-dihydroxy-dibenzofuran as major metabolites or via angular oxygenation which produced 2, 2', 3-trihydroxybiphenyl as minor metabolite (L'Abbee *et al.*, 2005). This chapter will investigate the impact of RpoN inactivation towards the ability of two *Burkholderia* species and their single gene

knockout mutants obtained in Chapter 4 to degrade dibenzofuran. As mentioned previously, *Burkholderia xenovorans* are expected to be able to degrade dibenzofuran as reported by L'Abbee *et al.*, (2005), Mohammadi and Sylvestre, (2006) and Nojiri and Omori, (2002). Additionally this chapter will also investigate the potential ability of *Burkholderia cenocepacia* J2315, a clinical isolate, in the degradation of dibenzofuran. Additionally, the impact of RpoN inactivation towards the ability of the NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NRPJ (*rpoN* mutant) in degradation of dibenzofuran will also be investigated. This chapter began with optimisation of the favourable conditions for both bacteria to utilise dibenzofuran as a sole carbon source.

Dibenzofuran is selected for this study since it is the basic structure for Polychlorinated dibenzofuran (PCDFs) and a model compound for degradation of PCDFs. Furthermore, dibenzofuran is less toxic compared to its isomeric compound, dibenzo-p-dioxin which is the basic structure for polychlorinated dibenzo-p-dioxin (PCDDs), whose member included 2,3,7,8-tetrachlorinated dibenzo-p-dioxin (TCDD) that is considered as one of the most toxic chemical.

The optimisation of the concentration of dibenzofuran as sole carbon source is important to ensure the optimum growth of the bacteria while utilising the compound efficiently. If the concentration is too low, bacteria would not be able to multiply as they use the available carbon source to generate energy for cells activity that vital to sustain their survival. However, if the concentration is too high, it could be toxic to the cells or lead to accumulation of metabolites to the level that is toxic to the cells. In addition to the concentration of the compound of interest, environmental parameters such as pH and

temperature are also important for the optimal growth of bacterial cells thus increase the efficiency of the degradation process.

Extraction and purification of dibenzofuran prior to quantification analysis is important. The fastest and simplest method is by using Solid Phase Extraction (SPE). In this chapter, SPE 18-Envi (Supelco), a silica octadecyl sorbent-based column which usually utilised for purification and extraction of PAHs, PCBs and dioxins is used. The correct choice of solvent is also crucial as the filter depends on polarity of the compounds. The extracted dibenzofuran will be analysed using Gas Chromatography with Mass Spectrometry detector equipped with HP-5MS column for separation of the molecules.

After degradation process, the remaining dibenzofuran will be detected and the amount measured. The comparison between the two wildtype *Burkholderia* species and their single gene knockout mutants for the inactivation of *rpoN* genes which might involve in transcription of *bph* gene that encodes for biphenyl dioxygenase, a key enzyme in degradation of dibenzofuran via biphenyl dioxygenase pathway will be determined.

6.1 Effect of dibenzofuran concentration on growth of wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single-gene knockout mutants

Degradation of dibenzofuran was performed in 250mL baffled flask containing 150mL M9 minimal medium supplemented with dibenzofuran as sole carbon source. Bacterial growth was measured towards different concentration of dibenzofuran. In addition, bacterial growth was also observed under different incubation temperatures and pH levels.

Three different concentration of dibenzofuran were supplemented in M9 minimal media as sole carbon source. Figures 6.1 to 6.3 demonstrated that wildtype *Burkholderia xenovorans* LB400 and its mutant derivatives, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were able to grow in M9 minimal media by utilising dibenzofuran as sole carbon and energy source.

The wild-type of *Burkholderia xenovorans* LB400 did not show significant difference in growth rate when supplemented with 1µg/mL, 5µg/mL or 10µg/mL dibenzofuran with growth rate (μ) ranging from 1.45 to 1.47 (Figure 6.1). The NRPLB (*rpoN1* mutant) showed only slight growth compared to the wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) with maximum cell numbers of less than 2000×10^3 cfu/mL (compared to 8000×10^3 cfu/mL for wildtype *Burkholderia xenovorans* LB400 and 11000×10^3 cfu/mL for NRP2LB NRP2LB (*rpoN2* mutant) (Figure 6.2). NRP2LB NRP2LB (*rpoN2* mutant) showed no significant difference when cultured in 1µg/mL,

5 μ g/mL and 10 μ g/mL dibenzofuran with growth rate, $\mu = 0.51$. However NRP2LB NRP2LB (*rpoN2* mutant) cannot sustain its growth after 24 hours in 1 μ g/mL and 10 μ g/mL dibenzofuran, whereas in 5 μ g/mL dibenzofuran, the cell number remain unchanged until 72 hours of incubation and subsequently reduced (Figure 6.3)

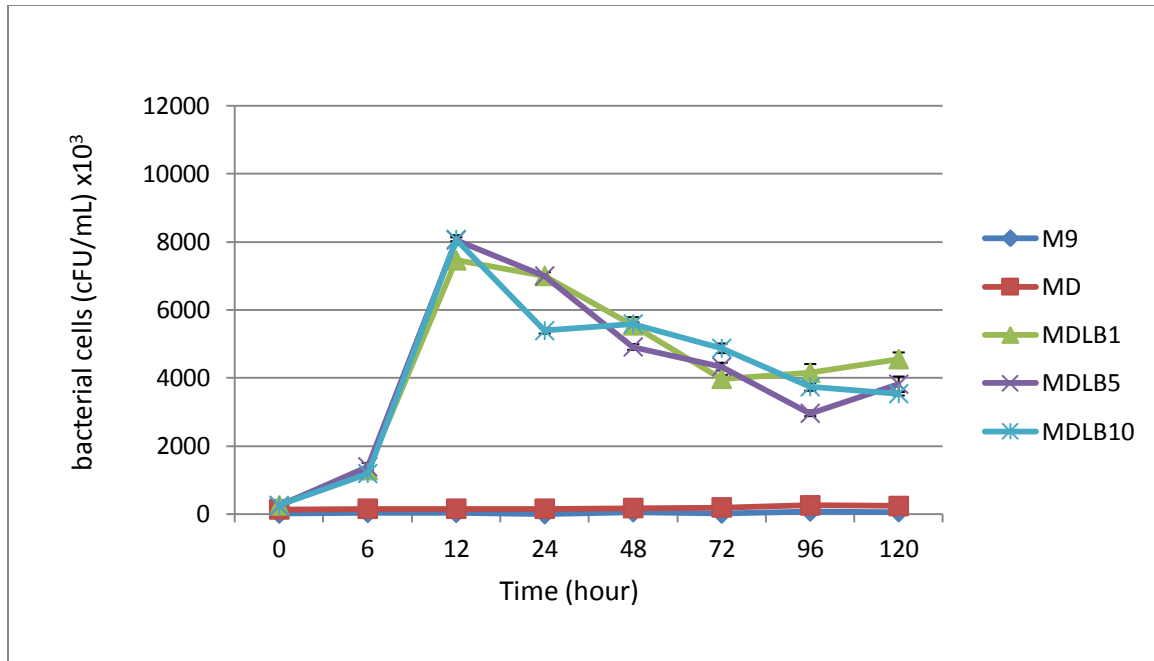


Figure 6.1: Growth of wildtype *Burkholderia xenovorans* LB400 in minimal media supplemented with different concentration of dibenzofuran as sole carbon source. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDLB1: Wildtype *Burkholderia xenovorans* LB400 in 1 μ g/mL dibenzofuran

MDLB5: Wildtype *Burkholderia xenovorans* LB400 in 5 μ g/mL dibenzofuran

MDLB10: Wildtype *Burkholderia xenovorans* LB400 in 10 μ g/mL dibenzofuran

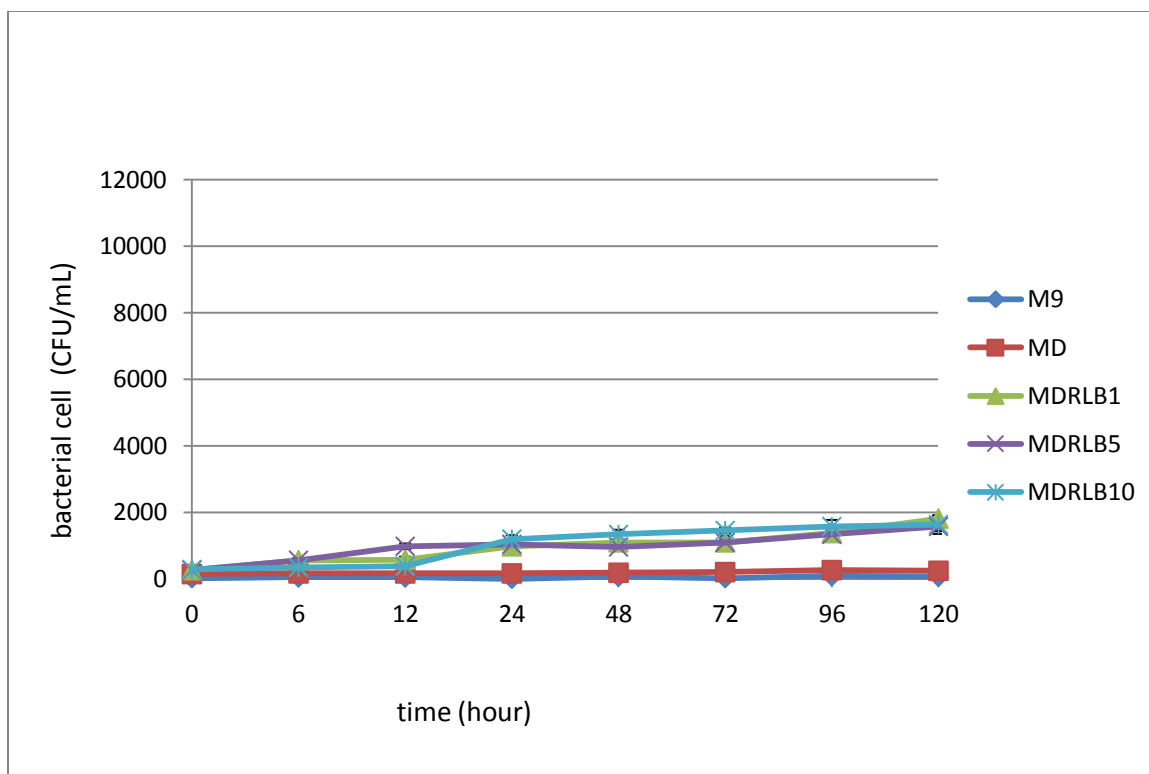


Figure 6.2: Growth of NRPLB (*rpoNI* mutant) in minimal media supplemented with different concentration of dibenzofuran as sole carbon source. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDRLB1: NRPLB (*rpoNI* mutant) in 1 μ g/mL dibenzofuran.

MDRLB5: NRPLB (*rpoNI* mutant) in 5 μ g/mL dibenzofuran

MDRLB10: NRPLB (*rpoNI* mutant) in 10 μ g/mL dibenzofuran

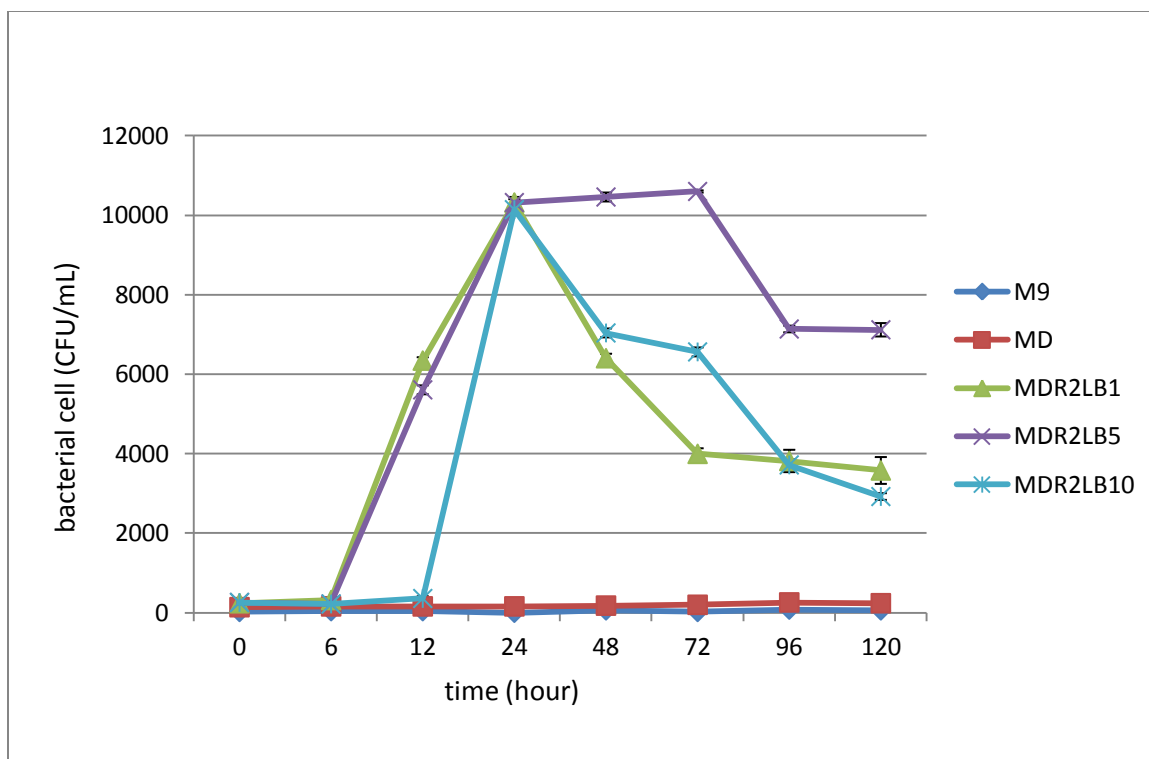


Figure 6.3: Growth of NRP2LB (*rpoN2* mutant) in minimal media supplemented with different concentration of dibenzofuran as sole carbon source. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD : M9 minimal media supplemented with 5 $\mu\text{g}/\text{mL}$ dibenzofuran (control)

MDR2LB1: NRP2LB (*rpoN2* mutant) in 1 $\mu\text{g}/\text{mL}$ dibenzofuran.

MDR2LB5: NRP2LB (*rpoN2* mutant) in 5 $\mu\text{g}/\text{mL}$ dibenzofuran

MDR2LB10: NRP2LB (*rpoN2* mutant) in 10 $\mu\text{g}/\text{mL}$ dibenzofuran

Wildtype *Burkholderia cenocepacia* J2315 showed slight growth when supplemented with dibenzofuran as sole carbon and energy source with maximum cell number achieved was 3000×10^3 cfu/mL approximately. Wildtype *Burkholderia cenocepacia* J2315 showed highest growth rate when supplemented with 5 μ g/mL dibenzofuran ($\mu=1.32$) compared to 1 μ g/mL ($\mu=1.27$) and 10 μ g/mL ($\mu= 1.30$). From the observation, NRPJ (*rpoN* mutant) showed better growth than wildtype *Burkholderia cenocepacia* J2315 in all three different concentrations of dibenzofuran. However, the highest growth rate shown by NRPJ (*rpoN* mutant) was in 5 μ g/mL dibenzofuran ($\mu=1.43$) compared to 1 μ g/mL dibenzofuran ($\mu= 1.40$) and 10 μ g/mL dibenzofuran ($\mu=1.39$). Both wildtype *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant) achieved their highest cell number within 12 hours of incubation (Figure 6.4).

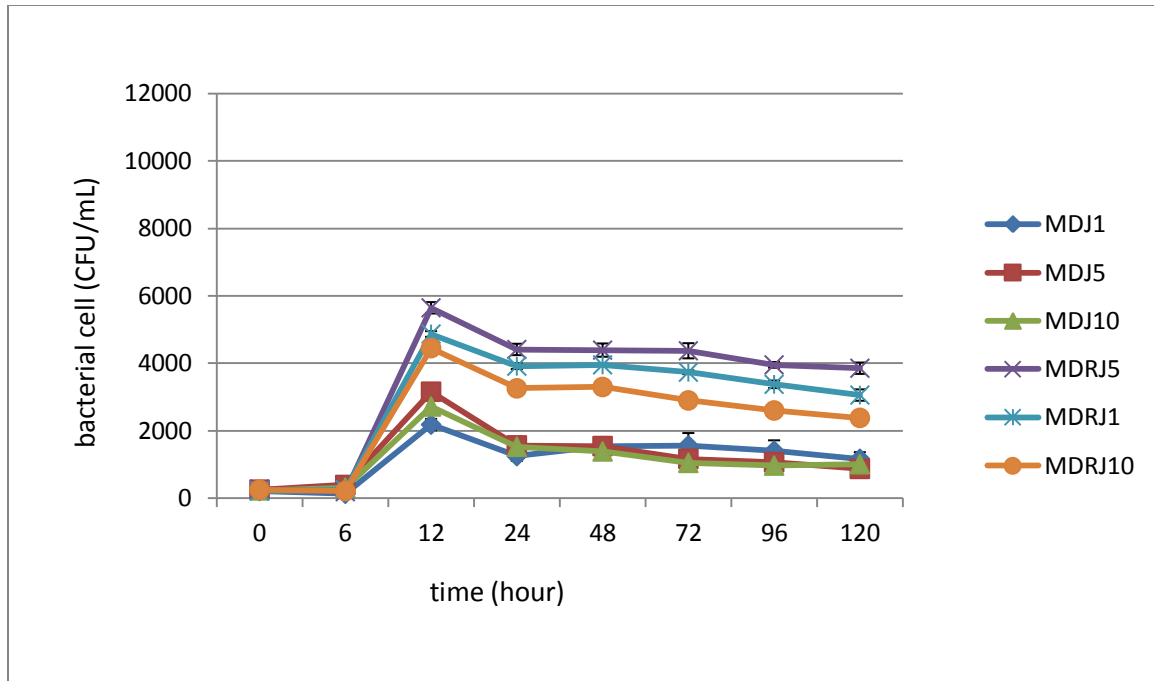


Figure 6.4: Growth of wildtype *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant) in minimal media supplemented with different concentration of dibenzofuran as sole carbon source. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

MDJ1: Wildtype *Burkholderia cenocepacia* J2315 in 1 μ g/mL dibenzofuran

MDJ5: Wildtype *Burkholderia cenocepacia* J2315 in 5 μ g/mL dibenzofuran

MDJ10: Wildtype *Burkholderia cenocepacia* J2315 in 10 μ g/mL dibenzofuran

MDRJ1: NRPJ (*rpoN* mutant) in 1 μ g/mL dibenzofuran

MDRJ5: NRPJ (*rpoN* mutant) in 5 μ g/mL dibenzofuran

MDRJ10: NRPJ (*rpoN* mutant) in 10 μ g/mL dibenzofuran

6.2 Effect of temperature on the growth of wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single gene knockout mutants

Figures 6.5 to 6.7 showed the growth of wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) in M9 minimal media supplemented with 5µg/mL dibenzofuran as sole carbon and energy source with three different temperatures of incubation; 30°C, 37°C and 42°C. Wildtype *Burkholderia xenovorans* LB400 showed highest growth rate ($\mu=1.35$) when incubated at 30°C. However, the growth rate was reduced to $\mu=1.25$ and $\mu=1.30$ when incubated at 37°C and 40°C, respectively. NRPLB (*rpoN1* mutant) did not show any significant difference in its cell number when cultured in all three different temperatures. However, NRPLB (*rpoN1* mutant) showed higher growth rate ($\mu=1.10$) at 37°C compared to 30°C and 40°C with $\mu= 1.07$ and $\mu= 1.03$, respectively. The cell numbers observed was lower than 2000×10^3 cfu/mL as demonstrated in Figure 6.6. In contrast to NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) grew better than the wildtype *Burkholderia xenovorans* LB400 when supplemented with dibenzofuran as observed with maximum cell numbers which was 5500×10^3 cfu/ml (Figure 6.7). The result also showed that NRP2LB (*rpoN2* mutant) had slightly higher growth rate ($\mu= 1.43$) when incubated at 37°C compared to at 30°C ($\mu=1.42$) when cultured in minimal media supplemented with 5µg/mL dibenzofuran.

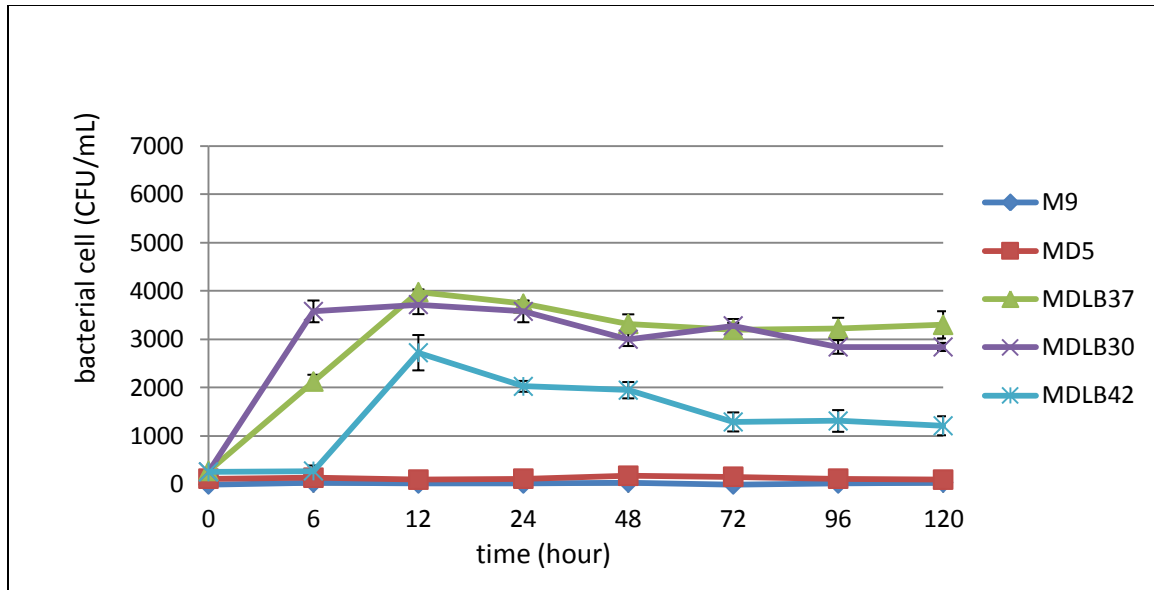


Figure 6.5: Growth of wildtype *Burkholderia xenovorans* LB400 in minimal media supplemented with 5 μ g/mL dibenzofuran and incubated at 30°C, 37°C and 40°C. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD5 : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDLB30: Wildtype *Burkholderia xenovorans* LB400 in MD5 incubated at 30°C

MDLB37: Wildtype *Burkholderia xenovorans* LB400 in MD5 incubated at 37°C

MDLB42: Wildtype *Burkholderia xenovorans* LB400 in MD5 incubated at 42°C

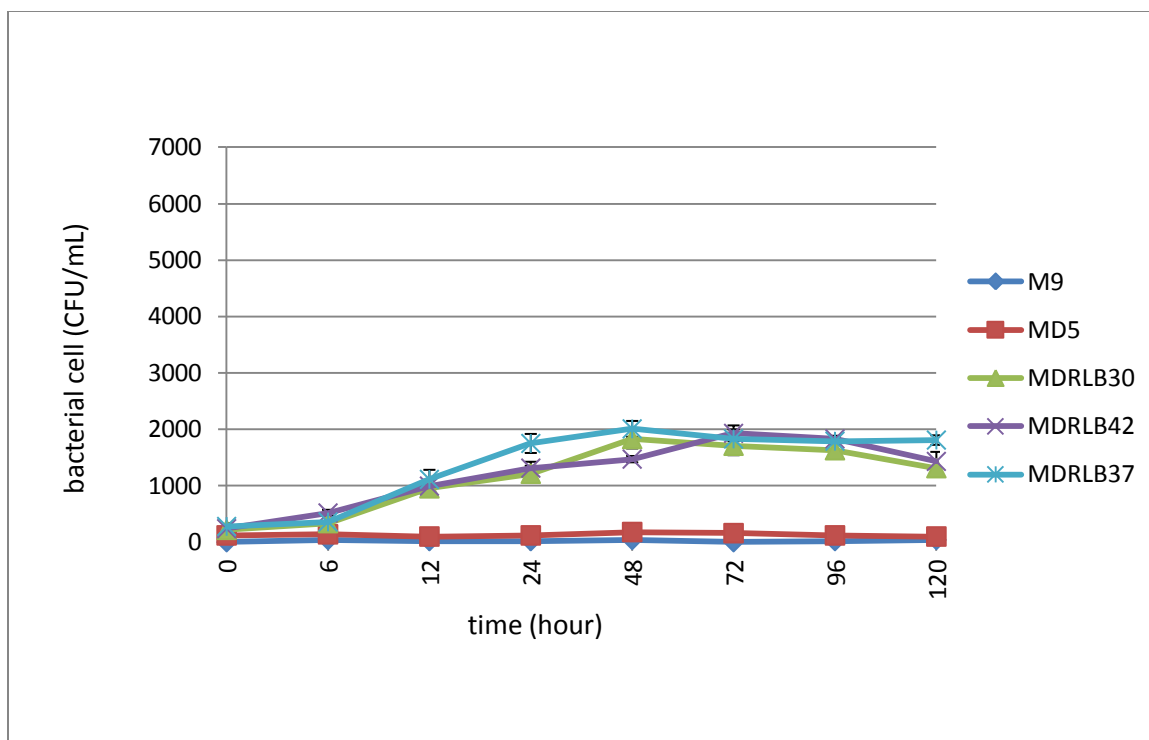


Figure 6.6: Growth of NRPLB (*rpoN1* mutant) in minimal media supplemented with 5 μ g/mL dibenzofuran and incubated at 30 $^{\circ}$ C, 37 $^{\circ}$ C and 40 $^{\circ}$ C. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD5 : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDRLB30: NRPLB (*rpoN1* mutant) in MD5 incubated at 30 $^{\circ}$ C

MDRLB37: NRPLB (*rpoN1* mutant) in MD5 incubated at 37 $^{\circ}$ C

MDRLB42: NRPLB (*rpoN1* mutant) in MD5 incubated at 42 $^{\circ}$ C

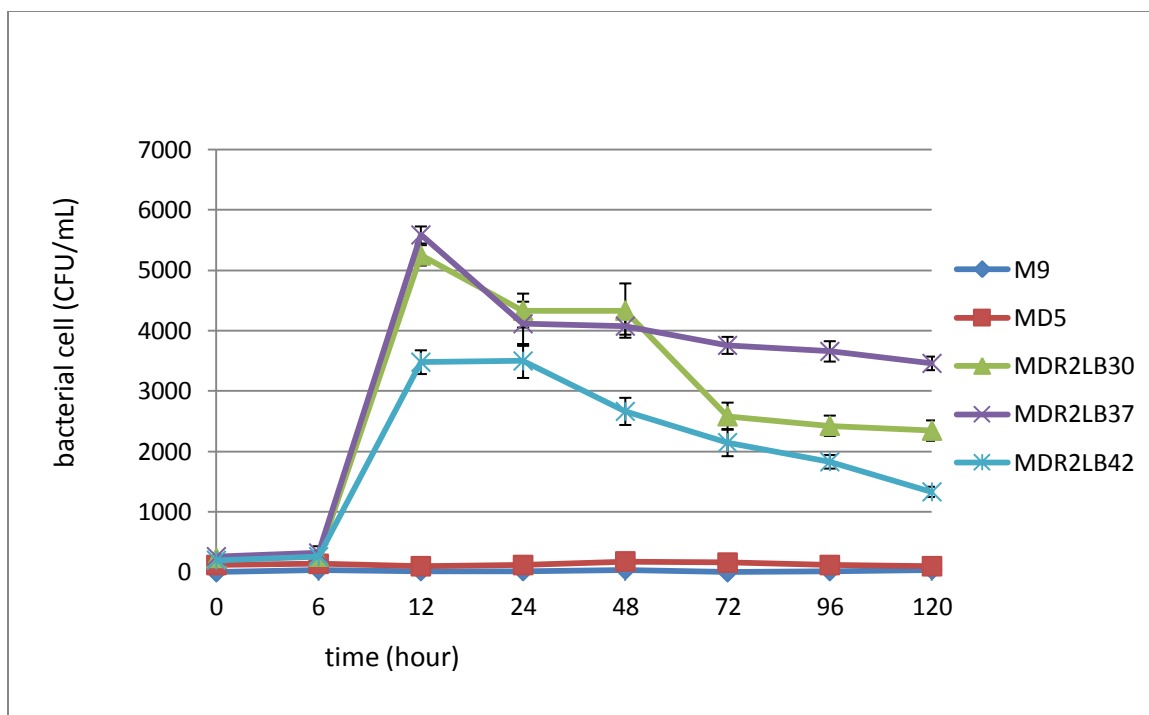


Figure 6.7: Growth of NRP2LB (*rpoN2* mutant) in minimal media supplement with 5 μ g/mL dibenzofuran and incubated at 30°C, 37°C and 40°C. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

M9 : M9 minimal media (control)

MD5 : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDR2LB30: NRP2LB (*rpoN2* mutant) in MD5 incubated at 30°C

MDR2LB37: NRP2LB (*rpoN2* mutant) in MD5 incubated at 37°C

MDR2LB42: NRP2LB (*rpoN2* mutant) in MD5 incubated at 42°C

Figure 6.8 showed that wildtype *Burkholderia cenocepacia* J2315 grew better at 30°C with growth rate of $\mu = 1.26$ compared at 37°C ($\mu = 1.20$) and 42°C ($\mu = 1.13$). When incubated at 30°C, wildtype *Burkholderia cenocepacia* J2315 reached cell numbers of 2000×10^3 cfu/ml within 12 hours but declined afterwards. As observed for wildtype *Burkholderia xenovorans* LB400, the wildtype *Burkholderia cenocepacia* J2315 was also demonstrated to be able to grow better at 30°C.

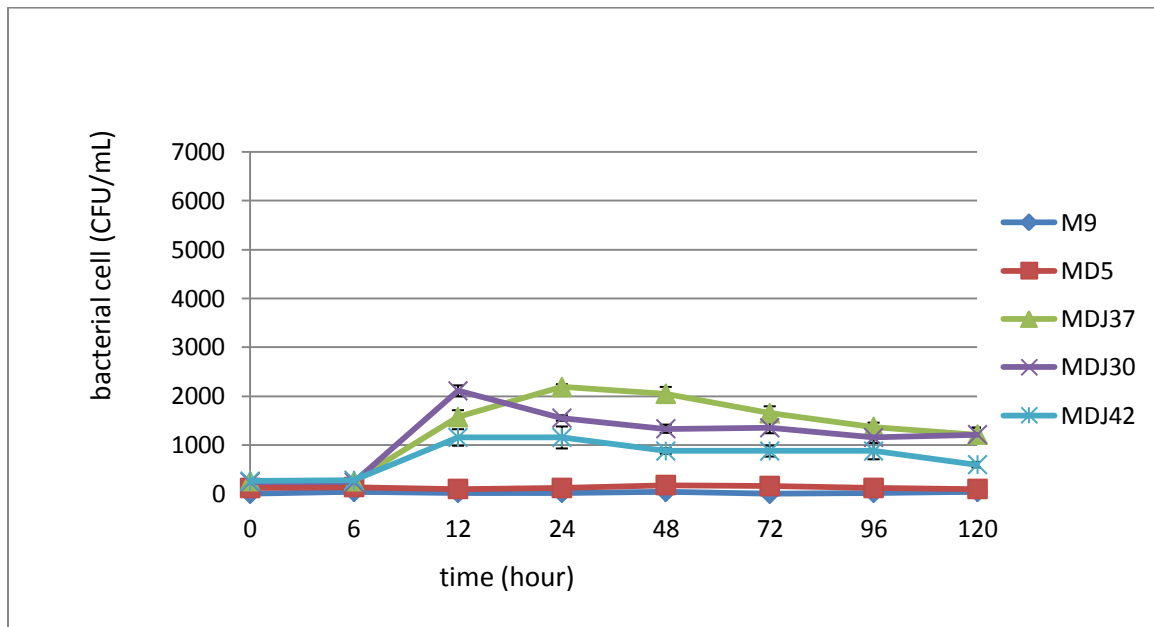


Figure 6.8: Growth of wildtype *Burkholderia cenocepacia* J2315 in minimal media supplemented with 5 μ g/mL dibenzofuran and incubated at 30°C, 37°C and 40°C. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean

M9 : M9 minimal media (control)

MD5 : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDJ30 : Wildtype *Burkholderia cenocepacia* J2315 in MD5 incubated at 30°C

MDJ37 : Wildtype *Burkholderia cenocepacia* J2315 in MD5 incubated at 37°C

MDJ42 : Wildtype *Burkholderia cenocepacia* J2315 in MD5 incubated at 42°C

The growth rate of NRPJ (*rpoN* mutant) was slightly higher when incubated at 37°C with growth rate of $\mu = 1.41$ compared at 30°C ($\mu=1.40$) and 42°C ($\mu= 1.37$) (Figure 6.9). However, the cell numbers were reduced after 12 hours of incubation which may be due to lack of carbon source or accumulation of toxic metabolite.

NRPJ (*rpoN* mutant) enter the exponential phase within 6 hours of incubation in all three different incubation temperatures (Figure 6.9). NRPJ (*rpoN* mutant) showed no significant difference when incubated at 30°C and 37°C with slight different of growth rate of $\mu = 1.40$ and $\mu = 1.41$, respectively.

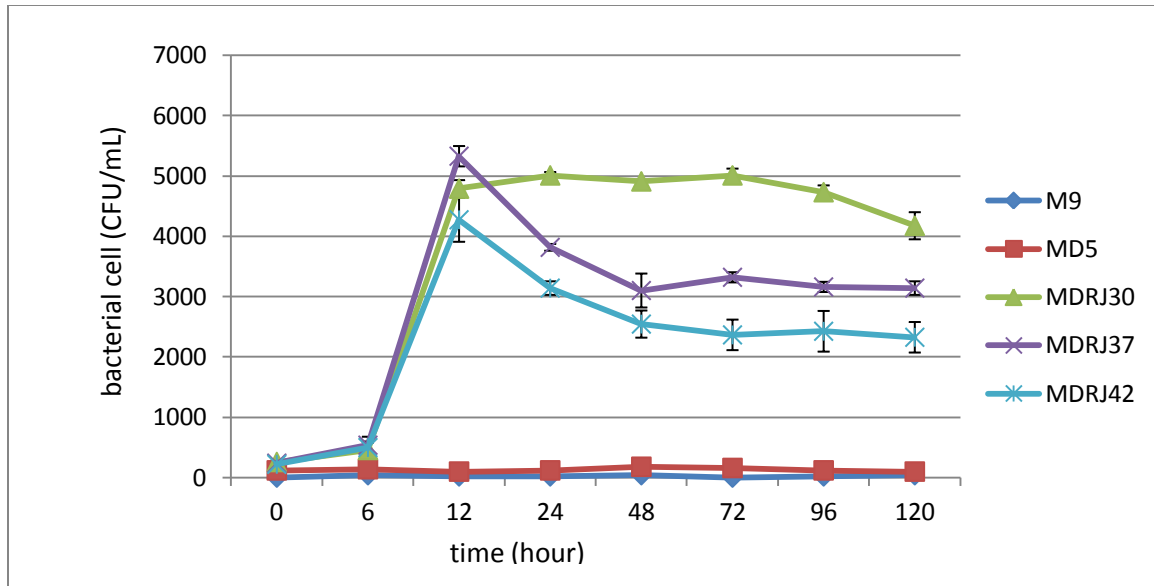


Figure 6.9: Growth of NRPJ (*rpoN* mutant) in minimal media supplemented with 5 μ g/mL dibenzofuran and incubated at 30°C, 37°C and 40°C. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean

M9 : M9 minimal media (control)

MD5 : M9 minimal media supplemented with 5 μ g/mL dibenzofuran (control)

MDRJ30: NRPJ (*rpoN* mutant) in MD5 incubated at 30°C

MDRJ37: NRPJ (*rpoN* mutant) in MD5 incubated at 37°C

MDRJ42: NRPJ (*rpoN* mutant) in MD5 incubated at 42°C

From the results, it was observed that wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single gene knockout mutants are able to grow in both 30°C and 37°C, however the wildtype *Burkholderia xenovorans* LB400 and wildtype *Burkholderia cenocepacia* J2315 grew better in 30° unlike the NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NRPJ (*rpoN* mutant) that grew better in 37°C.

6.3 Effect of pH on growth of wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single gene knockout mutants

Figures 6.10 to 6.12 showed the growth of wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) when incubated in three different pH levels. Suitable pH is an important factor for optimal growth of microorganisms especially bacteria. Three pH levels (pH 5, pH 7 and pH 9) were tested for the optimal growth of the bacteria. It was observed that both wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) showed better growth in pH 7 while no significant difference was obtained for growth rate of NRPLB (*rpoN1* mutant) in the different pH levels.

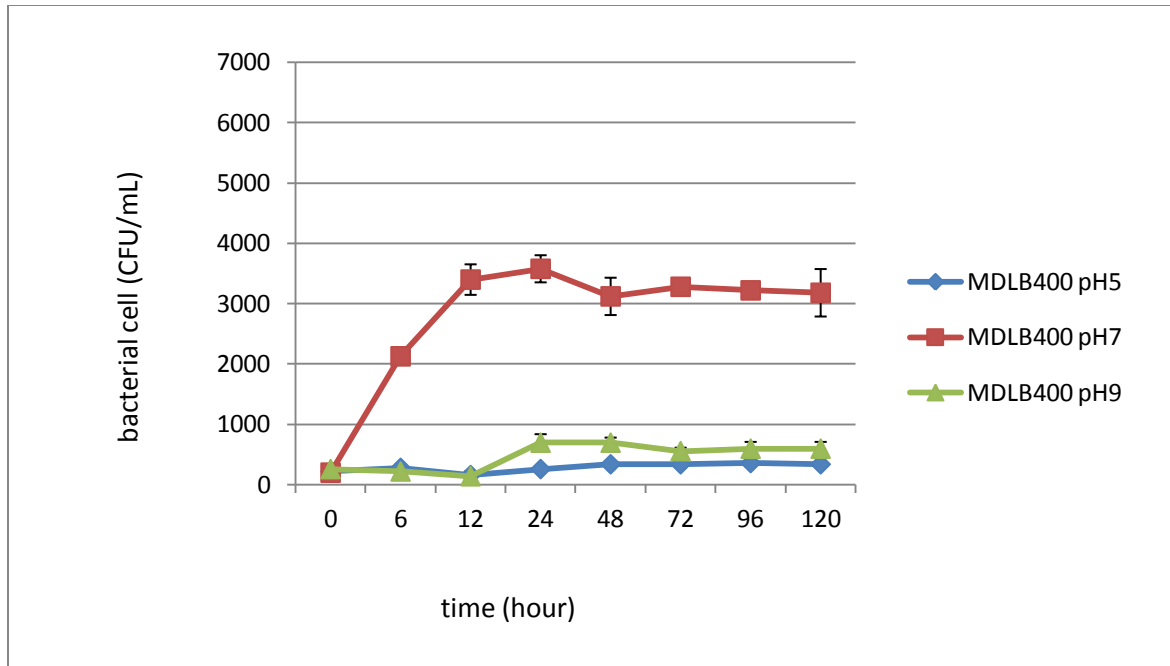


Figure 6.10: Effects of pH levels on growth of wildtype *Burkholderia xenovorans* LB400 in minimal media supplemented with 5 μ g/mL dibenzofuran. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean

MDLB400pH5: Wildtype *Burkholderia xenovorans* LB400 in MD5 with pH 5

MDLB400pH7: Wildtype *Burkholderia xenovorans* LB400 in MD5 with pH 7

MDLB400pH9: Wildtype *Burkholderia xenovorans* LB400 in MD5 with pH 9

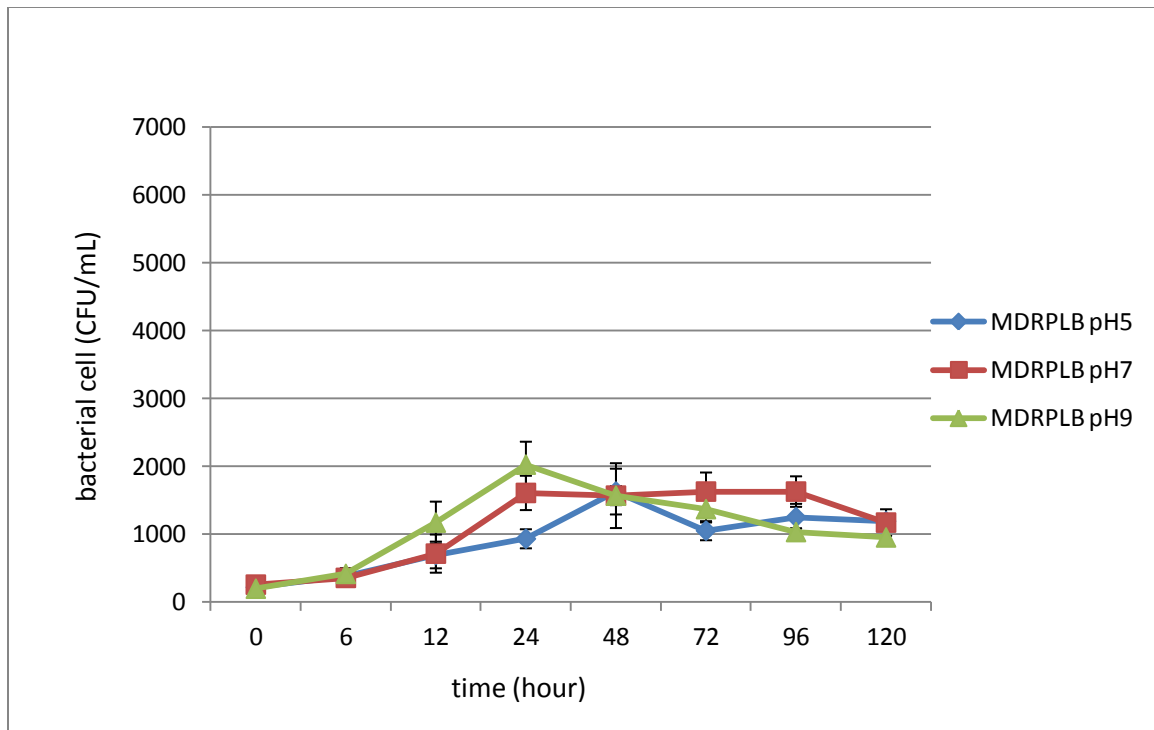


Figure 6.11: Effects of pH levels on growth of NRPLB (*rpoN1* mutant) in minimal media supplemented with 5 μ g/mL dibenzofuran. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean

MDRPLBpH5: NRPLB (*rpoN1* mutant) in MD5 with pH 5

MDRPLBpH7: NRPLB (*rpoN1* mutant) in MD5 with pH 7

MDRPLBpH9: NRPLB (*rpoN1* mutant) in MD5 with pH 9

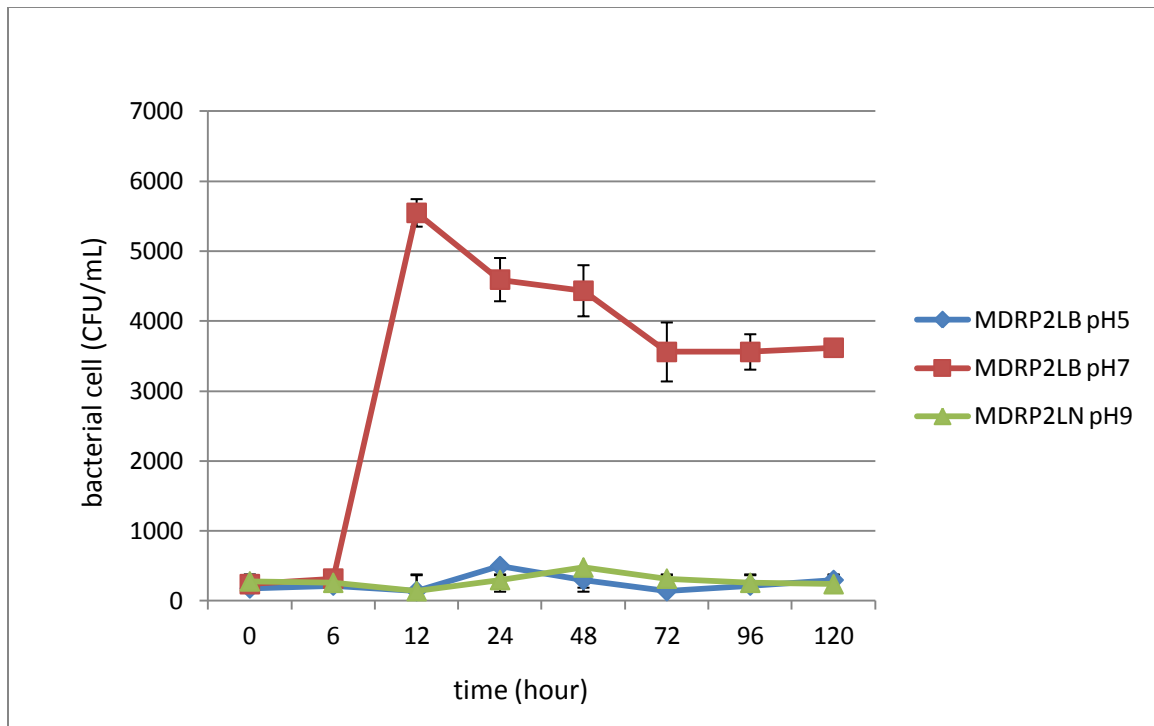


Figure 6.12: Effects of pH level on growth of NRP2LB (*rpoN2* mutant) in minimal media supplemented with 5 μ g/mL dibenzofuran. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

MDRP2LBpH5: NRP2LB (*rpoN2* mutant) in MD5 with pH 5

MDRP2LBpH7: NRP2LB (*rpoN2* mutant) in MD5 with pH 7

MDRP2LBpH9: NRP2LB (*rpoN2* mutant) in MD5 with pH 9

On the other hand, the wildtype *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant) were observed to grow better in pH 7 (with $\mu= 1.20$ for wildtype *Burkholderia cenocepacia* J2315 and $\mu=1.41$ for NRPJ [*rpoN* mutant]) compared to pH 5 ($\mu= 0.5$ for wildtype *Burkholderia cenocepacia* J2315 and $\mu=0.96$ for NRPJ [*rpoN* mutant]) and pH 9 ($\mu= 0.80$ for wildtype *Burkholderia cenocepacia* J2315 and $\mu= 1.12$ for NRPJ [*rpoN* mutant]) (Figures 6.13 and 6.14).

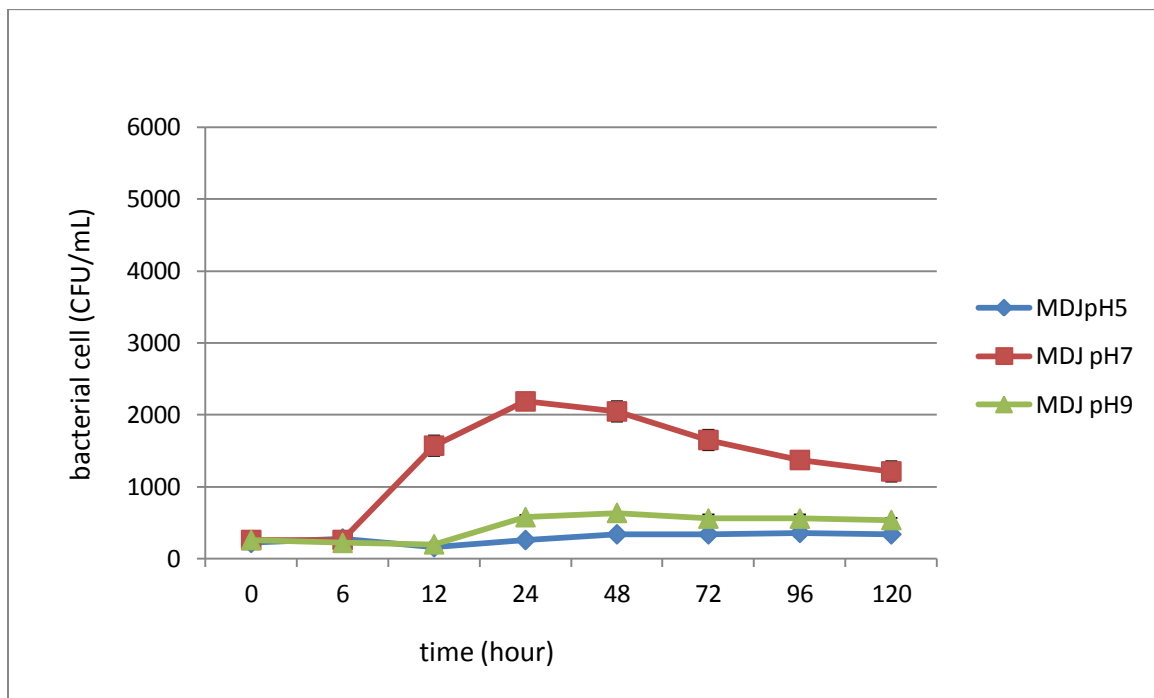


Figure 6.13: Effects of pH levels on growth of wildtype *Burkholderia cenocepacia* J2315 in minimal media supplemented with 5 μ g/mL dibenzofuran. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

MDJpH5: wildtype *Burkholderia cenocepacia* J2315 in MD5 with pH 5

MDJpH7: wildtype *Burkholderia cenocepacia* J2315 in MD5 with pH 7

MDJpH9: wildtype *Burkholderia cenocepacia* J2315 in MD5 with pH 9

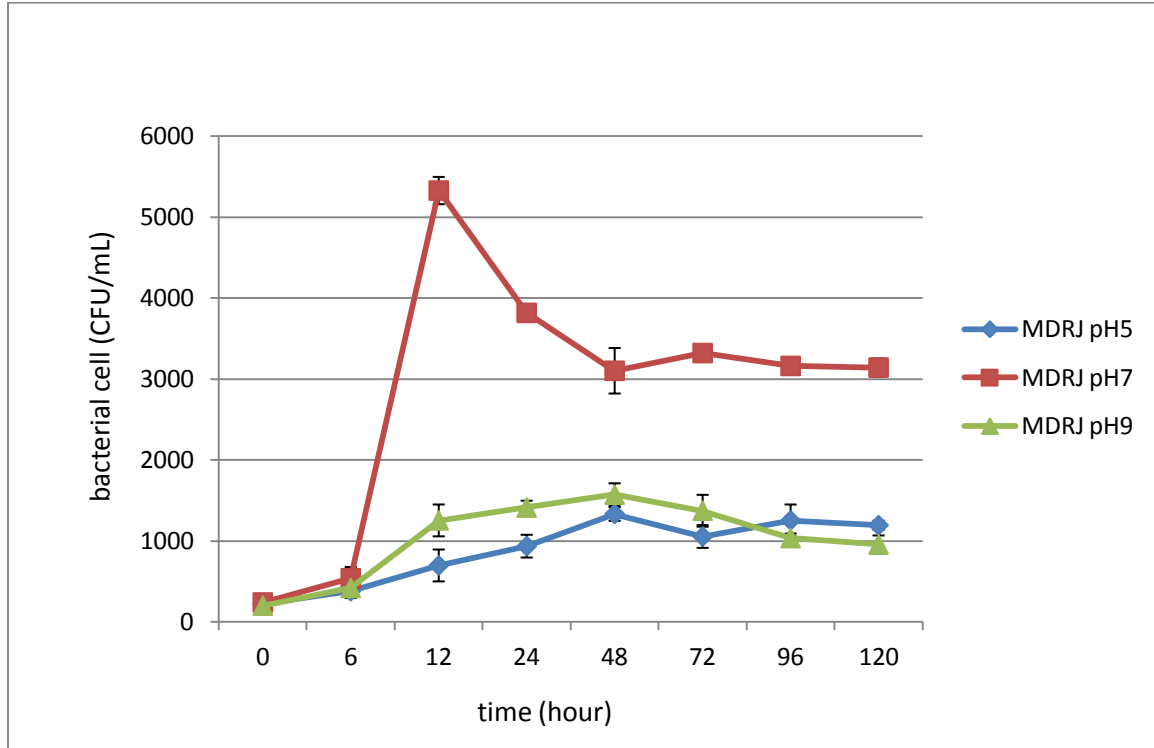


Figure 6.14: Effects of pH level on growth of NRPJ (*rpoN* mutant) in minimal media supplemented with 5 μ g/mL dibenzofuran. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

MDRJpH5: NRPJ in MD5 with pH 5 and incubated at 37°C

MDRJpH7: NRPJ in MD5 with pH7 and incubated at 37°C

MDRJpH9: NRPJ in MD5 with pH9 and incubated at 37°C

From the results obtained in the optimisation of bacterial growth conditions, this study continued with culturing both *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 and their *rpoN* mutants in M9 minimal media (pH 7.0) supplemented with 5 μ g/mL dibenzofuran and incubated at 37°C for degradation study.

6.4 Analysis of dibenzofuran degradation using Gas Chromatography-Mass Spectrometry (GC-MS).

Degradation study was conducted in 250mL baffled flask with optimised growth conditions obtained previously (see subsection 6.3) for 96 hours. Colony forming unit for each sampling time was compared to degradation of dibenzofuran based on abundance of the ion in the peak area of the chromatogram. Selected samples were analysed using GC-MS for quantitative analysis. Dibenzofuran from aqueous samples were extracted using SPE ENVI-18 which is a silica-based hydrophobic octadecyl bonded (C18) column. The samples were concentrated in hexane using gentle blow of nitrogen gas and portions were aliquoted to 1.5mL vial for GC-MS analysis.

External standards for dibenzofuran were prepared in hexane. The chromatogram of the standard solutions 1 μ g/ml, 5 μ g/ml, 10 μ g/ml and 20 μ g/ml dibenzofuran in hexane is shown in Figures 6.15A, 6.15B, 6.15C and 6.15D, respectively. Each peak at retention time of 27.8 represented spectrum of 139 (m/z) and 168 (m/z) which was identified as dibenzofuran using MassHunter Qualitative Analysis software (Agilent Technology).

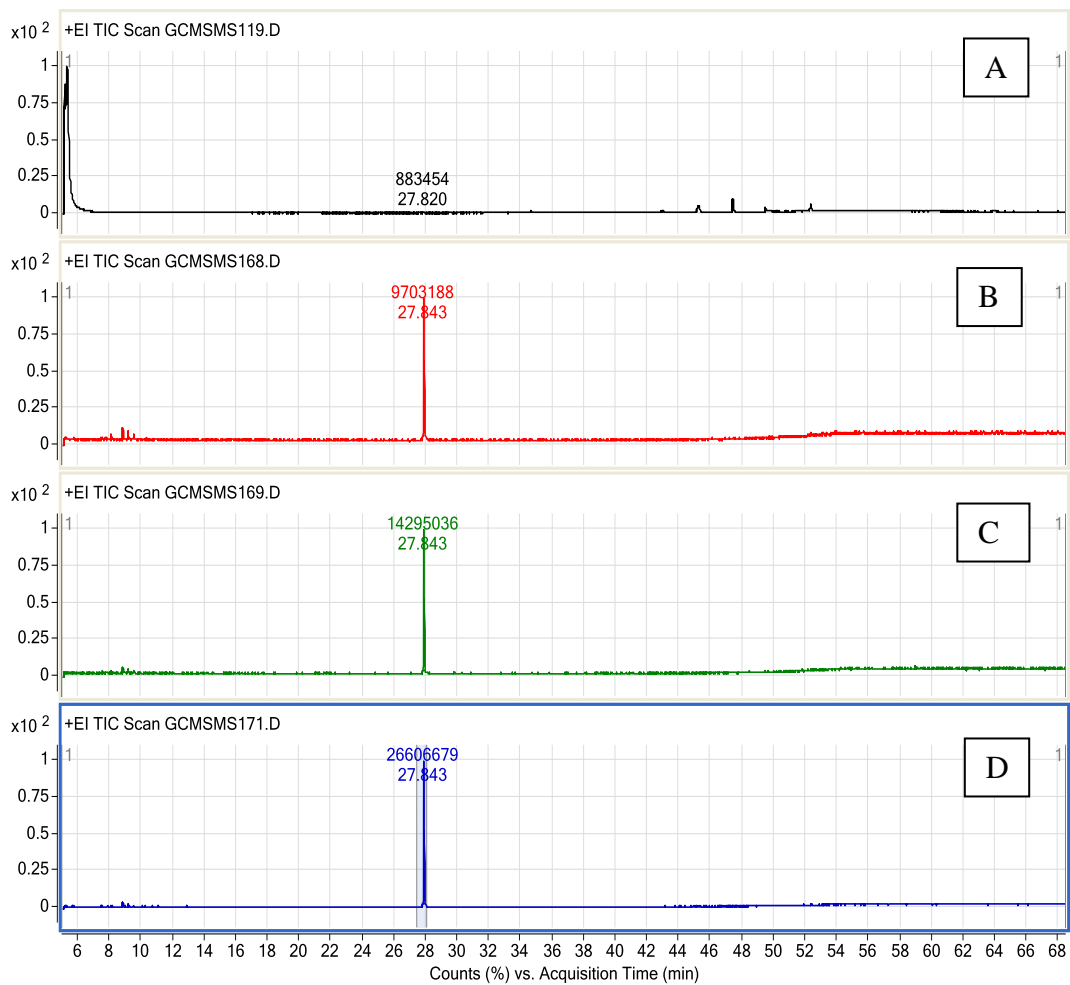


Figure 6.15: Chromatograms of standard solutions for dibenzofuran in hexane.

Panel A: 1µg/mL of dibenzofuran

Panel B: 5µg/mL of dibenzofuran

Panel C: 10µg/mL of dibenzofuran

Panel D: 20µg/mL of dibenzofuran

The standard curve for dibenzofuran was also established based on the abundance of ions in the peak area. R^2 value of 0.981 was generated from the standard curve of dibenzofuran (Figure 6.16)

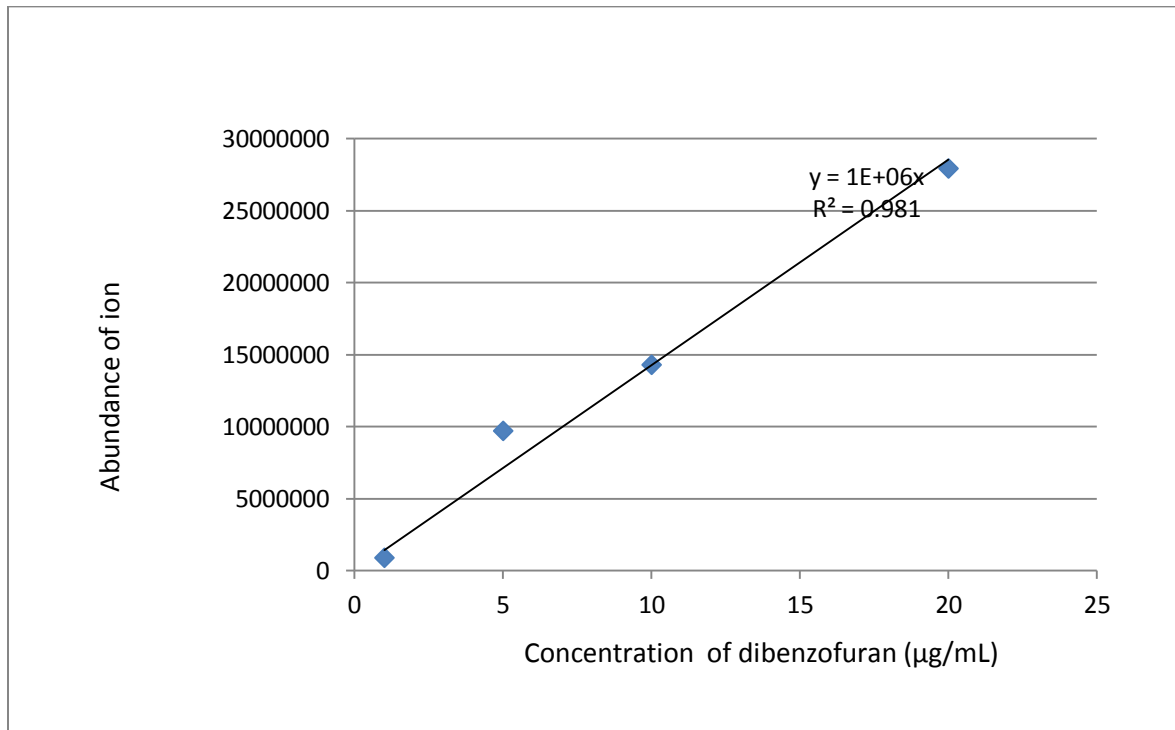


Figure 6.16: Standard curve of dibenzofuran based on the abundance ion.

Figures 6.17A, 6.17B and 6.17C show the degradations of dibenzofuran by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant), respectively. In general, all three strains showed significance degradation of dibenzofuran with the increase of cell numbers. The reduction of dibenzofuran is higher in NRP2LB (*rpoN2* mutant) compared to wildtype *Burkholderia xenovorans* LB400 and NRPLB (*rpoN1* mutant). This can be seen after 96 hours of incubation where the amount of dibenzofuran detected by GC-MS in both wildtype *Burkholderia xenovorans* LB400 and NRPLB (*rpoN1* mutant) was 2-fold and 5 fold higher than the remaining dibenzofuran in NRP2LB (*rpoN2* mutant), respectively.

Better growth was shown by NRP2LB (*rpoN2* mutant) with the highest cell number ($>10000 \times 10^3$ cfu/mL) compared to wildtype *Burkholderia xenovorans* LB400 (8000×10^3 cfu/mL). NRPLB (*rpoN1* mutant) showed the least growth among all three strains with the highest cell number was lower than 2000×10^3 cfu/mL. Rapid degradation of dibenzofuran was observed in NRP2LB (*rpoN2* mutant) culture indicated the potential of NRP2LB (*rpoN2* mutant) as excellent dibenzofuran degrader.

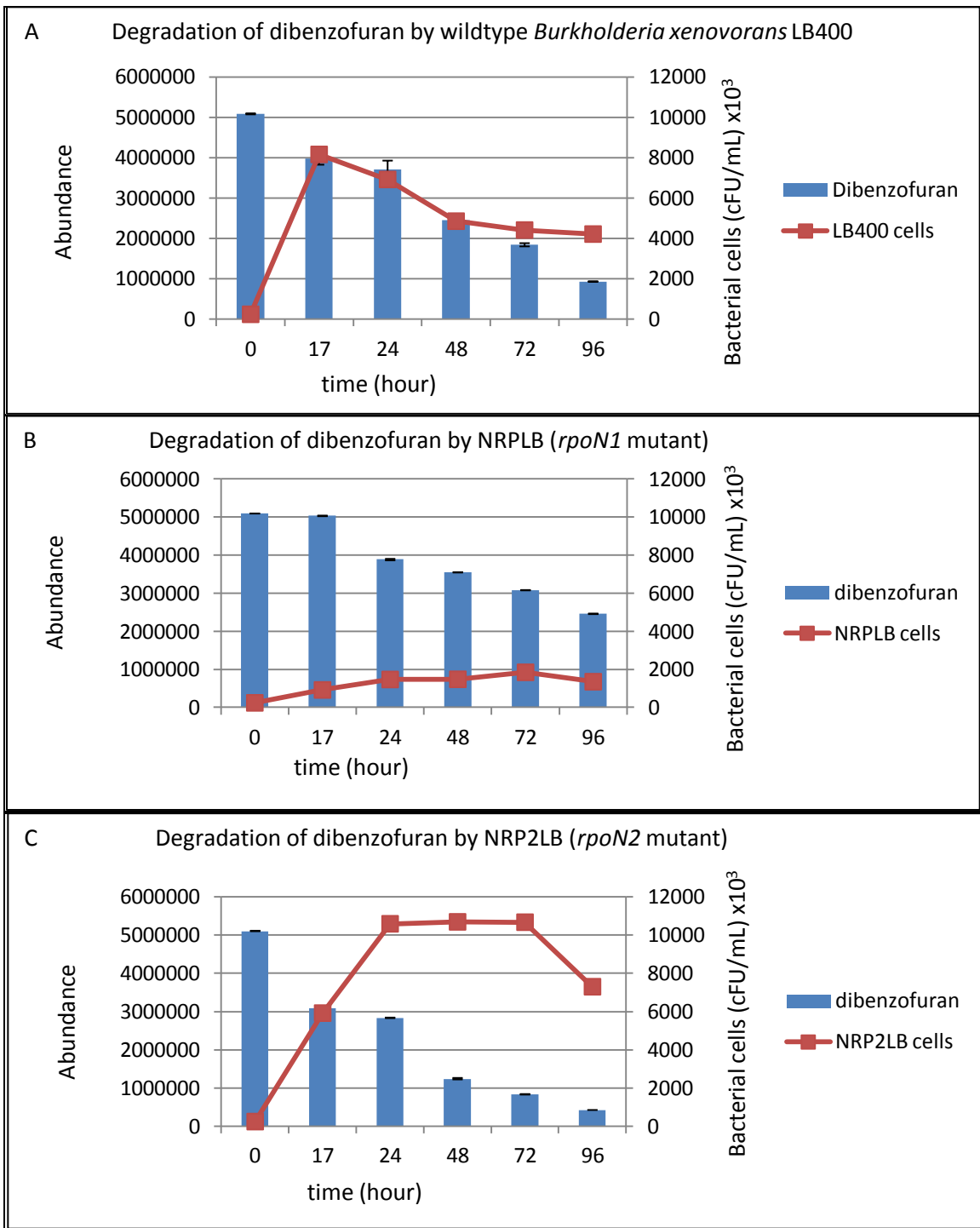


Figure 6.17: Degradation of dibenzofuran by wildtype *Burkholderia xenovorans* Lb400 and its single gene knockout mutants.

Panel A: wildtype *Burkholderia xenovorans* LB400

Panel B: NRPLB (*rpoN1* mutant)

Panel C: NRP2LB (*rpoN2* mutant).

The degradation of dibenzofuran by the clinical isolate *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant) were also conducted. Bacterial cells were also grown in the optimum growth condition (concentration of dibenzofuran of 5 µg/mL at pH 7 and incubated at 37°C). Both strains showed no significant difference in their ability to degrade dibenzofuran. However the growth of NRPJ (*rpoN* mutant) is higher compared to wildtype *B. cenocepacia* J2315 when dibenzofuran was supplemented as sole carbon source (Figures 6.18A and 6.18B).

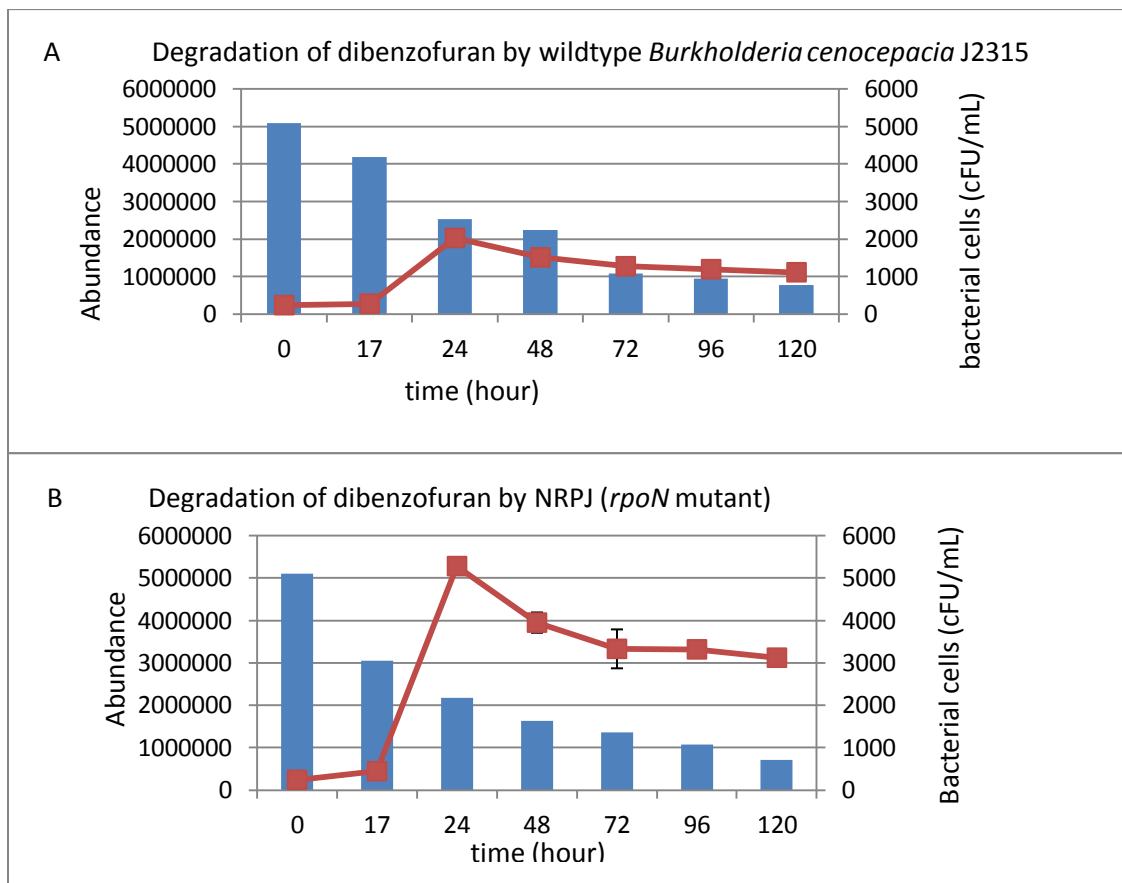


Figure 6.18: Degradation of dibenzofuran by wildtype *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant).

Panel A: wildtype *Burkholderia cenocepacia* J2315

Panel B: NRPJ (*rpoN* mutant)

Previously L'Abbee and co workers (2005) demonstrated that dibenzofuran degradation yielded a yellow metabolite from lateral dioxygenation that is toxic to bacterial cells and inhibit the further degradation of dibenzofuran. However during the degradation of dibenzofuran by both wildtype *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 and their single gene knockout mutants, no yellow metabolite was observed in this study indicating that dibenzofuran was degraded via angular dioxygenation. This outcome suggested that dibenzofuran was not degraded via lateral dioxygenation. In the previous study by L'Abbee and colleagues (2005), it was demonstrated that biphenyl dioxygenase that responsible in dibenzofuran degradation by *Burkholderia xenovorans* LB400 can act via both pathways either angular or lateral dioxygenation. Furthermore, the absence of yellow metabolites might also be due to prolonged incubation as demonstrated by Kubota *et al.* (2005) when growing *Nocardioides aromaticivorans* in DBF-BSV liquid medium where all of the isolates able to degraded dibenzofuran completely within 96 hours of incubation and the yellow metabolite that produced before the logarithmic phase of growth disappeared with the time of prolonged incubation.

6.5 Gene expression analysis of RpoN-dependent *bphA* gene that involve in dibenzofuran degradation

Expression of *bphA* gene was observed in both wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) during dibenzofuran utilisation but not during utilisation of glucose or glucose-dibenzofuran mixture (Figure 6.19). The Normalized Fold Expression of *bphA* gene was automatically generated by the Gene Expression Analysis program based on the expression of the *gyrB* gene (internal control gene). Normalisation was done in order to normalise the expression level of *bphA* gene to that of an internal control gene in each sample so that the level of *bphA* gene in one sample can be compared to another sample. The normalisation process controls the unequal RNA loading and other differences introduced during Reverse Transcription reaction.

The result showed that *bphA* gene was only expressed with the presence of dibenzofuran as sole carbon source but not as co-substrate suggesting the involvement of *bphA* gene in degradation of dibenzofuran.

The expression of *bphA* gene in glucose-dibenzofuran mixture was not detected as the wildtype *Burkholderia xenovorans* LB400 and its single gene knockout mutant strains may have the tendency to utilise simple compound such as glucose as the primary carbon and energy source rather than complex compound such as aromatic compounds (Basu *et al.* 2005), thus the enzymes required for the utilisation of the dibenzofuran as the carbon source was repressed.

The expression of *bphA* gene was not detected during the growth of NRPLB (*rpoN1* mutant) when glucose, dibenzofuran or glucose-dibenzofuran mixture were supplemented as carbon source(s), indicating that *rpoN2* gene was not involved in expression of *bphA* gene. In contrast with NRPLB (*rpoN1* mutant), the *bphA* gene was overexpressed with the presence of only *rpoN1* gene in NRP2LB (*rpoN2* mutant) during the dibenzofuran utilisation compared to wildtype *Burkholderia xenovorans* LB400 when both *rpoN1* and *rpoN2* genes are present. Moderate expression of *bphA* gene in wildtype *Burkholderia xenovorans* LB400 might be due to negative autoregulation by RpoN2 as suggested by Moris *et al.* (2004).

Furthermore, the expression of *bphA* gene was increased after 24 hours of incubation (Figure 6.20) where the cell numbers of NRP2LB (*rpoN2* mutant) is the highest (refer to subsection 6.4) indicating that expression of *bphA* gene is correlatively regulated by the abundance of RpoN2.

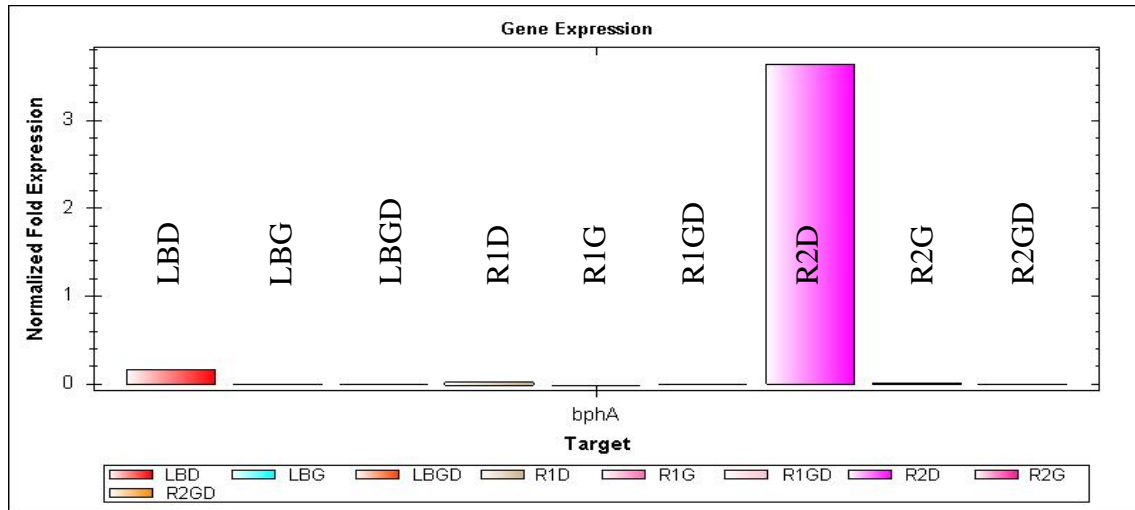


Figure 6.19: Expression of *bphA* gene in wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) after 12 hours incubation in M9 minimal media when supplemented with dibenzofuran.

LBD = wildtype *Burkholderia xenovorans* LB400 in dibenzofuran

R1D = NRPLB (*rpoN1* mutant) in dibenzofuran

R2D = NRP2LB (*rpoN2* mutant) in dibenzofuran

LBG = wildtype *Burkholderia xenovorans* LB400 in glucose

R1G = NRPLB (*rpoN1* mutant) in glucose

R2G = NRP2LB (*rpoN2* mutant) in glucose

LBGD = wildtype *Burkholderia xenovorans* LB400 in glucose-dibenzofuran mixture

R1GD = NRPLB (*rpoN1* mutant) in glucose-dibenzofuran mixture

R2GD = NRP2LB (*rpoN2* mutant) in glucose-dibenzofuran mixture

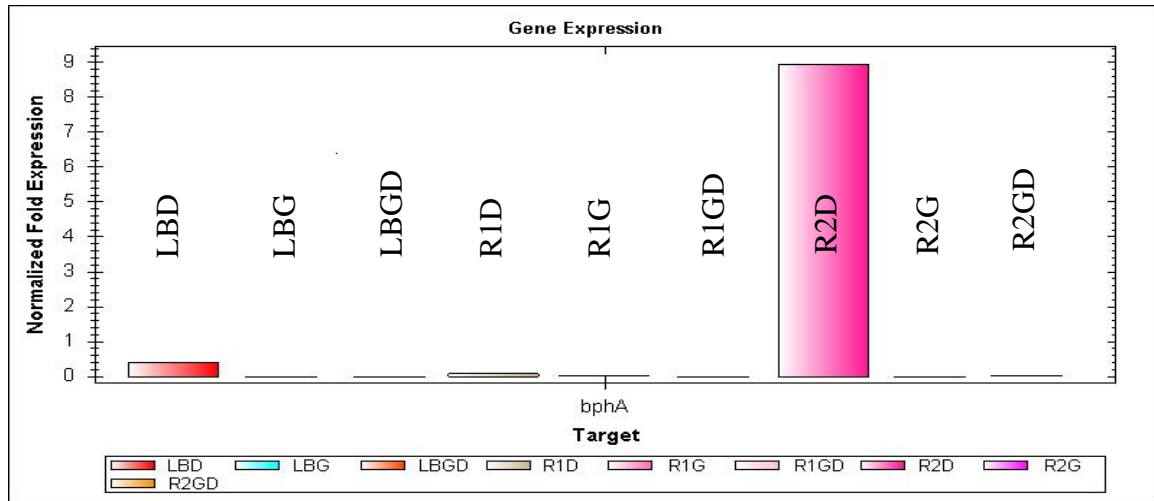


Figure 6.20: Expression of *bphA* gene in wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) after 24 hours incubation in M9 minimal media when supplemented with dibenzofuran

LBD = wildtype *Burkholderia xenovorans* LB400 in dibenzofuran

R1D = NRPLB (*rpoN1* mutant) in dibenzofuran

R2D = NRP2LB (*rpoN2* mutant) in dibenzofuran

LBG = wildtype *Burkholderia xenovorans* LB400 in glucose

R1G = NRPLB (*rpoN1* mutant) in glucose

R2G = NRP2LB (*rpoN2* mutant) in glucose

LBGD = wildtype *Burkholderia xenovorans* LB400 in glucose-dibenzofuran mixture

R1GD = NRPLB (*rpoN1* mutant) in glucose-dibenzofuran mixture

R2GD = NRP2LB (*rpoN2* mutant) in glucose-dibenzofuran mixture

6.6 Discussion

Wildtype *Burkholderia xenovorans* LB400 and its single gene knockout mutants (NRPLB and NRP2LB) were able to grow on dibenzofuran. Different concentration of dibenzofuran did not affect the growth of wildtype *Burkholderia xenovorans* LB400 and NRPLB (*rpoN1* mutant) even though the cell numbers of NRPLB (*rpoN1* mutant) was low compared to wildtype *Burkholderia xenovorans* LB400. This might be due to the inactivation of *rpoN1* gene in *Burkholderia xenovorans* LB400, thus reduced the ability of wildtype *Burkholderia xenovorans* LB400 to utilise dibenzofuran efficiently. On the other hand, NRP2LB (*rpoN2* mutant) grew faster in both 1µg/mL and 5µg/mL dibenzofuran containing culture where the time taken for the strain to overcome the lag phase was shorter (~6 hours) while at the higher concentration (10µg/mL) the time taken was longer (~12 hours). The growth reduction was observed for NRP2LB (*rpoN2* mutant) after 24 hours of incubation when supplemented with 1µg/mL and 10µg/mL dibenzofuran. The growth reduction in 1µg/mL dibenzofuran might be due to the limitation of carbon source for further growth of wildtype *Burkholderia xenovorans* LB400. Nevertheless the reduction of bacterial growth in 10µg/mL dibenzofuran may be due to the toxicity of the dibenzofuran or accumulation of toxic metabolites.

In *Burkholderia cenocepacia* J2315, the inactivation of *rpoN* gene enhanced the growth of the NRPJ (*rpoN* mutant) in dibenzofuran compared to the wildtype *Burkholderia cenocepacia* J2315. No information on the degradation of dibenzofuran by *B. cenocepacia* J2315 is available in the literature especially regarding the regulation by *rpoN* gene of *B. cenocepacia* J2315.

The effect of temperature on the growth of wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single gene knockout mutants were also studied. The results obtained showed that although no significant difference was observed in cell numbers when incubated at 30°C or 37°C for wildtype *Burkholderia xenovorans* LB400 and wildtype *Burkholderia cenocepacia* J2315, the growth was faster at 30°C compared to 37°C. The growth of wildtype *Burkholderia cenocepacia* J2315 was delayed by 6 hours compared to that of wildtype *Burkholderia xenovorans* LB400. This was expected as PCBs and its ortho-substituted compound including dibenzofuran were readily available for degradation by wildtype *Burkholderia xenovorans* LB400 but that was not the case for wildtype *Burkholderia cenocepacia* J2315.

No significant difference was observed in NRPLB (*rpoN1* mutant) growth when incubated at 30°C, 37°C and 42°C, respectively. Even though there was no significant difference in growth rate for incubation of NRP2LB (*rpoN2* mutant) at 30°C and 37°C during the exponential phase, the cell number was reduced after 48 hour of incubation at 30°C but not for NRP2LB (*rpoN2* mutant) cells that were incubated at 37°C. For NRPJ (*rpoN* mutant), the cell number was highest at 37°C but was reduced to almost half within 36 hours but constant at 30°C. The results suggested that the optimum incubation temperature was at 37°C as most strains were able to achieve the highest cell number when incubated at 37°C. Additionally, wildtype *Burkholderia xenovorans* LB400 and its single gene knockout mutants (NRPLB and NRP2LB) were able to sustain their growth at 37° compared to at 30°C.

The effect of pH on the growth of wildtype *Burkholderia xenovorans* LB400, wildtype *Burkholderia cenocepacia* J2315 and their single gene knockout mutants were also studied. All the bacterial strains were observed to grow better in pH 7 compared to pH 5 and pH 9, except for NRPLB (*rpoN1* mutant) which showed no significant difference for all three different pH (pH 5, pH 7 and pH 9).

Dibenzofuran biodegradation capabilities for all the strains were further analysed. The remaining dibenzofuran at the end of the degradation process (under the optimal growth conditions for each strain) was extracted and purified and subsequently identified using Gas Chromatography/ Mass Spectrometry (GC/MS). Dibenzofuran was detected at retention time of 27.8 where the spectrum shows 139 m/z and 168 m/z. Generally all the strains used in this study were able to utilise dibenzofuran. The growth of NRPLB (*rpoN* mutant) was low compared to both wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant), suggesting the involvement of *rpoN1* gene in degradation of dibenzofuran since its inactivation lead to significant reduction of dibenzofuran degradation. Total dibenzofuran loss due to degradation by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN* mutant) and NRP2LB (*rpoN2* mutant) were 81%, 51% and 92%, respectively. The higher growth of NRP2LB (*rpoN2* mutant) indicated that *rpoN2* gene was not involved in degradation of dibenzofuran. Information on how the *rpoN1* and *rpoN2* genes were connected to each other in the regulatory system is limited. This study has successfully demonstrated that by knocking out the *rpoN2* gene, the activity of *rpoN1* gene will be enhanced.

High rates of microbial activity affect the successfulness of biodegradation or bioremediation processes. However, there are a lot of environmental factors that are involved in activation or repressions of gene expression of which modulate the activity of the microbes (Daubars and Chakrabarty, 1992). Several studies had successfully demonstrated that gene expression can be measured at transcription level either using a reporter gene (King *et al.* 1990; Burlage *et al.* 1994), gene probe (Fleming *et al.* 1993; Sanseverino *et al.* 1993; Jeffery and Barkay, 1996) or RT-PCR (Ogram *et al.* 1994; Selvartnam *et al.* 1995; Wilson *et al.* 1999).

Previous study by Parnell (2007) demonstrated that several pathways associated with PCBs degradation are influenced by sigma-54 (σ^{54}). Since degradation of dibenzofuran was reported via the same pathways as degradation of biphenyl and PCBs (Seeger *et al.*, 2001), this study has also investigated the involvement of *bph* genes in the degradation of dibenzofuran by wildtype *Burkholderia xenovorans* LB400 and its single gene knockout mutant strains (NRPLB and NRP2LB). The expression of *bphA* gene in *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) suggested the involvement of *rpoN1* gene in transcription of *bphA* gene during dibenzofuran degradation and not *rpoN2* gene. Furthermore the *bphA* gene was over-expressed in NRP2LB (*rpoN2* mutant) when only *rpoN1* gene was active compared to moderate expression of *bphA* gene in wildtype *Burkholderia xenovorans* LB400 when the *rpoN1* and *rpoN2* genes were active. This finding suggested that negative auto-regulation by RpoN2 in wildtype *Burkholderia xenovorans* LB400 may occurred as demonstrated by Moris *et al* (2004) in *Ralstonia etli*.

6.8 Summary

The wildtype strains (*Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315) and their single gene knockout mutants (NRPLB, NRP2LB and NRPJ) were able to utilise dibenzofuran as sole carbon source. The growth of *Burkholderia xenovorans* LB400 and its single gene knockout mutants (NRPLB and NRP2LB) were higher than *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant). NRP2LB (*rpoN2* mutant) was observed to have better growth and degradation capabilities towards dibenzofuran compared to wildtype *Burkholderia xenovorans* LB400. This result was supported by gene expression analysis in which the *bphA* gene that involved in dibenzofuran degradation was expressed higher in NRP2LB (*rpoN2* mutant) compared to wildtype *Burkholderia xenovorans* LB400.