EFFECT OF PROTEIN CRUDE EXTRACT ON OXIC/ANOXIC DIAUXIC GROWTH OF A NAP-DEFICIENT MUTANT OF *PARACOCCUS PANTOTROHPUS*

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To my mom, Qiu Xia, and my dad, Yin Qingsheng

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
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Paracoccus pantotrophus has two nitrate reductases: membrane bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap). The mutant KD102 (mutant from *P. Pantotrophus*, napEDABC::Km) does not express Nap and exhibits a significant diauxic lag when growth is switched from aerobic (oxygen present) to anoxic conditions. In this study, it was shown that addition of crude protein extract from either wild type *P. Pantotrophus* or KD102 positively affected the growth of KD102 after switching from aerobic to anoxic growth. The diauxic lag was reduced or, in some cases, eliminated. Furthermore, the final specific growth rate was linearly related with the dosage of crude extract added.

CHAPTER 1
INTRODUCTION

Denitrification is a respiratory process that reduces oxidized forms of nitrogen in response to the oxidation of an electron donor, resulting in removal of nitrogen from water in form of N2. Some forms of nitrogen in water are harmful to human health and high levels of nitrogen in wastewater can harm aquatic life, create bad odors, and have other adverse effects. Thus nitrogen removal is important. The most widely used methods for removing nitrogen from wastewater are biological using a mixed culture of nitrifying and denitrifying bacteria (Grady et al., 1999).

To remove both ammonia and nitrate in a biological wastewater treatment plant, a mixed culture of bacteria is exposed to alternating aerobic (dissolved oxygen present) and anoxic (dissolved oxygen absent, nitrate present) conditions. When the conditions are aerobic nitrifying bacteria oxidize ammonia to nitrate, and when oxygen is absent, denitrifying bacteria reduce nitrate to nitrite and ultimately nitrogen gas. This combined balanced process reduces nitrogen to acceptably low concentrations. In this process, the denitrifiers continually switch from aerobic to anoxic conditions and vice versa. Under aerobic conditions, denitrifiers grow utilizing oxygen as terminal electron acceptor, and only switch to nitrate when anaerobic conditions prevail. Many denitrifiers, however, experience periods without growth during the transition from aerobic to anoxic conditions, termed diauxic lag. These unproductive periods negatively affect nitrogen removal.

The reason for existence of diauxic lag is that one of the enzymes for denitrification, membrane bound nitrate reductase (Nar), is not synthesized under aerobic conditions and it takes time after switching to anoxic conditions to be

synthesized to levels high enough to support growth. A few denitrifying bacteria express a second reductase, periplasmic nitrate reductase (Nap), that is expressed under aerobic conditions. It has been shown that Nap eliminates or reduces the diauxic lag [1]. Due to the autocatalytic nature of nitrate reductase, a method that jump starts its production could have significant impact in shortening diauxic lag. This work investigates whether crude extract from denitrifying bacteria can accomplish this. It is not expected that a large protein (nitrate reductase units typically range from 20-200 kDa, [2]) can penetrate the cell wall of gram-negative bacteria to relocate in either the periplasmic space (Nap) or the inner membrane and cytoplasm (Nar), because for the natural transport channel the maximum size for passive free-locating protein should be in the order of 600 Da [3]. However it is possible that crude extract contains factors that have stimulatory effects, such as cell-penetration peptide, which can help in delivering useful molecules that cannot otherwise penetrate the cell wall, or simply peptides that can be utilized for protein synthesis material, e.g. n-valyl-L-valyl-L-valine which stimulates growth of Streptococcus faecalis [4], or inducers that will induce biosynthesis of Nar by denitrifying bacteria.

CHAPTER 2
BACKGROUND

Anaerobic Respiration

The basic nitrogen removal pathway is indicated in Figure 1. In the presence of oxygen (aerobic conditions), nitrifiers oxidize ammonia to nitrate, whereas in the absence of oxygen (anoxic conditions) denitrifiers switch the terminal electron acceptor from oxygen to nitrate, which usually leads to a period of growth stagnation (which is called diaxiuc lag). The respiration of denitrifying bacteria mainly includes the electron transport system, aerobic or anaerobic acceptors, ATP synthase, and the proton gradient. Both aerobic and anaerobic respirations begin with the establishment of a proton gradient by a complex electron transport system. For anaerobic respiration unsing nitrate as the terminal electron acceptor, the net reaction is $2H^{+}+2e^{-}$ $+1/2O_2 \rightarrow H_2O$ with concomitant production of ATP from ADP and inorganic phosphorous. For anoxic respiration, the net reaction is $NO_3^{-}+ 2H^{+}+2e^{-} \rightarrow NO_2^{-} + H_2O$ with simultaneous production of ATP. Nitrate reductase is the catalyst .

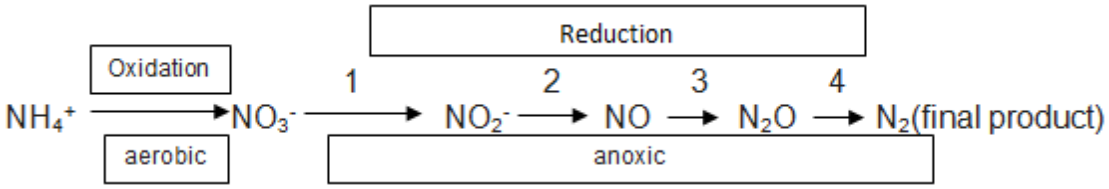

Figure 2-1. Nitrogen removal pathway

In Figure 2-1 each of the numbered reduction steps is catalyzed by a different enzyme. These enzymes are listed in Table 2-1. Nitrate reductase starts the reduction sequence and without it nitrogen removal would cease. It is apparent that nitrate reducatse starts the first step of nitrogen removal , which means it plays an important role in the denitrifican.

Table 2-1. Enzyme that catalyze the nitrogen removal chain reactions

Enzyme	Abbreviation	Relate Reaction Corresponding to Figure 2-1
Membrane bound Nitrate reductase	Nar	1
Periplasmic Nitrate reductase	Nap	
Nitrite reductase	Nir	2
Nitric oxide reductase	Nor	3
Nitrous oxide reductase	Nos	4

Characteristics and Properties of Nitrate Reductase

Considerable research has been conducted on nitrate reductase to elucidate characteristics such as stabilization, the regulation system, and kinetics (e.g., [5-9], [21-24]). There are two kinds of nitrate reductase utilized: Membrane bound nitrate reductase (Nar) and Periplasmic nitrate reductase (Nap).

Nitrate reductase exists in many bacterial strains, such as *E.coli K12*, *Paracoccus pantotrophus*, *Rhodobacter sphaeroides DSM 158*, *Penicillium chrysogenum* or even in plants [5-9], [20-24] here. Although different bacterial strains contain different subunits of nitrate reductase, the overall function and structure of certain main subunits are similar.

In *E.coli K12*, nitrate reductase consists of both the inducible membrane–bound (Nar) and periplasmic reductase (Nap) [5]. After the whole genome sequencing of E.coli, structural genes of NapFDAGHBC (encode NapABC as well as electron transfer components) and also of NarGHJI as well as NarZYWV operon were detected.

Nap in *E.coli K12* is reported to support anaerobic respiration, containing Mo-molybdopterin guanine dinucleotide, one iron-sulfur cluster and diheme cytochrome C_{552}

as well as a tetraheme cytochrome c [5]. Nap can be expressed both under aerobic and anoxic conditions. Also, Darwin (ref) proved that Nap is preferentially utilized under low nitrate concentrations. The reason of that is hypothesized to be that lower energy is required to reduce nitrate in the periplasmic area as excess nitrate does not need to be transported into the cell.

Nar in *E.coli K12* can co-operate with quinol oxidation associated with proton translocation and gradient formation. In *E.coli K12*, Nar consists of a Mo-molybdopterin guanine dinucleotide, a five iron-sulfur cluster and diheme cytochrome b_{556}(ref). The structure of Nar in *E.coli* can be divided in to a large subunit and a small one, the weights of which are 142,000 and 58,000 Da respectively, and the subunits are tightly packed. Also, except for the effect of azide, which is an inhibitor of Nar, it is proved that Nar is stable under a wide temperature range, except under sudden freezing conditions. Other bacterial nitrate reductases having similar characteristics with that in E.coli, are the *Micrococcus* and the *Azotobacer* enzymes [6].

The nitrate redutases in *Paracoccus pantotrophus* (Nar and Nap) are of similar function and structures as those in *E.coli*. Their location is shown in Figure 2-2.

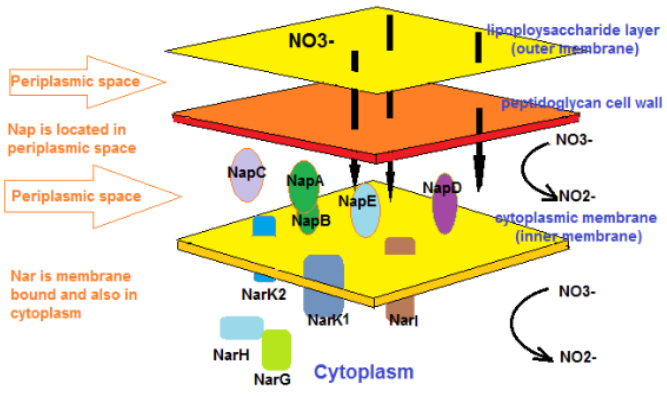

Figure 2-2. Location of Nar and Nap in gram-negative bacteria

A brief working reducing pathways and structures of both Nap and Nar is shown in Figure 2-3 [7].

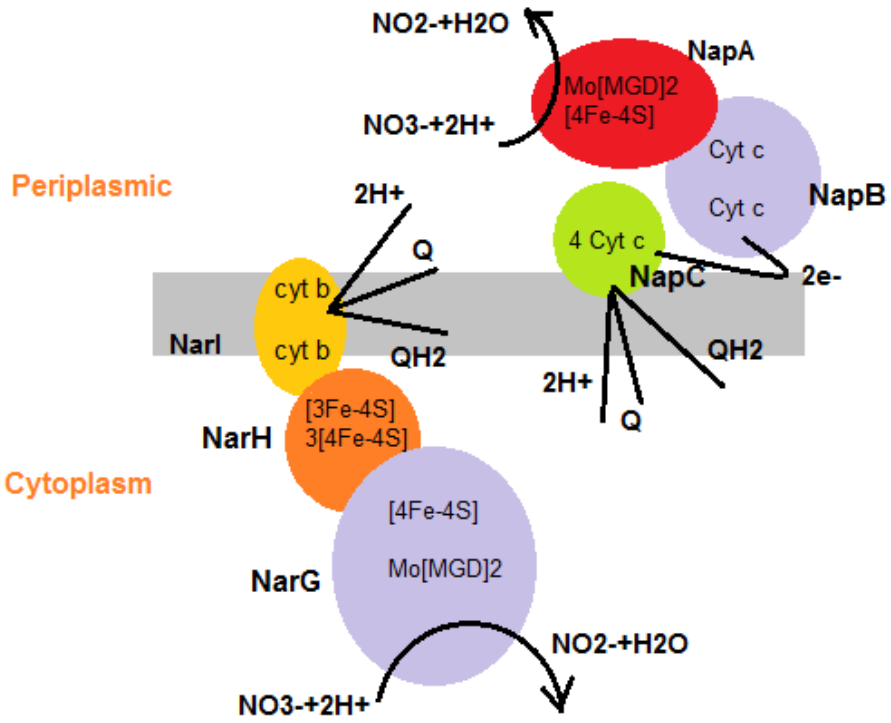

Figure 2-3. Structure of Nar and Nap and nitrate reducing pathways

It is reported that for *P. Pantotrophus* [8], best pH for the highest Nap activity is between 7 to 7.5, and also Nap will be stable and of high activity at 37℃, which match the growth condition of bacteria well and also are considered to be the operating conditions for crude extract from *P. Pantotrophus.* Nar has activity over a wide range of pH (2-9) [9].

Aerobic/Anoxic Diauxic Lag

Under aerobic conditions, the Nar-catalyzed nitrate reduction respiration cannot be achieved because the movement of nitrate across the cytoplasmic membrane to the active site of Nar is inhibited [9]. Nar is an inducible enzyme that is only expressed

under anoxic denitrifying conditions. Nap can be expressed when cells are grown in both aerobic and anoxic conditions. However, most denitrifying bacteria do not contain Nap, while they should all have the gene for Nar. The reason for the existence of diauxic lag after the anoxic switch is that bacteria need time to synthesize and activate Nar. A study comparing the growth of Nap-positive and Nap-deficient bacteria reported that after switching from aerobic to anoxic conditions the Nap-negative had large lags while the Nap-positive had either no lag or short lags [1]. Also, diauxic growth of a Nap deficient mutant, KD102 (Km::NapEDABC), and the Nap-positive parent wild type strain of *Paracoccus pantotrophus* were investigated. As the Nap-deficient mutant exhibited a long diauxic lag while the wild type exhibited none under the same conditions and nearly identical aerobic growth, the assumption that Nap eliminates or shortens diauxic lag after an anoxic switch was supported [1]. Part of the designed plasmid (pKD100) used in the transformation of Paracoccus is shown in Figure 2-4.

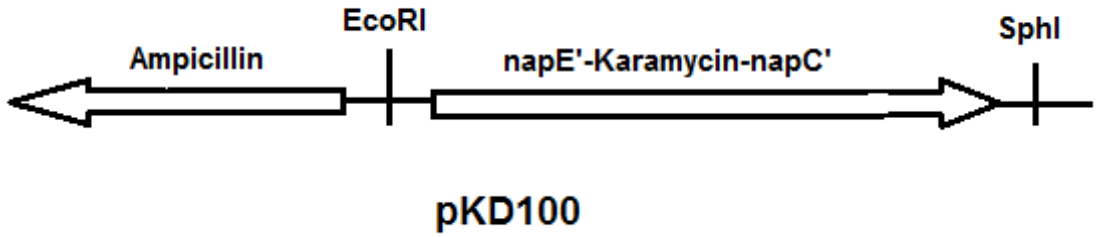

Figure 2-4. Partial designed vector used in transformation

Transport across Bacteria Cell Wall and Cell Penetration

There are two kinds of bacteria divided by cell wall structures: gram negative bacteria and gram positive bacteria. The cell wall is selectively- permeable to ions and organic molecules, and also, it can control the substrate movement in and out of cells. The gram positive bacteria cell wall has better permeability than the gram negative

bacteria cell wall (including the outer membrane) for most substances. A better permeability can enlarge the range of substances coming as nutrients and also facilitates utilization of higher-molecular substances into the surrounding medium with the intention of improving the conditions of bacterial growth and multiplication. But it also makes the bacteria more susceptible to harmful substances such as toxins . Gram-negative bacteria possess a thinner peptidoglycan wall but also a complex outer cell membrane with a hydrophobic interior which can block the penetration of hydrophilic molecules, while hydrophobic substances such as O_2, N_2, and CH_4 easily penetrate. Transport of hydrophilic molecules in gram-negative bacteria can be achieved via: 1, Open, water- filled channels (only valid when the size is less than 600 Da); 2, Fast diffusion channels when substance stereo-specific binding sites exist; 3, Active transport when specific binding sites exist while energy is provided. However, the permeability of cell wall can be improved by applying chemicals such as EDTA, lysozyme as well as NH_4^+, which help to weaken the cell wall [3].

Due to the property that cell wall is selectively permeable, it is not clear which substances can penetrate gram-negative cells. For example, β-lactamase antibiotics species, such as penicillin, can penetrate the cell wall into the periplasmic area and then work by breaking bridges connecting peptides, which are essential component of the cell membrane, leading to cell death.

There are also cell-penetrating peptides, which can serve as carriers of useful molecules into cells. This was first found when it was discovered that some cells can take up the trans-activating transcriptional activator from HIV-1 efficiently. Based on the assumption that higher efficacy of drugs will result if they penetrate the cell wall, several

cell-penetration peptides are designed and used in pharmaceutical industry and biochemistry research. For instance, M918 [10], a novel cell-penetrating peptide, is designed to deliver proteins as well as need nucleic acids efficiently. A summary of currently known penetration peptides [11] is listed in Table 2-2.

Table 2-2. Currently know penetration peptides

Tat	HIV-1
Oigoarginine	Tat derivative
p-Antp	Antermapedia homeodomain
pIsl	Igl-1 homeodomain
Transportan	Galanin-mastoparan
Pβ	gp41-SV40
Pα	gp41-SV40
Pep-1	Trip-rich motif-SV40

Also, small molecule proteins, such as Crotamine, can also penetrate the cell wall [12].

Uptake and utilization of Protein, Peptide and Free Amino Acid in Bacteria Growth

Bacterial growth is an important criterion for judging if environmental factors (such as addition, media, or pH, temperature) will be favorable to bacteria. In the biological industry, such as enzyme fermentation, a faster bacterial growth rate is usually considered to be more productive.

Among the factors that may affect bacterial growth are addition of peptides, amino acids and proteins. Addition of free protein and corresponding peptides as well as amino acids to *Lactobacillus delbrueckii 730*, improved its growth with peptide being most beneficial and free protein have the least positive impact . The difference may due to the ability of these substances to penetrate the cell wall [13]. Similiarly, n-valyl-L-valyl-L-valine addition can be utilized by *Streptococcus faecalis* [4], while growth of *Pediococcus cerevisiae* was stimulated by peptides [14], too.

It is also reported that peptide Micrococcus Isolate F4, has positive multiple effects on lactic acid bacteria mixed cultures, such as *Streptococcus lactis* 5S, *lactis* C10 and also *Streptococcus cremoris* Cl, either by increasing the growth rate or final yield. The addition of F4 helped to remove H2O2 that was produced in the fermentation, which inhibited the growth of bacteria. The mechanism is similar with that of catalase. However, the effects of isolate F4 on bacteria are more complex [15].

So it has been established that the growth of bacteria can be stimulated by the presence of small peptides. However, Pittman used a C14 labeled oligopeptide uptaken by *Bacteroides ruminicola* to prove the utilization of a big size of peptide. It is likely that the mechanism of peptide uptake by *B. ruminicola* is quite general for larger peptides. It is reported to be active transport in this case. In addition to the active transport, oligopeptides can be utilized as growth factors in a vitamin or hormone like manner [16].

In contrast to positive effects on growth, certain kinds of peptides, such as N4-(L-Valyl-L-valyl)), can be inhibitors of bacterial growth. These peptides can act by interfering with the uptake of essential amino acids (such as PABA) which is needed to facilitate bacterial growth. N4-(L-Valyl-L-valyl)) affects the growth *of Pediococcus cerevisiae, Lactobacillus plantasum, andLeuconostoc mesenteroides* [17]. These peptides can be used in the food industry as a preservative.

Also, certain bacteria will change their expression balance in response to amino acid addition. In *Prevotella albensis*, addition of free amino acid will lead it to adopt its own nutritional strategy in order to adapt to the environmental conditions of the ruminal ecosystem by slowing its growth rate [18].

Up till now, it is reported that by simply adding extract as small molecular protein, amino acid as well as peptides, growth of bacteria will be affected, either positively or negatively. Some peptides or amino acids can either diffuse into bacteria for synthesis supporting bacterial growth or as inducers that lead to certain regulation change in bacteria. Due to the reason for diauxic lag, which is the lack of inducible nitrate reductase—Nar, it was investigated whether Nap or Nar extract will stimulate bacterial after an aerobic/anoxic switch.

CHAPTER 3
MATERIALS AND METHODS

Bacteria

The two bacterial species used in this research were *Paracoccus pantotrophus* (strain ATCC #35512), which is a Nap expressing bacterium, and a Nap-deficient mutant of *P. Pantotrophus* (KD102; napEDABC::Km, from the University of Florida).

Growth of Bacteria

Bacteria were grown for 3 purposes: 1, Aerobic growth of the Nap-positive bacteria*, P. Pantotrophus*, to obtain a crude extract containing Nap; 2, Aerobic/Anoxic growth of the Nap-negative mutant, KD102, to obtain a crude extract containing Nar and no Nap. 3. Growth of KD102 switching from aerobic to anoxic conditions to investigate effects of crude extract on diauxic lag and growth rate.

P. Pantotrophus or KD102 were incubated overnight at 37°C in minimal media (Table 3-1) with sodium acetate trihydrate as the carbon source and ammonium chloride as the nitrogen source.

Table 3-1. Minimal Media recipe for *P. Pantotrophus*

Chemicals	Concentration (g/L)
Sodium acetate trihydrate	1.36
Sodium phosphate dibasic	4.2
Potassium phosphate monobasic	1.5
Ammonium chloride	0.3
Magnesium sulfate heptahydrate	0.1
Potassium nitrate	2.88
Vishniac and Santer trace element solution	4 drops (2mL)

The bacteria were grown aerobically in either a 1L or a 250 mL Erlenmeyer flask containing 500 mL or 125 mL minimal medium, respectively, at 170 rpm in an incubator-shaker. The bacteria were grown anaerobically (without head space) in 15 mL Falcon

culture tubes or in 4mL rectangular plastic cuvettes in an incubator, with shaking at 170 rpm or without shaking.

Table 3-2. Recipe for Vishniac and Santer trace element solution

Chemicals	Concentration (g/L)
EDTA	50
$ZnSO_4 \cdot 7H_2O$	22
$CaCl_2$	5.54
$MnCl_2 \cdot 4H_2O$	5.06
$FeSO_4 \cdot 7H_2O$	4.99
$CoCl_2 \cdot 6H_2O$	1.61
$CuSO_4 \cdot 5H_2O$	1.57
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.1

Crude Protein Extraction

P. Pantotrophus was incubated aerobically and then harvested when the optical density (OD) reached approximately 0.6 (A550 nm). KD102 was grown aerobically until exponential growth was confirmed and then switched to anoxic conditions. The crude protein extract was obtained by modifying the procedure of Berks [11] Bacteria were harvested at an OD of 0.3~0.4, following the resumption of growth after the anoxic switch. The harvested bacterial suspension was centrifuged at 11,380 rcf and 4°C for 20 min. The supernatant was carefully poured off and then the bacterial pellet was suspended in 10 mL of extraction buffer (Table 3-3) and then 0.25 g lysozyme (as a powder) was added and vortexed until the lysozyme was dissolved and the pellet was thoroughly disrupted. Then the suspension was incubated at 30°C for 20 min in order to break the cell membranes and release the expressed protein into the solution. The

suspension was centrifuged at 11,380 rcf and 4°C for 4 min. The supernatant was carefully poured off into a new tube and 3.7 g of ammonium sulfate (powder) was added and vortexed until the powder was dissolved and the solution became cloudy (protein was precipitated). The suspension was centrifuged at 11,380 rcf and 4°C for 4 min and the supernatant was poured off and the pellet (crude protein extract) was suspended in 10 mL of 20 mM Tris-HCl (pH=7). The extract was used immediately or stored at 4°C for a maximum of 3 days.

Table 3-3. Extraction buffer

Ingredient	Concentration
Tris/HCl	100mM/L
Na_2EDTA	3mM/L
Sucrose	0.5M/L
Adjust PH to 8 with KOH	

Experiments with Addition of Crude Extract to KD102 Eultures

KD102 was taken out from a -80°C deep freezer and incubated in Luria Broth (Bacto, city, state) at 37°C at 170rpm in a shaking incubator overnight and then transferred to minimal media and grown for 24 hrs, followed by another transfer to minimal media till the initial absorbance (1cm path length; 550nm) of the culture became 0.1. After the 2nd transfer, the culture was grown until the absorbance doubled. The culture was sparged for 4mins with nitrogen gas to strip out the dissolved oxygen. It was at this point that a volume of 1.0 (usually) or 0.5 or 0.25 mL of crude extract was added to 100mL of culture volume. One of the controls was culture with no addition of extract. Another control was the addition of 1mL extract per 100mL media containing no bacteria. Following addition of extract, the culture was mixed on a magnetic mixer for 1min and then poured into 15ml Falcon culture tubes or 4mL plastic cuvettes, leaving no

head space, and then vortexed. The culture was then incubated in an incubator (with or without shaking). The test of the absorbance was made after certain time interval. A culture in a Falcon tube was sacrificed for measurement, whereas repeated measurements were made on cuvettes.

CHAPTER 4
RESULTS AND DISCUSSION

In order to investigate the effect of crude extract from *P. Pantotrophus* and KD102 on the growth of KD102, certain groups of experiments were performed. Initially, the growth patterns of *P. Pantotrophus* and KD102 without enzyme extract addition are shown. Subsequently, the effects of adding enzyme extract harvested from either P. Pantotrophus or KD102, grown under different conditions (aerobic vs. anoxic) are shown. Finally, the effect of extract dosage is displayed.

Typical Aerobic/anoxic Growth Pattern of *P. Pantotrophus* and KD102

The figure below indicates the growth of *P. Pantotrophus* (wild type) and KD (a mutant of *P. Pantotrophus* that lacks the Nap gene) before and after anoxic switch. Upon removal of oxygen (indicated by vertical lines in the figure), *P. Pantotrophus* kept growing while the mutant stopped growing. The reason for the growth difference is that the terminal electron acceptor for the process has been changed from oxygen to nitrate. This means that the bacteria need to gain a certain amount of Nar for respiration. It has been suggested that Nap can be utilized to facilitate the adaptation of metabolism from aerobic to anoxic (Moreno-Vivián et al., 1999). Subsequently, it was demonstrated by Durvasula et al. that deletion of the gene for Nap from *P. Pantotrophus* caused the Nap-deficient mutant to exhibit significant diauxic lag, whereas the Nap-containing wild type exhibits no such lag.

Durvasula showed growth curves in which the KD mutant and P. Pantotrophus wild strain had similar characteristics under aerobic conditions. In contrast, Figure 4-1 shows considerably slower growth for the mutant. This difference is attributed to the fact that D used complex LB with NO3- as growth media, while minimal media were used in

the current study. As a matter of fact, to enable growth in minimal media, KD had to be revived in LB.

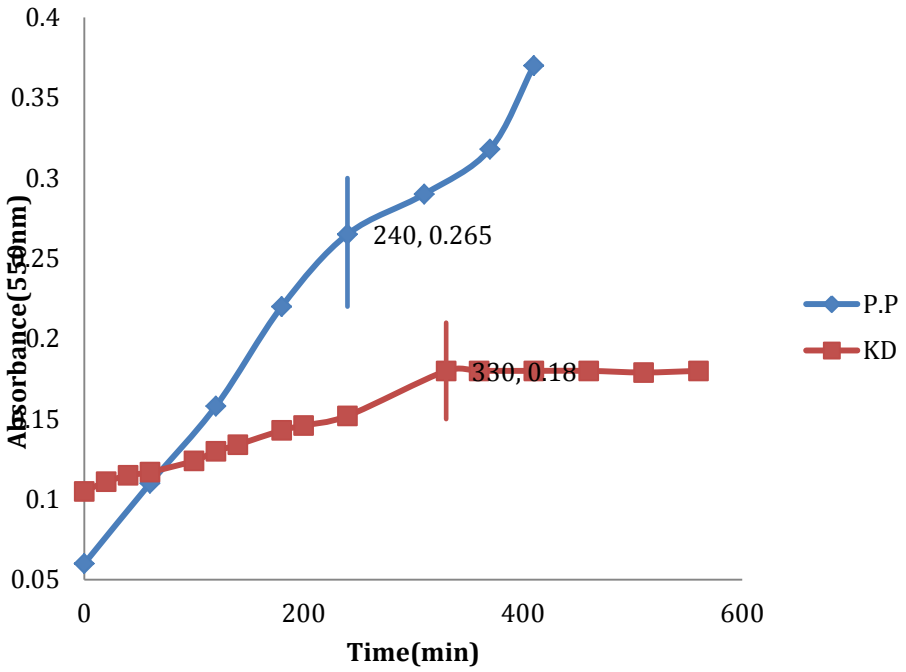

Figure 4-1. Growth curve of *P. Pantotrophus* and KD. (The switch from aerobic to anoxic conditions is indicated by a vertical line cutting through the plot for each bacterium.) .

Effect of Crude Extract from *P. Pantotrophus* on KD Growth

To begin this experiment, *P. Pantotrophus* was grown aerobically for the purpose of generating Nap. These bacteria were harvested and crude extract (containing Nap) was obtained. Subsequently, KD was grown aerobically. After about a 55% increase in biomass concentration, the dissolved oxygen was stripped and the crude extract was added to one set of Falcon tubes, whereas the other two sets contained either no addition or buffer without extract. That can be seen from the Figure 3. Without crude extract addition, growth ceased upon the switch from aerobic to anoxic condition. With crude extract addition, growth continued, albeit with a somewhat lesser slope.

To verify the previous results, the same sets of tests in Figure 4-2 were performed, except one additional control was used. The results shown in Figure 4-3 are similar with growth continuing only in the flasks in which *P. Pantotrophus* extract was added. The additional control (media and extract without bacteria) showed no growth.

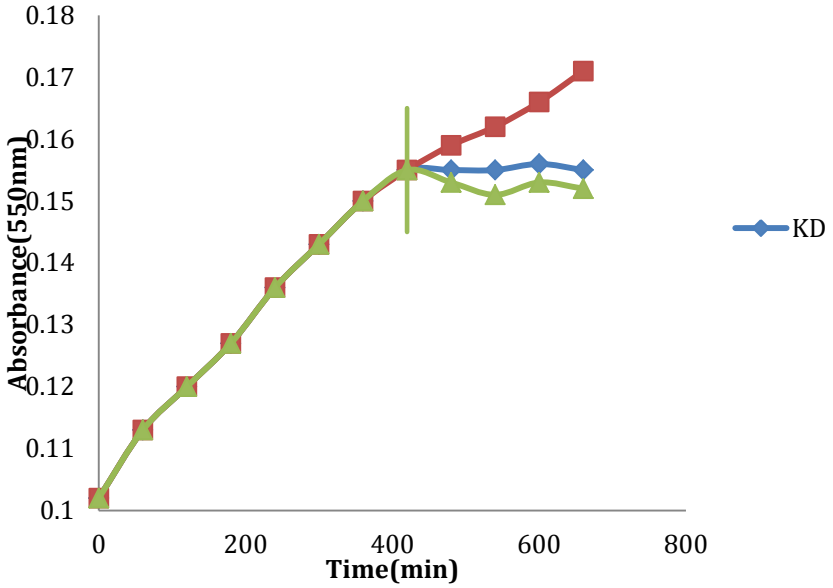

Figure 4-2. Effect of Crude extract from *P. Pantotrophus* under aerobic condition on growth of KD

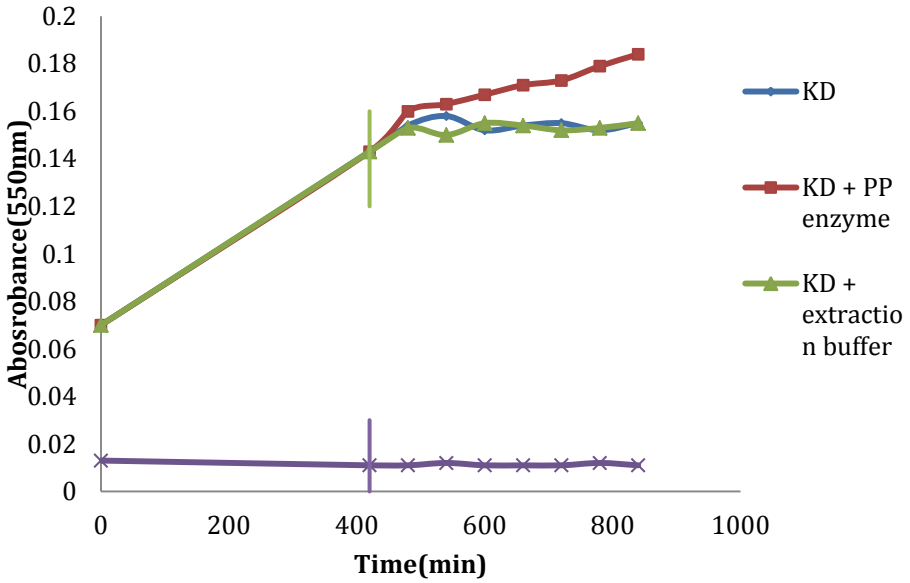

Figure 4-3. Effect of *P. Pantotrophus* extract under aerobic condition on growth of KD

Effect of Extract from KD and *P. Pantotrophus* on Growth of KD

Up till now, only extract from *P. Pantotrophus* was added for the purpose of demonstrating the effect of adding Nap to a Nap-deficient bacterium. However, the crude extract would also contain some Nar. Thus, it was of interest to investigate the effect of adding a Nap-deficient extract to KD. In order to accomplish this, we grew KD under anoxic conditions long enough so that the growth resumed (AxEP), presumably due to biosynthesis of Nar, as shown in Figure 4-4. A long diauxic lag of approximately 40 hours (AxLP) was also observed.

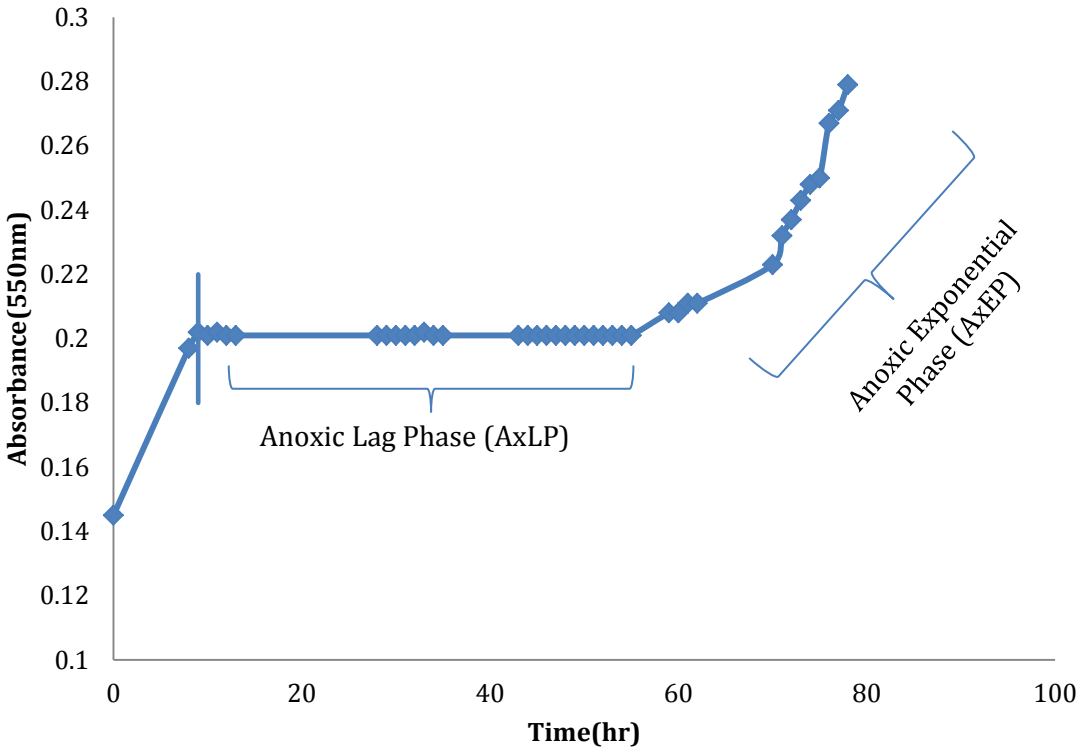

Figure 4-4. Growth curve of KD before and after the anoxic switch

Figure 4-5 shows the effect of adding KD extract obtained from the anoxic lag phase (10mL of extract suspension per liter culture). It is seen that adding the KD AxLP extract did not promote KD growth after the switch to anoxic conditions. As an extra control, it is shown that KD would have continued growing if it had not been switched to anoxic conditions.

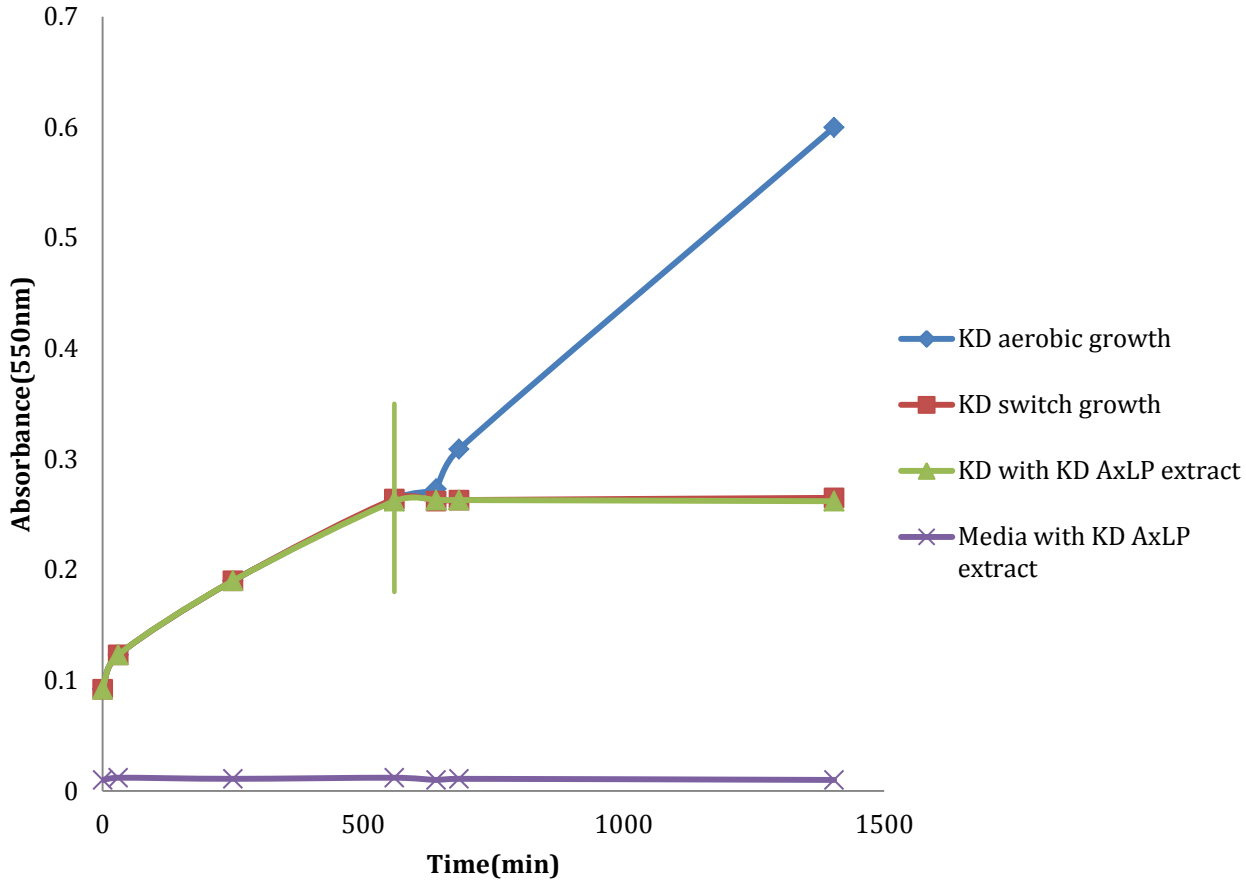

Figure 4-5. The effect of crude extract from KD obtained from the anoxic lag phase

Figure 4-6 shows that different results are obtained if added KD extract is obtained from the anoxic exponential phase (in this case, the last point in Figure 8). It is apparent that this KD AxEP extract (10mL of extract suspension per liter culture) causes the bacteria to immediately start growing after the anoxic switch. The figure shows a similar

effect by the addition of *P. Pantotrophus* extract, while the bacteria without extract addition showed practically no growth until the end of the diauxic phase, about 25 hours later.

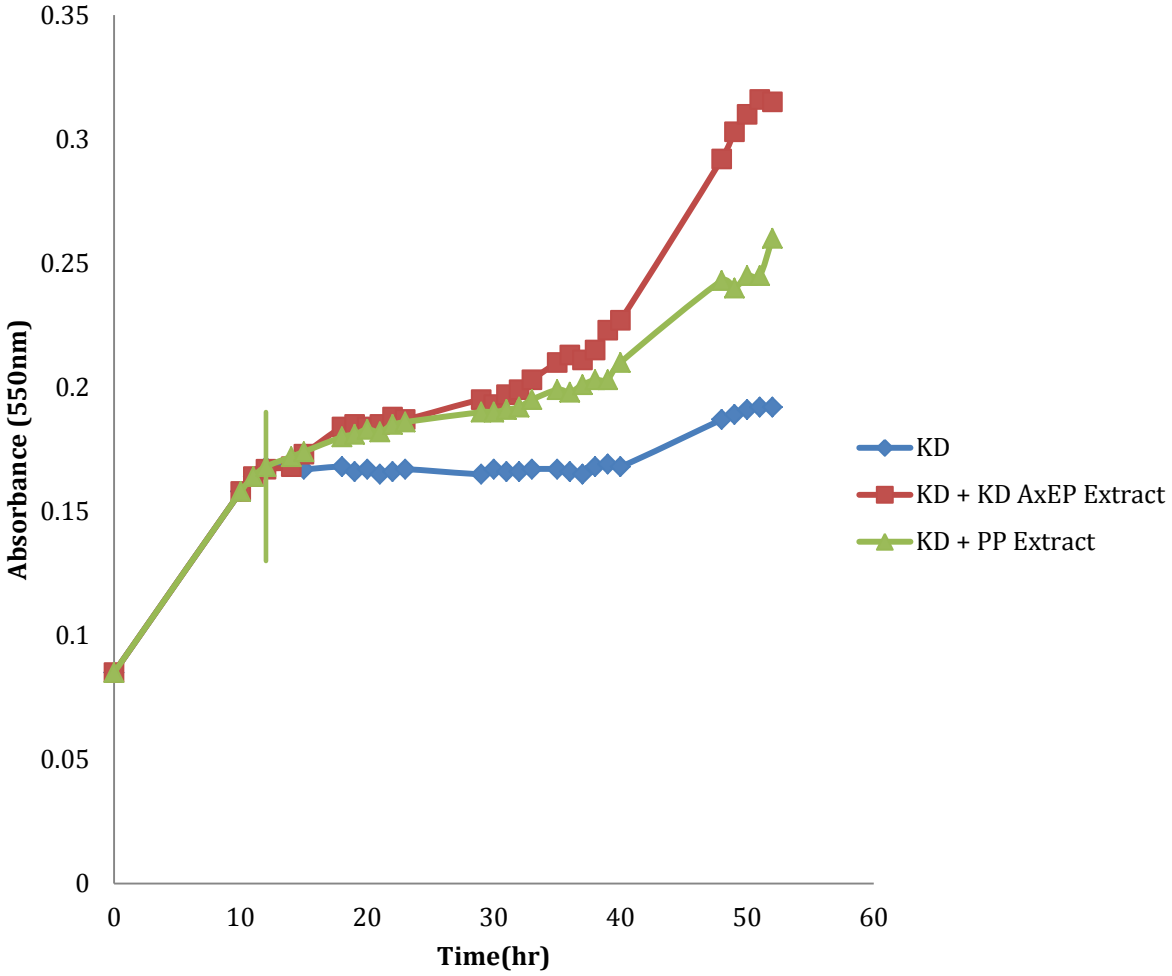

Figure 4-6. The effect of crude extract from KD under anoxic exponential phase and crude extract from *P. Pantotrophus* under aerobic exponential phase

The anoxic period data displayed in Figure 4-6 are replotted in Figure 4-7 in terms of the natural logarithm of absorbance versus time. It is apparent that a single exponential phase (fitting with a single straight line) would not provide good fits to the data from cultures in which enzyme extract was added (top two curves). Instead, these

data could be better characterized by dividing the growth into two exponential phases, the first with a lower specific growth rate (0.0060 hr^{-1} for KD AxEP extract and 0.0050 hr^{-1} for *P. Pantotrophus* enzyme extract) and the second with a higher specific growth rate (0.0273 hr^{-1} for KD AxEP extract and 0.0161 hr^{-1} for *P. Pantotrophus* enzyme extract). Without extract addition, the specific growth rate of KD is practically zero for 25 hours, after which exponential growth started with specific growth rate of 0.0118hr^{-1}.

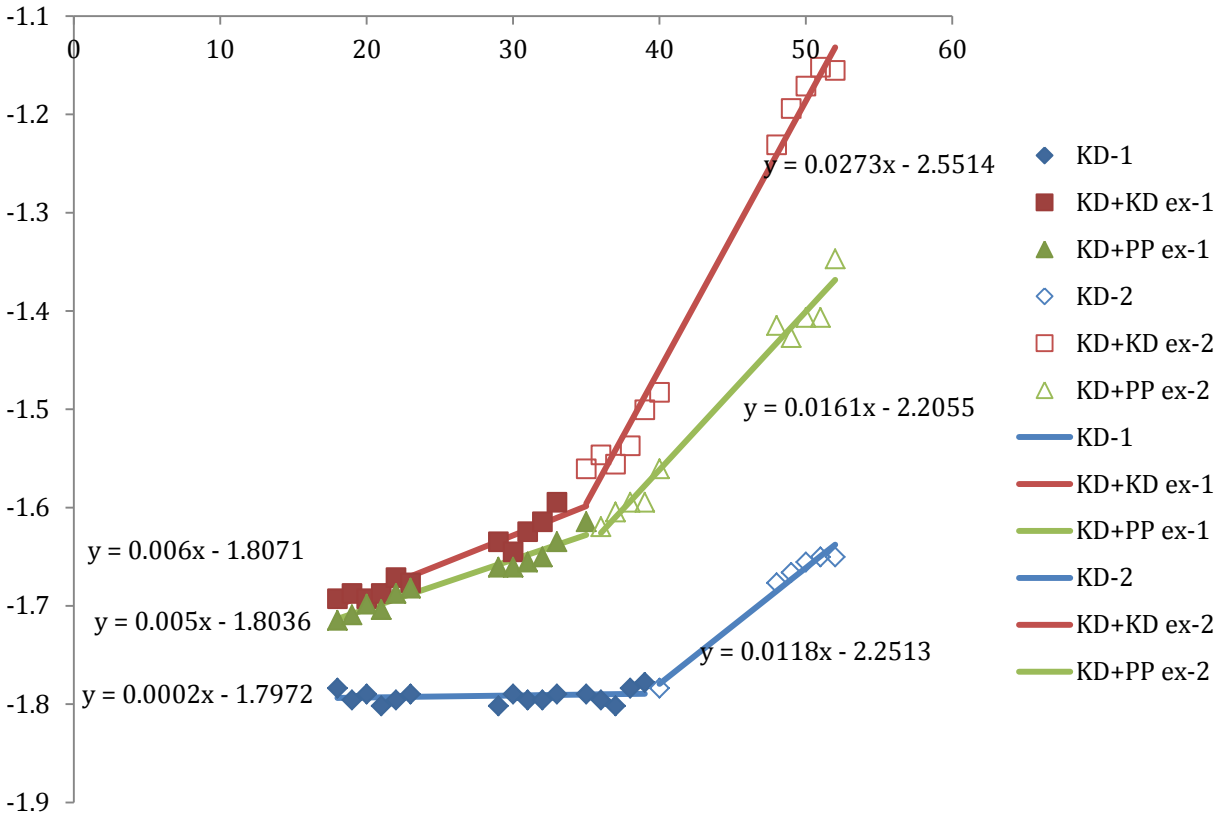

Figure 4-7. Effects of KD AxEP and *P. Pantotrophus* extracts on the anoxic specific growth rate of KD .

Different effect of Different dose of crude extracts from KD under anoxic exponential growth condition on anoxic growth was apparent in Figure 4-8. There was still no growth of KD under anoxic condition, while KD with addition of full dose extract, one-half dose extract and one-fourth dose extract started to grow with different growth

rates (slopes) after a short time of stationary growth. That indicates the crude extracts from KD under anoxic exponential growth condition can help to improve the diauxic lag.

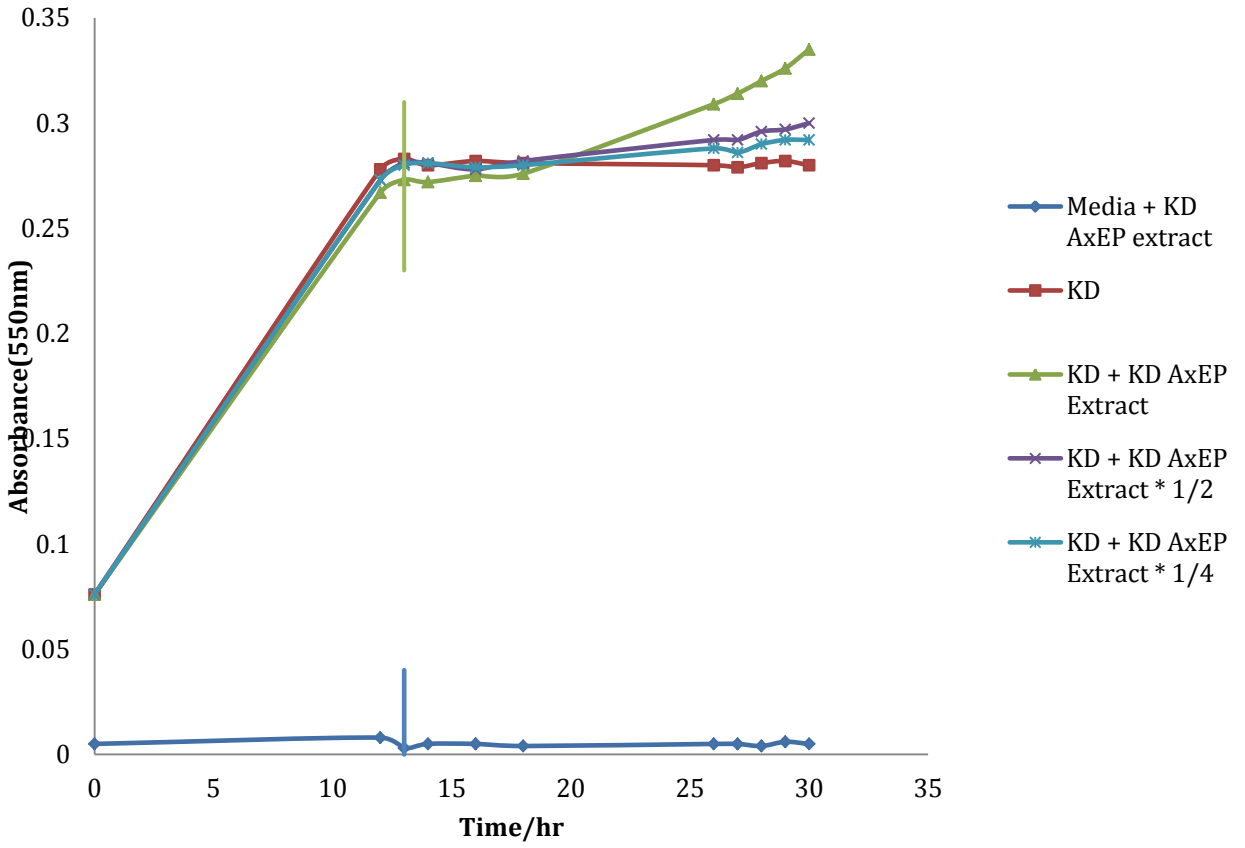

Figure 4-8. Effect of Different dose of extract from KD under anoxic exponential growth condition on anoxic growth of KD

The anoxic data from Figure 4-8 were replotted in terms of logarithm of culture absorbance versus time and are shown in Figure 4-9, along with the trend equations. Only the full dosage data exhibit the double exponential phase pattern observed in Figure 13. Half dose and quarter dose data exhibited a lag of approximately 10 hours, while without extract addition the lag is longer than 20 hours. The full dosage specific growth rates (0.0027hr^{-1} and 0.0199 hr^{-1}) were lower than the corresponding rates in Figure 4-6. This can perhaps be attributed to the fact that the extract used for the Figure 4-8 data was three days old while that used for the figure 4-6 data was fresh.

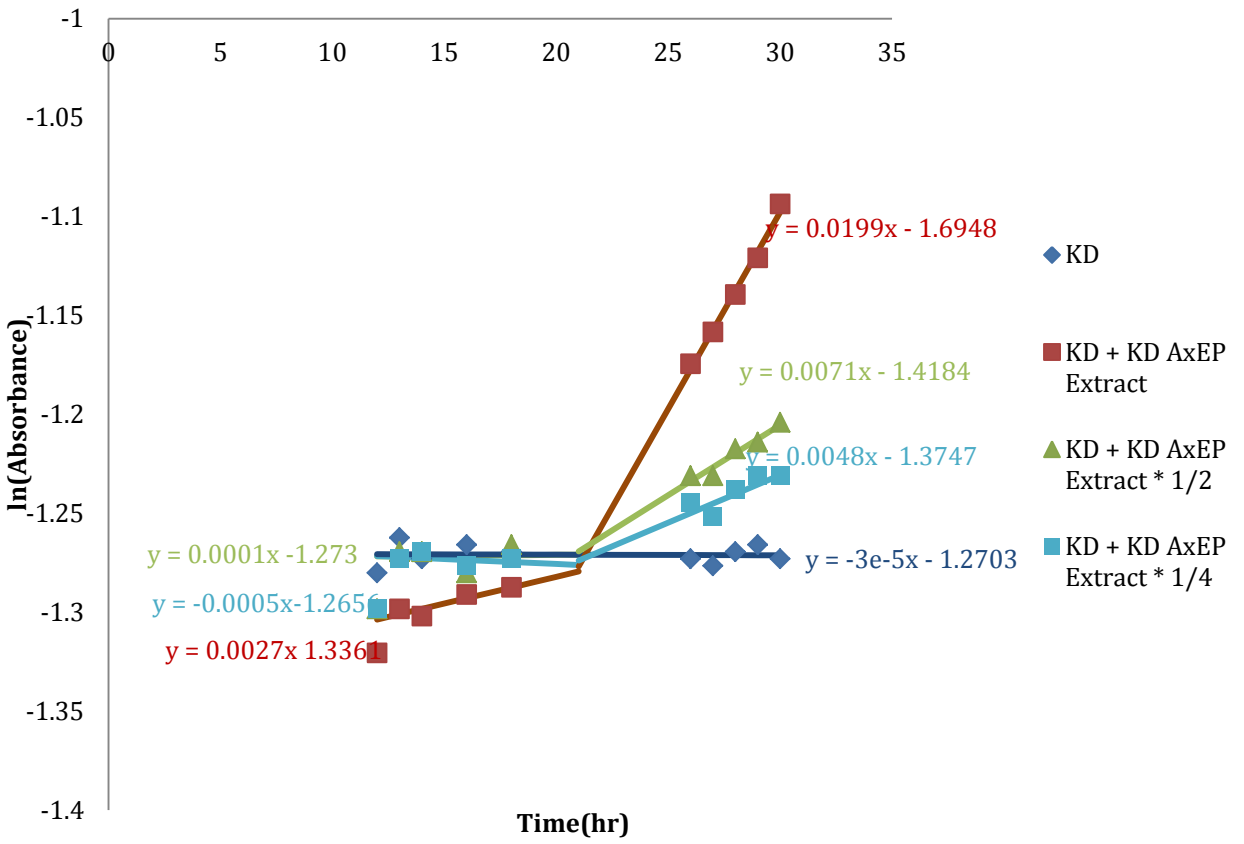

Figure 4-9. Effects of KD AxEP extract dosages on the anoxic specific growth rate of KD

Figure 4-9 also shows that the end of the first exponential phase with full dosage approximately coincides with the end of the lag phase with lower dosages. So perhaps the lower growth initial exponential phase should be considered as low-growth 39999lag phase. With this interpretation, it appears that extract dosage does not significantly affect the duration of the lag phase. However, after about 10 hours lag, the crude extract helped to increase the specific growth rate apparently, and the full dosage addition improved the specific growth rate right after the anoxic switch. Moreover, as Figure 4-10 shows, enzyme extract dosage significantly affects the final specific growth rate and does so linearly.

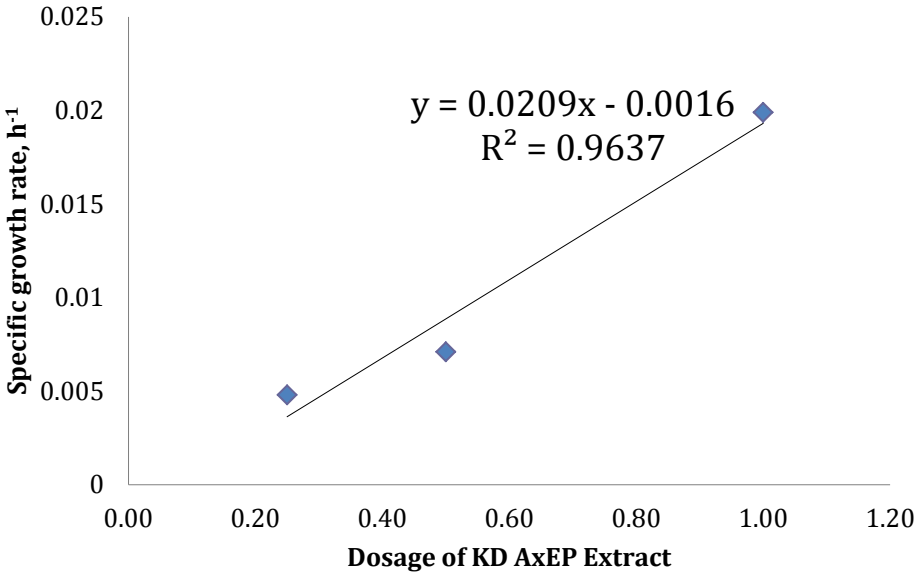

Figure 4-10. Effect of KD AxEP extract dosage on specific growth rates of the final exponential growth phase

CHAPTER 5
CONCLUSION

In this study it was shown that addition of crude protein extract from either the Nap-positive wild type *P. Pantotrophus* strain or the Nap-negative KD-102 mutant positively affected the growth of KD-102 after switching from aerobic to anoxic growth. The diauxic lag that was observed without extract addition was reduced or, in some cases, eliminated. Furthermore, the increase in growth rate varies linearly with the amount of crude extract added.

The most likely explanation of the observed effect is that Nar or Nap subunits did not penetrate the cell wall and membrane but smaller molecules (possibly created by degrading the enzymes) penetrated and served as precursors or inducers to speed up the production of Nar, which in turn resulted in the observed positive growth effects.

The beneficial effects of crude extract addition might be applicable to a wide range of bacterial cultures, so this work might provide an easy and low cost foundation for an effective mass production process for industrial application.

CHAPTER 6
FUTURE WORK

Repeat the experiments using LB growth medium and no EDTA. This will investigate whether EDTA caused cell wall penetration and whether the benefit of crude extract was mainly to overcome the lack of a growth factor in the minimal medium used.

Repeat the experiments with one or more wild type gram-negative denitrifiers. A positive outcome will prove that the benefits of crude protein extract addition can be widespread.

Subdivide the crude extract into parts of different size range and repeat the experiments with each of the parts. This will ellucidate which part of the extract is beneficial and will help improve our understanding of the mechanism.

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BIOGRAPHICAL SKETCH

Yin Jun was born in Henan, China. She received her bachelor's degree in pharmaceutical engineering from Xi'an university of technology in Xi'an, China, in 2004. She has been worked as a research assistant of pharmacokinetics in Hongkong polytech university, Shenzhen institution and then was accepted to the University of Florida to pursue a Master of Science. She currently is ph.D candidate of University of Florida, chemical engineering.