Journal of
Bioremediation & Biodegradation

Yin et al., J Bioremediat Biodegrad 2016, 7:5
DOI: 10.4172/2155-6199.1000367

Research Article Open Access

Effect of Protein Crude Extract on Oxic/Anoxic Diauxic Growth of a Nap-deficient Mutant of *Paracoccus pantotrophus*

Jun Yin[1]*, Wes Stevenson[1], Zhiling Guo[2], Ben Koopman[2] and Spyros A Svoronos[1]

[1]*Department of Chemical Engineering, University of Florida, Gainesville, FL 32611, USA*
[2]*Department of Environmental Engineering, University of Florida, Gainesville, FL 32611, USA*

Abstract

In this study it is shown that addition of crude protein extract containing either membrane bound nitrate reductase (Nar) or periplasmic nitrate reductase (Nap) shortens the diauxic lag of denitrifying bacteria switched from aerobic to anoxic conditions. The specific growth rate under anoxic conditions, following resumption of exponential growth, was found to be linearly related to the extract dose.

Keywords: Denitrification; Diauxic lag; Nitrate reductase; Nap; Nar; *Paracoccus pantotrophus*

Abbreviations: KD: KD102; Km: napEDABC; Nap: Periplasmic nitrate reductase; Nar: Membrane bound nitrate reductase; PP: *Paracoccus pantotrophus*

Introduction

Discharge of fixed nitrogen forms (NH_4^+, NO_3^-, Organic N) to the aquatic environment causes detrimental effects that include methemoglobinemia (if the water is used for drinking), ammonia toxicity, and eutrophication. Removal of nitrogen from wastewater is therefore an important means of improving water quality [1,2]. Removal of ammonia and nitrate can be achieved in a biological wastewater treatment plant by exposing a mixed culture of bacteria 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. When oxygen is absent, denitrifying bacteria reduce nitrate to nitrite and ultimately to nitrogen gas. This combined, balanced process can decrease nitrogen to concentrations that are sufficiently low for discharge to the environment.

Many denitrifying bacteria experience periods without growth during the transition from aerobic to anoxic conditions. These periods, which are termed diauxic lag, negatively affect the kinetics of nitrogen removal because without growth, nitrate is not metabolized. Diauxic lag occurs because the membrane-bound nitrate reductase (Nar) of these bacteria is synthesized only under anoxic conditions. During aerobic periods, Nar is diluted due to growth [3,4]. If the aerobic period is long, the level of Nar can drop sufficiently to reduce the rate of denitrification at the beginning of the subsequent anoxic period to nearly zero. Nar is responsible for transport of nitrate into the cell and, in turn, intracellular nitrate stimulates the biosynthesis of Nar. Thus, the biosynthesis of Nar is autocatalytic with respect to the intracellular Nar level [5]. A method that jump starts production of Nar, such as addition of nitrate reductase or its fragments to the culture medium, could jump start the process of Nar synthesis and thus shorten diauxic lag. Some denitrifying bacteria express a second reductase, periplasmic nitrate reductase (Nap), under aerobic conditions [6]. Nap has been shown to eliminate or reduce the diauxic lag [7].

This work investigates whether crude extracts from denitrifying bacteria containing Nap or Nar can shorten diauxic lag if added to cultures of denitrifying bacteria immediately following a switch from aerobic to anoxic conditions. The test bacterium was a mutant of *Paracoccus pantotrophus* that had been genetically modified to prevent expression of Nap [7]. Nar-containing extract was prepared from the test bacteria harvested while growing exponentially under anoxic conditions. Nap-containing extract was prepared from the wild type of *Paracoccus pantotrophus* growing exponentially under aerobic conditions.

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). Henceforth, the wild strain will be referred to as PP and the mutant as KD.

Growth of bacteria

Bacteria were grown for four purposes: 1. To obtain a crude extract containing only Nap or its fragments. 2. To obtain a crude extract containing only Nar and its fragments. 2. To obtain a crude extract containing neither Nap nor Nar. 4. To investigate effects of crude extract addition on diauxic lag and exponential anoxic specific growth rate.

PP or KD were incubated overnight at 37°C in minimal media [7] with sodium acetate trihydrate as the carbon source and ammonium chloride as the nitrogen source. The bacteria were grown aerobically in either a 1 L or a 250 mL Erlenmeyer flask containing 500 mL or 125 mL minimal medium, respectively, at 170 rpm in an incubator-shaker. Following aerobic growth, the bacteria were grown anaerobically in sealed 15 mL Falcon culture tubes or in sealed 4 mL rectangular plastic cuvettes, both with no head space, in an incubator with shaking at 170 rpm.

***Corresponding author:** Jun Yin, Department of Chemical Engineering, Nuclear Science Building, Room 402, University of Florida, Gainesville, FL 32611-6005, USA, Tel: +3522266574; E-mail: sarayj@ufl.edu

Received July 30, 2016; **Accepted** August 09, 2016; **Published** August 10, 2016

Citation: Yin J, Stevenson W, Guo Z, Koopman B, Svoronos SA (2016) Effect of Protein Crude Extract on Oxic/Anoxic Diauxic Growth of a Nap-deficient Mutant of *Paracoccus pantotrophus*. J Bioremediat Biodegrad 7: 367. doi:10.4172/2155-6199.1000367

Crude protein extraction

PP was incubated aerobically and then harvested when its biomass reached approximately 1.28 mg/mL. The bacteria at this stage would contain negligible Nar because Nar is not biosynthesized under aerobic conditions, but would contain abundant Nap because Nap is biosynthesized under aerobic conditions. KD was grown aerobically until exponential growth was confirmed and then switched to anoxic conditions. Bacteria were harvested immediately after the anoxic switch, at which point the cells would contain negligible levels of Nar (again, because of no Nar biosynthesis under aerobic conditions) and no Nap (because it is the Nap-deficient mutant) at approximately 0.47 mg/mL. KD was also harvested after the resumption of exponential growth when the biomass reached 0.68 ~ 0.88 mg/mL, when Nar levels were high, sufficient to support exponential growth.

The crude protein extract was obtained by modifying the procedure of Berks [8]. 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 (100 mM Tris/HCl, 3 mM Na_2EDTA, 0.5 M sucrose) and then 0.25 g lysozyme (as a powder) was added and the mixture was vortexed until the lysozyme was dissolved and the pellet was thoroughly disrupted. The suspension was then incubated at 30°C for 20 min, followed by centrifugation at 11,380 rcf and 4°C for 4 min. The supernatant was collected and mixed with 3.7 g of ammonium sulfate (powder). The mixture was vortexed until the powder was dissolved and the solution became cloudy (i.e., protein was precipitated), followed by centrifugation at 11,380 rcf and 4°C for 4 min. After pouring off the supernatant, the pellet was dissolved 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. Extract prepared with PP biomass harvested under aerobic conditions (with negligible Nar and abundant Nap) is referred to as Nap-extract. Extract prepared with KD harvested immediately after the anoxic switch is referred to as Nap-and-Nar-free extract. Extract prepared with KD harvested during anoxic exponential phase is referred to as Nar-extract.

Experiments with addition of crude extract to KD cultures

KD was taken out from a -80°C deep freezer and incubated in Luria Broth (BD science, New Jersey, NJ, USA) at 37°C at 170 rpm in a shaking incubator overnight and then transferred to minimal media and grown for 24 hrs, followed by another transfer to minimal media until the initial absorbance (1 cm path length; 550 nm) of the culture became approximately 0.1 (0.27 mg/mL). After the second transfer, the culture was grown until the absorbance approximately doubled. The culture was sparged for 4 min with nitrogen gas to strip out the dissolved oxygen. It was at this point that a specific volume (1.0, 0.5 or 0.25 mL) of crude extract was added to 100 mL of culture volume. Following addition of extract, the culture was mixed on a magnetic mixer for 1 min and then poured into 15 ml Falcon culture tubes or 4 mL plastic cuvettes, leaving no head space, and then vortexed. The culture was then incubated in a shaking incubator. The Falcon tubes were sacrificed at regular intervals for measurement of culture absorbance, whereas repeated measurements of culture absorbance were made from the cuvettes.

Experiments were conducted by adding to the culture Nap-extract, Nar-extract, or, for control, Nap-and-Nar-free extract. As a further control, in some experiments a culture of KD was passed through an anoxic switch followed by addition of extraction buffer or by no addition whatsoever. Another control was the addition, to media containing no bacteria, of 1 mL of Nap-extract or Nap-and-Nar-free extract per 100 mL.

Results and Discussion

Nap-extract positively affected the growth of KD after switching from aerobic to anoxic growth

Typical growth patterns of PP and the Nap-deficient mutant of PP (KD) when switched from aerobic to anoxic conditions are shown in Figure 1. As expected, PP kept growing upon removal of oxygen. In contrast, KD stopped growing upon the removal of oxygen. Figure 2 shows that, following the anoxic switch, KD resumes growth only after a very long lag (approximately 40 hours). The substantially longer length of this lag, in comparison to that reported by Durvasula [7], is attributed to the use of minimal media in the present study, as opposed to the use of rich media by Durvasula et al. [7].

If crude extract of PP (Nap-extract) is added to the KD culture at the same time that oxygen is removed, the bacteria continues growing, as seen in Figure 3A. Without such extract addition (i.e., no addition or addition of extraction buffer alone) growth stopped, as before. A repeat of the experiment, as shown in Figure 3B, generated nearly equivalent results. An additional control was included in this experiment to check whether the extract itself, rather than bacterial growth, gave the appearance of increasing culture absorbance. This possibility was disproven by the fact that addition of Nap-extract to growth media without bacteria causes no change in absorbance.

Figure 3C shows that adding Nap-and-Nar-free extract (i.e., extract prepared with KD harvested immediately after the anoxic switch) did not prevent diauxic lag after the switch to anoxic conditions. The aerobic control shows that KD remained viable throughout the experiment.

Nar-extract elevated the growth of KD after switching from aerobic to anoxic growth

In contrast to the results shown in Figures 3C and 4A shows that addition of Nar-extract (i.e., extract prepared from KD haversted during the exponential portion of the anoxic phase) eliminates the anoxic lag of KD. Similar improvement on KD growth was also observed with Nap-extract (i.e., extract obtained from PP harvested while growing exponentially under aerobic conditions). In contrast, the bacteria without extract addition showed practically no growth until the end of the diauxic lag phase, about 25 hours later.

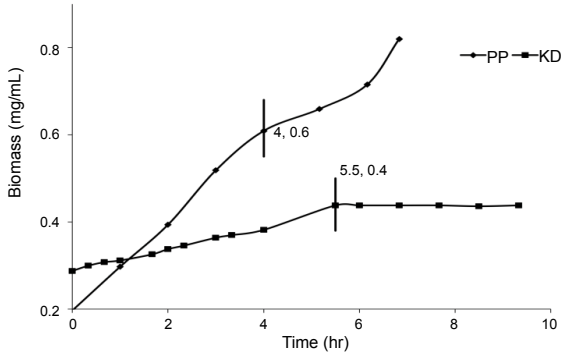

Figure 1: Growth curves of PP and KD. The switch from aerobic to anoxic conditions is indicated by a vertical line cutting through the plot for each bacterium.

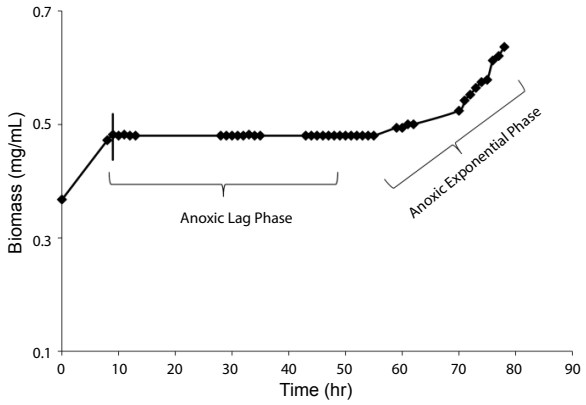

Figure 2: Complete growth curve of KD before and after the anoxic switch. The switch is indicated by the vertical line.

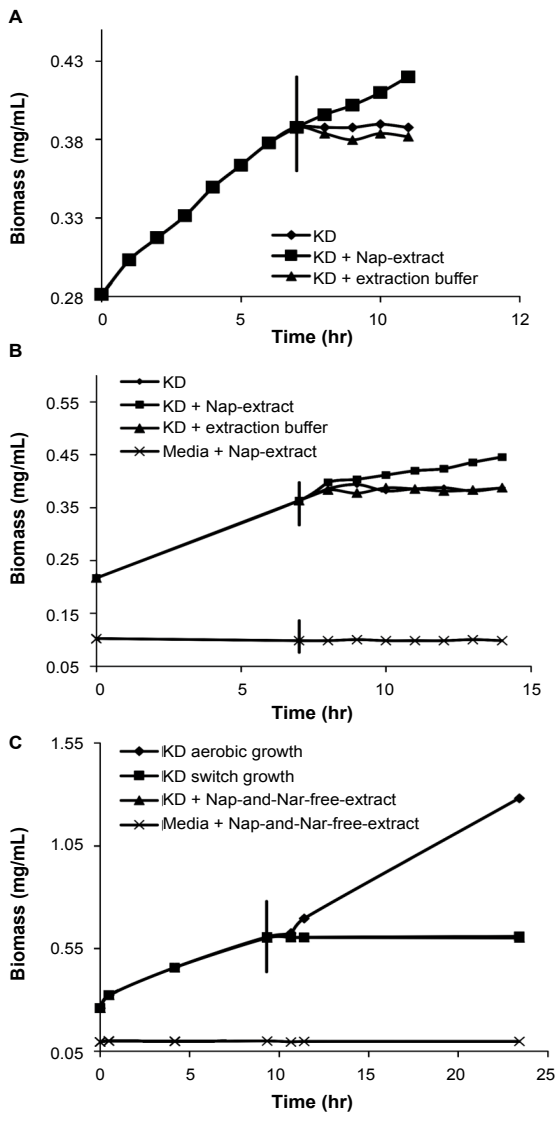

Figure 3: Effect of Nap-extract on growth of KD after anoxic switch. Each switch is shown by a vertical line. (A) and (B) compare the effect of Nap-extract addition to various controls; (C) Additional controls.

The anoxic period data displayed in Figure 4A are re-plotted in Figure 4B in terms of the natural logarithm of absorbance versus time. It is apparent that, during the anoxic conditions, 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 (0.005 hr^{-1} for Nar-extract and 0.0042 hr^{-1} for Nap-extract) and the second with a higher specific growth rate (0.0238 hr^{-1} for Nar-extract and 0.038 hr^{-1} for Nap-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 0.0097 hr^{-1}.

The specific growth rate of the final exponential phase of Nar extract was positively correlated with the dosage of Nar extract

To investigate the dose-response effect, the KD culture was dosed with various concentrations of Nar-extract. The response in terms of specific growth rate of KD following the anoxic switch was clearly dependent on Nar-extract dose, as shown in Figure 5B. The response in terms of diauxic lag of KD following the anoxic switch was also dependent on the dosage.

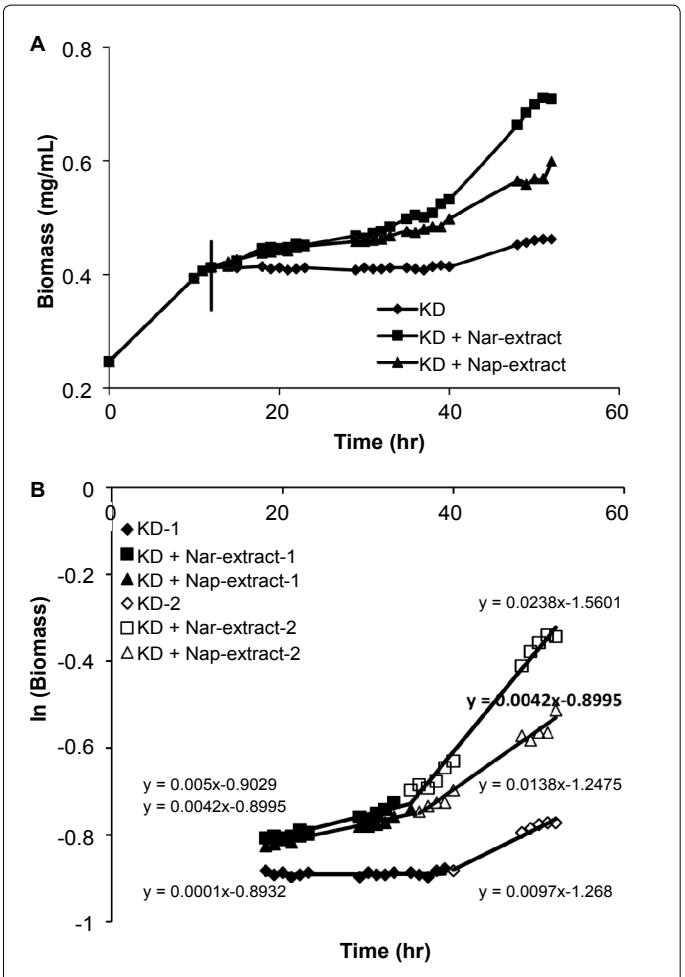

Figure 4: Effect of Nar-extract and effect of Nap-extract on growth of KD after anoxic switch. Data in (A) are replotted on a semilog scale in (B) and the results of linear regression analyses are shown.

Only the full dosage data exhibited the double exponential phase pattern that was shown in Figure 5B. Half dose and quarter doses of extract led to resumption of exponential growth after a lag of approximately 10 hours (Without extract addition, the anoxic lag is at least 20 hours). The full dosage specific growth rates (0.0041 hr^{-1} and 0.0178 hr^{-1}) were lower than the corresponding rates in Figure 4B. This may be due to the use of extract that had been stored for 3 days, whereas the extract for the experimental data shown in Figures 4A and 4B was prepared immediately before the experiement.

The correlation of specific growth rates of the final exponential phase with extract dose is shown in Figure 6. As indicated, there is a linear relationship between the dose and the response.

Figure 5B 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 lag phase. Even if the lower growth phase is considered as lag, it is considerably shorter than the lags of 40 hours and 25 hours shown in Figures 2 and 4B, respectively.

Nap and Nar free extract added to KD culture at the beginning of the anoxic switch did not eliminate or even shorten diauxic lag of the culture. On the other hand, the diauxic lag of KD was eliminated by addition of extract from either PP or KD growing exponentially under anoxic conditions. It is notable that, whereas there is no diauxic lag in KD culture supplemented with a sufficient amount of Nar-containing extract, the anoxic phase was characterized by two different specific growth rates: The first, and lower, specific growth continued for a period slightly shorter than the lag of the control, followed by a second period of higher specific growth rate.

KD exhibited a slower anoxic exponential phase specific growth rate with addition of 3 day old extract in comparison to culture dosed with fresh extract, as shown by comparing Figure 5B-4B. This could suggest that degradation of certain components of the extract made it less effective for eliminating the diauxic lag.

Finally, the strongly linear relationship between the specific growth rate of the second anoxic exponential phase and dosage of Nap-extract clearly indicates that the extract not only eliminates diauxic lag, but the effect is also directly proportional to dose. We hypothesize that increasing the extract dose resulted in faster transport of nitrate into the cell, thus hastening the onset of Nar biosynthesis [9]. Furthermore, the higher specific growth observed with higher dosage of extract suggests that more energy is available for growth, and the origin of energy resource is due to enhanced electron transport rate that is linked to the intrecellular Nar concentration [10,11].

The most likely explanation of the observed effect is that Nar or Nap subunits did not penetrate the cell wall [12] and membrane but smaller molecules (possibly created by degrading the enzymes [13,14] penetrated and served as precursors or inducers [15,16] to speed up the production of Nar, which in turn resulted in the observed positive growth effects.

Conclusions

Our study showed that addition of either Nap-extract or Nar-extract can positively affect the growth of KD after switching from aerobic to anoxic growth. The diauxic lag observed with extract addition was reduced or, in some cases, eliminated. Furthermore, the specific growth rate varied linearly with the amount of crude extract added. The beneficial effects of crude extract addition might be applicable to a wide range of bacterial cultures.

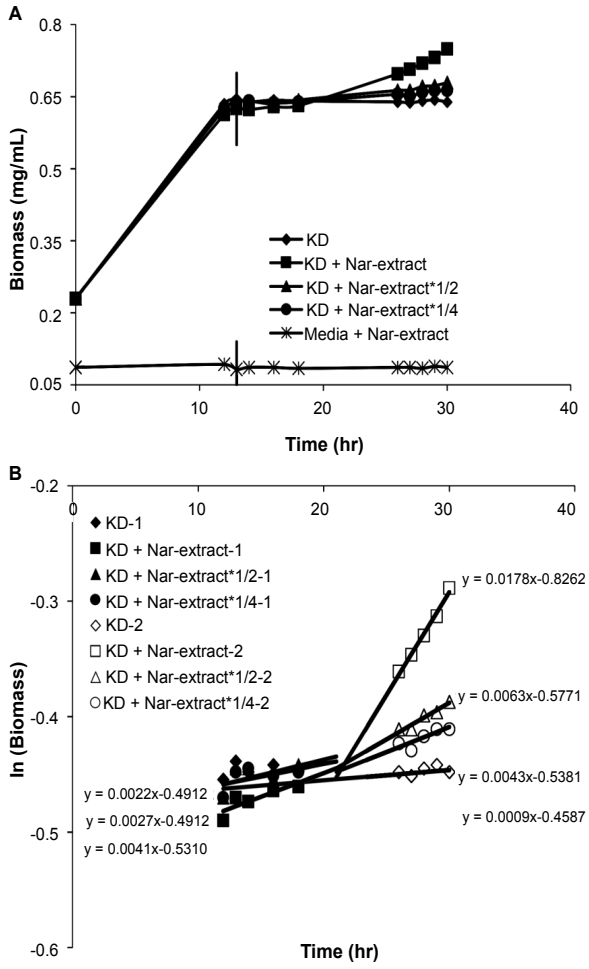

Figure 5: Effect of Nar-extract dosages on the anoxic specific growth rate of KD. Data in (A) are replotted on a semilog scale in (B) and the results of linear regression analyses are shown.

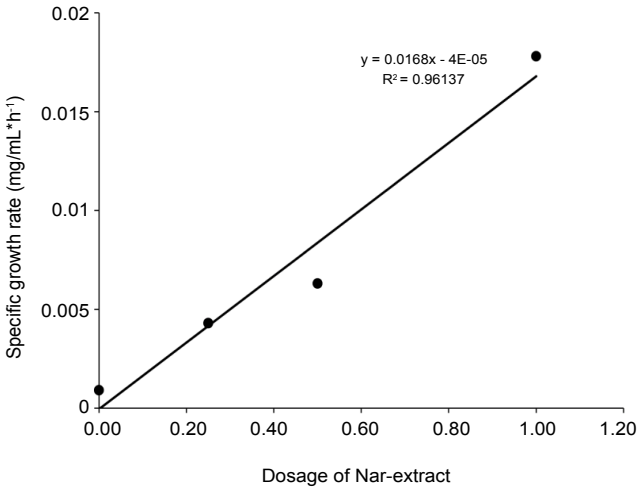

Figure 6: Dose-response relationship for Nar-extract.

Acknowledgements

The present research was carried out at the Academic Interphase Laboratory of the University of Florida Water Reclaimation Facility.

References

1. Park JY, Yoo YJ (2009) Biological nitrate removal in industrial wastewater treatment: which electron donor we can choose. Appl Microbiol Biotechnol 82: 415-429.
2. Mohseni-Bandpi A, Elliott DJ, Zazouli MA (2013) Biological nitrate removal processes from drinking water supply-a review. J Environ Health Sci Eng 11: 35.
3. Gouw M, Bozic R, Koopman B, Svoronos SA (2001) Effect of nitrate exposure history on the oxygen/nitrate diauxic growth of *Pseudomonas denitrificans*. Water research 35: 2794-2798.
4. Liu PH, Svoronos SA, Koopman B (1998) Experimental and modeling study of diauxic lag of *Pseudomonas denitrificans* switching from oxic to anoxic conditions. Biotechnology and bioengineering 60: 649-655.
5. Casasus AI, Hamilton RK, Svoronos SA, Koopman BA (2005) A simple model for diauxic growth of denitrifying bacteria. Water research 39: 1914-1920.
6. Bell LC, Richardson DJ, Ferguson SJ (1990) Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*. FEBS letters 265: 85-87.
7. Durvasula K, Jantama K, Fischer K, Vega A (2009) Effect of periplasmic nitrate reductase on diauxic lag of *Paracoccus pantotrophus*. Biotechnology progress 25: 973-979.
8. Berks BC, Richardson DJ, Robinson C, Reilly A (1994) Purification and characterization of the periplasmic nitrate reductase from *Thiosphaera pantotropha*. European journal of biochemistry 220: 117-124.
9. Hamilton R, Casasus A, Rasche M, Narang A (2005) Structured model for denitrifier diauxic growth. Biotechnology and bioengineering 90: 501-508.
10. MacGregor CH, Schnaitman CA, Normansell DE (1974) Purification and properties of nitrate reductase from *Escherichia coli* K12. The Journal of biological chemistry 249: 5321-5327.
11. Enoch HG, Lester RL (1975) The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. The Journal of biological chemistry 250: 6693-6705.
12. Clegg RA (1975) The size of nitrate reductase in *Escherichia coli*. Biochemical Society transactions 3: 691-694.
13. Johansson HJ, El-Andaloussi S, Holm T, Mäe M, Jänes J, et al. (2008) Characterization of a Novel Cytotoxic Cell-penetrating Peptide Derived From p14ARF Protein. Molecular Therapy 16: 115-123.
14. Pittman KA, Lakshmanan S, Bryant MP (1967) Oligopeptide uptake by *Bacteroides ruminicola*. Journal of bacteriology 93: 1499-1508.
15. Sebbage V (2009) Cell-penetrating peptides and their therapeutic applications. Bioscience Horizons 2: 64-72.
16. El-Andaloussi S, Johansson HJ, Holm T, Langel U (2007) A novel cell-penetrating peptide, M918, for efficient delivery of proteins and peptide nucleic acids. Molecular therapy 15: 1820-1826.