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Effects of Temperature on Denitrifying Growth and Nitrate Reduction End Products of *Comamonas testosteroni* Isolated from Estuarine Sediment

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Predictions of seasonal changes in N_2O emission that occur in natural estuaries are important to anticipate the future implications of global warming. This study showed the effect of temperature on denitrifying growth and nitrate reduction end product of *Comamonas testoteroni* isolated from estuarine sediment using both batch and continuous cultures. The μ_{max} values of *Comamonas testosteroni* grown in anaerobic batch culture were increased with increasing temperature, and the highest μ_{max} was found at 26 °C. Concentrations of nitrate reduced (mg^{-1} dried weight cells) were higher at low temperature. Concentrations of N_2 produced were higher at low temperature and the production of N_2 was higher than both NO_2^- and N_2O productions.

Key words: denitrification, nitrate reduction, nitrous oxide, *Comamonas testosteroni*

Denitrification and dissimilative nitrate reduction to ammonium are the major processes of nitrate removal in aquatic environments. Both processes have an implication for global warming, due to their emission of N_2O, a potential greenhouse gas. N_2O concentration is currently increasing at 0.2-0.3% per year (Conrad 1995), and 90% of global N_2O emission comes from biotic processes. Many studies have been conducted to determine whether denitrification or dissimilative nitrate reduction to ammonium is the process more responsible for nitrate removal and N_2O production. *In situ* measurement of microbial activities can give valid information about undisturbed microbial activities in their natural condition, but this approach cannot describe the physiological process of microbial interaction due to the complexity of microbial communities. Microbial activities may also be studied under laboratory conditions, thereby allowing us to control experimental conditions. This laboratory experiment may help to get better understanding of the effect of temperature on denitrifying growth and end product of its nitrate reduction of *Comamonas testoteroni* isolated from estuary sediment. Characterization of denitrifying bacteria isolated from estuary sediment indicated that *C. testosteroni* has complete denitrification enzymes. It has *nar*G, *nap*A, *nir*S, and *nos*Z genes. The end-product of nitrate/nitrite reduction in 20 °C was 100% N_2 (Rusmana 2006).

Predictions of seasonal changes in dissimilative nitrate reduction process outcome that occur in natural environments are important to anticipate the future implications of global warming, this is especially so as denitrification can produce N_2O. Some *in situ* measurement studies suggested that denitrification was the dominant process at low temperature, whereas dissimilative nitrate reduction to ammonium at high temperature (King & Nedwell 1984; Herbert & Nedwell 1990; Ogilvie *et al.* 1997). In contrast, Zimmerman and Benner (1994) and Nowicki *et al.* (1997) showed the opposite results. Other experiments suggested that denitrification was the predominant process at low C/N ratio, whereas DRNA predominated at high C/N ratio environments (Koike & Hatori 1978; Nedwell 1982). In additional Kandeler *et al.* (2006) sugested that nutrient availability may have contributed more to nitrate-reducing activities than to the density of the nitrate reducer community. Therefore this study was conducted to determine the effect of temperature on denitrifying growth and end product of its nitrate reduction of *C. testoteroni* isolated from estuary sediment.

MATERIALS AND METHODS

Effect of Temperature on End Products of Nitrate/Nitrite Reduction by *C. testosteroni* in Batch Culture. The medium composition comprise as follow (g l^{-1}): NaCl 20.0, KCl 0.5, Na_2HPO_4 5.5, NH_4Cl 0.5, K_2SO_4 1.75, NaH_2PO_4 0.775, Na_2EDTA 0.078, $MgSO_4 \cdot 7H_2O$ 0.01, Na-acetate 0.68, KNO_3 0.17, Wolin's trace elements (7 ml).

Sterile medium (10 ml) was dispensed aseptically into sterile optically standardized tubes, triplicate for each temperature, capped with sterile suba-seals, and then gassed with OFN to make anaerobic by inserting a syringe needle through the suba-seal into the medium. An in-line sterile membrane filter (0.2 µM pore size, Sartorius) was connected to the needle for sterilizing the OFN. To one of the three tubes was added 10% (v/v) C_2H_2 to inhibit nitrous oxide reductase enzyme for determining total N_2 end product. The tubes were placed in the holes across the temperature gradient block with triplicate tubes in each temperature. The temperature range was set between 6-32 °C with approximately 2 °C increments. After temperature equilibration, the tubes were inoculated with 200 µl of exponential phase starter culture. Bacterial growth was monitored periodically using a nephelometer (EEL Unigalvo DS29, Diffusion Systems, London) to measure the OD in each tube. When the culture had reached stationary phase, 200 µl of the headspace gas was injected into the GC to measure N_2O gas. The culture was then centrifuged at 12,000 x g for 15 min, and the supernatant was frozen and placed in

the -20 °C freezer for further analyses of nitrate, nitrite, and ammonium.

The maximum specific growth rate (μ_{max}) of the bacterium for each temperature was calculated by least square regression analysis of the slope of the linear part of semilogaritmic plot of turbidity versus time (Pirt 1975; Nedwell & Rutter 1994).

End-Products of Nitrate Reduction by *C. testosteroni* in Steady State Chemostats. The continuous culture was conducted at four different temperatures, 5, 10, 15, and 20 °C, using a chemostat that consisted of a glass vessel with a volume of 500 ml, sealed with a rubber bung. To minimize adherence of bacteria, "repelcote" (BDH) was used to coat the vessel glass wall. Sterile medium was pumped continuously using a peristaltic pump (multiperpex; LKB, Broma, Sweden) with dilution rate 0.02 h^{-1} for incubation at 10, 15, and 20 °C, and 0.01 h^{-1} for incubation at 5 °C. A double line breaker connecting the reservoir vessel with the chemostat was used to prevent back contamination of sterile medium in the flow line. An aerobic condition was maintained by gassing the vessel and medium with sterile OFN. The OFN flow rate, 6 ml min^{-1}, was monitored by a calibrated flow meter (RSI type) and the OFN was passed through a chromous acid trap to remove out any O_2 contained in trace amounts. A magnetic stirrer mixed the chemostat culture and the temperature in the chamber was maintained by circulating coolant through a glass jacket around the growth vessel that regulated by a thermo-circulator (Grant CFC25, Grant instruments, Cambridge Ltd.) attached to a FH15 flow heater (Grant instruments, Cambridge Ltd.).

To calculate the μ_{max}, the dilution rate was increased so that the cells were washed out from the vessel faster than bacterial growth. The OD was monitored using a spectrophotometer at 550 nm. The slope of a plot of ln OD_{550} versus time was calculated, then the μ_{max} was also calculated using the following formula (Pirt 1975):

$$\mu_{max} = D_{washout} - \text{slope}$$

where $D_{washout}$ = the dilution rate during washout. The half saturation constant for nitrate (K_{NO3}-), was calculated using the following formula (Pirt 1975):

$$K_{NO3}- = s^*(\mu_{max} - D)/D$$

Where s* = residual nitrate concentration at steady state, D = dilution rate during steady state. The specific affinity (a_A) for nitrate was calculated for each temperature of chemostat run using the following formula:

$$a_{A\ -} = (\mu_{max} / K_{NO3}-)$$

Nitrous Oxide Analysis in Acetylene Block Samples. The gas chromatograph was used to analyse N-$_2$O as describe in Trimmer *et al.* (1998), the presence of C_2H_2 in injected samples can damage the ECD of the gas chromatograph, and therefore it was necessary to separate C_2H_2 from N_2O using an external pre-column. A stainless steel (2 m x 2 mm i.d.) packed with Paropak Q (80-100 mesh) was used at 25 °C, (Millipore Corporate, Milliford, U.K.), with OFN as a carrier gas (80 ml min^{-1}). The retention time of N_2O was shorter than C_2H_2, and therefore N_2O could be collected using a liquid nitrogen cryo-trap (-196 °C, 200 µl stainless steel sample loop) before the C_2H_2 came off the pre-column. After N_2O was eluted, the C_2H_2 was switched to waste and the pre-column brought on-line into the main GC column. The cryo-trap N_2O was mobilized into the main GC column by immersing the pre-column loop in warm water (Trimmer *et al*. 1998).

RESULTS

Growth Kinetics of *C. testosteroni* Batch Culture in Temperature Gradient Block. The μ_{max} values of *C. testosteroni* batch culture in the temperature gradient block with acetate as a substrate is shown in Figure 1. The μ_{max} values were increased with increasing the temperatures of incubation, and the highest μ_{max} was found at 26 °C. The natural log of μ_{max} plotted with the reciprocal of the absolute temperature shows that the plot was linear between 12 °C and 22 °C ($p < 0.001$) (Figure 2). These results indicated that *C. testosteroni* is a mesophilic bacterium.

Concentrations of nitrate reduced mg^{-1} dried weight cells were higher at low temperature, < 10 °C. Similar with nitrate reduced, concentrations of N_2 produced at temperature < 10 °C (Figure 3) were higher at low temperature. N_2 production from *C. testosteroni* was higher than both NO_2^- and N_2O productions. However, there was a peak of high nitrite concentration at 10 °C compared with the other temperatures. N_2O was only produced by *C. testosteroni* in small amount in all temperatures.

The percentages of nitrate reduced to N_2 gas were higher than that reduced to NO_2^- and N_2O gas, between 35.46 and 65.26%, at 30 and 10 °C respectively (Figure 4 & Table 1).

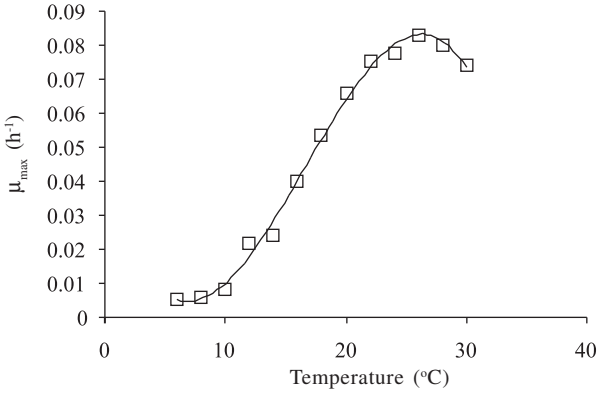

Figure 1 The maximum specific growth rate (μ_{max}) values of *Comamonas testosteroni* batch cultures with acetate as a substrate in a temperature gradient block incubator.

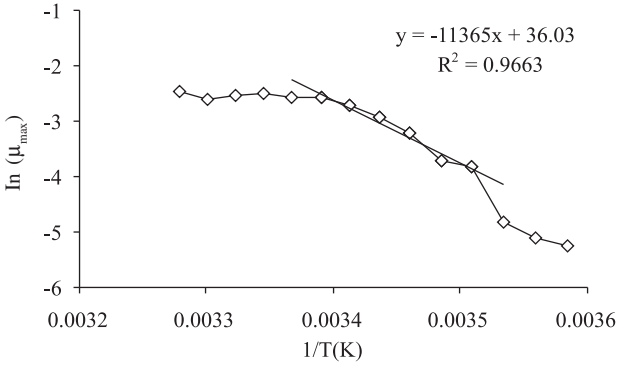

Figure 2 An Arrhenius semilogarithmic plot of the ln μ_{max} of *Comamonas testosteroni* versus the reciprocal of the absolute temperatures. A least square regression line was fitted to a linear section of the plot.

Nitrate reduced to nitrite was between 0.15 and 8.69%, at 28 and 10 °C respectively, and only between 0.78% (at 26 °C) and 2.34% (at 8 °C) were reduced to N_2O. The total nitrate recovery was between 36.58 and 75.70%, at 30 and 10 °C respectively (Table 1).

Growth Kinetics of *C. testosteroni* in Nitrate-Limited Continuous Culture with Acetate as a Substrate at 5, 10, 15, and 20 °C. Cell density at steady state in anaerobic continuous cultures with acetate as a substrate is shown in Figure 5. The ell density at steady state increased with increasing temperature, which were 1.58 ± 0.12, 2.70 ± 0.57, 7.77 ± 1.04, and 29.75 ± 3.21 x 10^6 CFUs ml^{-1}, at 5, 10, 15, and 20 °C respectively. Total concentrations of nitrate reduced also increased with increasing temperature, which were 40.00 ± 8.02, 253.94 ± 69.54, 816.96 ± 39.88, and 995.31 ± 2.74 µM at 5, 10, 15, and 20 °C (Figure 6). However, the highest amount of nitrate reduced per bacterial cell basis was at 15 °C, which

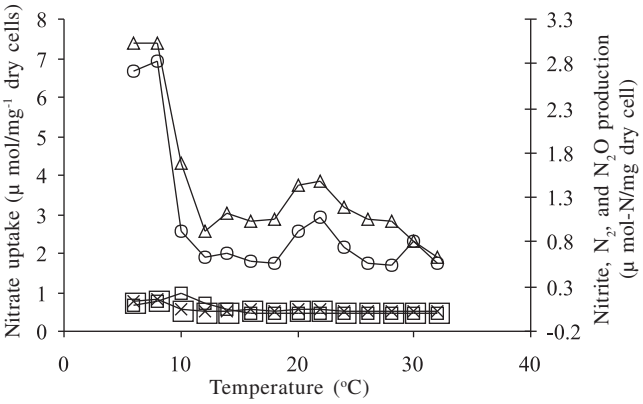

Figure 3 Temperature effect on nitrate (○) uptake and nitrite, (□) dinitrogen (△), and nitrous oxide (×) production per dried weight cells from *Comamonas testosteroni* batch cultures with acetate as a substrate.

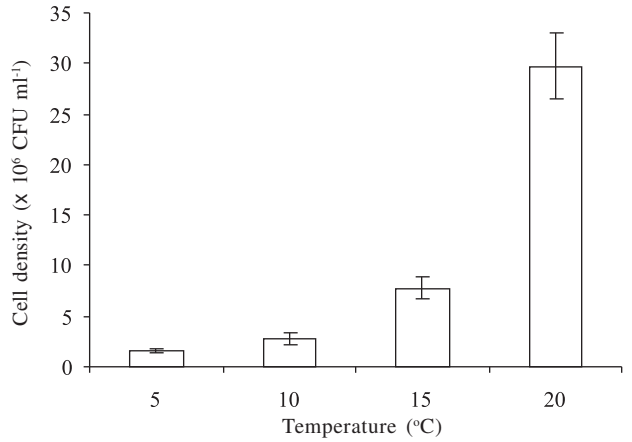

Figure 5 Cell density of *Comamonas testosteroni* continuous cultures in steady state at 5, 10, 15, and 20 °C. Bars indicate standard errors.

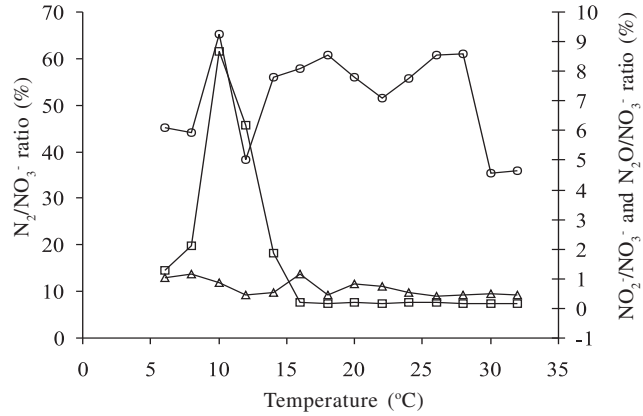

Figure 4 Percentage ratios of the nitrite (○), dinitrogen, (□) and nitrous oxide (△) production per nitrate removal basis from *Comamonas testosteroni* batch culture in a temperature gradient block incubator.

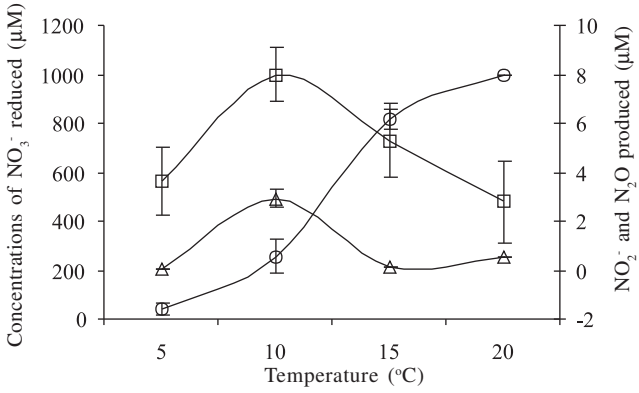

Figure 6 Concentrations of nitrate (○) reduced, nitrite (□) and N_2O (△) produced in steady state condition of *Comamonas testosteroni* continuous culture at 5, 10, 15, and 20 °C. Bars indicate standard errors.

Table 1 Cell dried weight, nitrate utilisation, and production of nitrite, dinitrogen, and nitrous oxide from *C. testosteroni* batch culture in temperature gradient block (± standard error)

Temperature (°C)	Cell dried weight (mg)	Amounts of								Cellular N from ammonium (µmol)	NO_3^- recovery (%)
		NO_3^- reduced		NO_2^- produced		N_2 produced		N_2O produced			
		(µmol)	(%)	(µmol)	(%)	(µmol-N)	(%)	(µmol-N)	(%)		
32	17.33 ± 0.02	29.84 ± 1.20	99.47	0.05 ± 0.002	0.16	10.74	35.98	0.28 ± 0.036	0.94	7.54 ± 0.008	37.09
30	13.00 ± 0.04	29.89 ± 3.47	99.63	0.05 ± 0.001	0.16	10.60	35.46	0.28 ± 0.029	0.96	5.65 ± 0.016	36.58
28	17.67 ± 0.03	29.93 ± 1.28	99.77	0.04 ± 0.004	0.15	18.28	61.04	0.26 ± 0.018	0.86	7.68 ± 0.012	62.09
26	17.33 ± 0.03	29.97 ± 0.90	99.90	0.06 ± 0.009	0.19	18.20	60.72	0.24 ± 0.001	0.39	7.54 ± 0.012	61.69
24	14.00 ± 0.06	29.97 ± 2.12	99.90	0.06 ± 0.013	0.19	16.74	55.86	0.32 ± 0.010	1.08	6.09 ± 0.024	57.14
22	10.33 ± 0.002	29.95 ± 2.68	99.83	0.05 ± 0.005	0.16	15.42	51.50	0.46 ± 0.010	1.52	4.49 ± 0.002	53.17
20	11.67 ± 0.02	29.96 ± 1.58	99.88	0.06 ± 0.005	0.18	16.75	56.02	0.50 ± 0.007	1.64	5.07 ± 0.008	57.88
18	17.33 ± 0.01	29.91 ± 3.94	99.69	0.05 ± 0.001	0.18	18.18	60.80	0.28 ± 0.007	0.94	7.54 ± 0.004	61.91
16	16.67 ± 0.04	29.99 ± 0.69	99.96	0.06 ± 0.002	0.21	17.36	57.88	0.68 ± 0.013	2.28	7.25 ± 0.016	60.38
14	15.00 ± 0.01	30.00 ± 0.00	100.00	0.56 ± 0.101	1.85	16.78	55.94	0.32 ± 0.080	1.08	6.52 ± 0.004	57.87
12	15.67 ± 0.01	30.00 ± 0.00	100.00	1.85 ± 0.405	6.18	14.28	48.28	0.28 ± 0.011	0.92	6.82 ± 0.004	55.38
10	7.67 ± 0.001	19.80 ± 1.47	66.00	1.72 ± 0.131	8.69	12.92	65.26	0.34 ± 0.033	1.76	3.34 ± 0.002	75.70
8	2.67 ± 0.001	18.40 ± 2.25	61.33	0.39 ± 0.134	2.11	8.12	44.08	0.44 ± 0.006	2.34	1.16 ± 0.002	48.53
6	2.33 ± 0.01	15.60 ± 1.65	52.00	0.20 ± 0.014	1.29	7.03	45.20	0.32 ± 0.016	2.06	1.01 ± 0.004	48.55

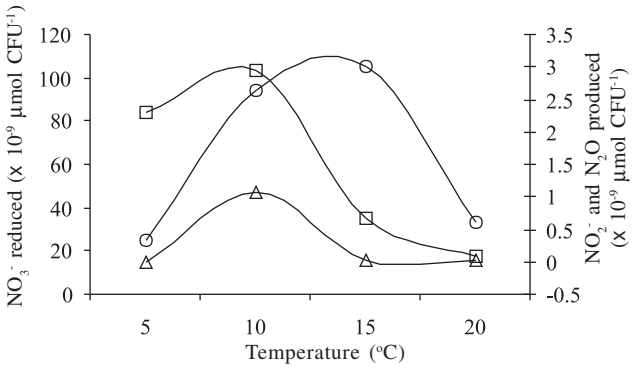

Figure 7 Amounts of nitrate (o) reduced, nitrite (□) and N_2O (Δ) produced per bacterial cell basis (CFU^{-1}) in steady state condition of *Comamonas testosteroni* continuous culture at 5, 10, 15, and 20 °C.

Table 2 Kinetic growth rate parameters of *C. testosteroni* in nitrate-limited continuous culture experiments at 10, 15, and 20 °C

Temperature (°C)	μ_{max} (h^{-1})	Residual nitrate (µmol l^{-1})	$K_{(no3-)}$ (µmol l^{-1})	$a_{A(NO3-)}$ (1 µmol^{-1} h^{-1})
20	0.22176	104.69	1056.09	0.00021
15	0.09276	283.03	1029.67	0.00009
10	0.03276	846.05	539.78	0.00006

was 105.18 x 10^{-9} µmol CFU^{-1} (Figure 7). The highest concentrations of nitrite and N_2O produced in both total and per bacterial cell basis were found at 10 °C, which were 77.99 ± 1.11 µM or 2.96 x 10^{-9} µmol CFU^{-1} and 2.92 ± 0.34 µM or 1.08 x 10^{-9} µmol CFU^{-1} respectively (Figure 5 & 7).

The kinetic growth rate parameters calculated using the formula reported by Pirt (1975) are shown in Table 2. The maximum specific growth rate (μ_{max}) values at 10, 15, and 20 °C were 0.03276, 0.09276, and 0.22176 h^{-1} respectively, and the specific affinity (a_A) for nitrate at 10, 15, and 20 °C were 0.00006, 0.00009, and 0.000211 µmol^{-1} h^{-1} respectively (Table 3).

DISCUSSION

The μ_{max} values of *C. testosteroni* grown in anaerobic batch culture with acetate as a substrate indicated that *C. testosteroni* is a mesophilic bacterium with optimum temperature for anaerobic growth at 26 °C (Figure 1). The Arrhenius plot of ln μ_{max} with 1/T°A shows that the linear/normal range temperature was between 12 and 22 °C. In this linear range the microbial growth can be predicted by extrapolation of the Arrhenius relationship (Ingraham *et al*. 1983) as follow:

$$\ln \mu = (-\Delta E^*/\text{gas constant})\ (1/T) + \text{constant}$$

where, µ is microbial growth rate, ΔE* is the activation energy, and T is temperature in Kelvin.

The temperature characteristic value, which is the slope of the Arrhenius plot of *C. testosteroni* in acetate/nitrate, indicated that *C. testosteroni* has small response of growth to increasing temperature. There is a correlation of temperature characteristic with the energy activation as a following formula (Ingraham *et al*. 1983):

$$\text{Temperature characteristic} = (-\Delta E^*/\text{gas constant})$$

where ΔE* is the activation energy.

Table 3 The Specific affinity (a_A) for nitrate by nitrate respiring bacteria in anerobic nitrate-limited chemostats

Substrate	Organism	a_A (1 µmol^{-1} h^{-1}) at a temperature (°C)			
		5	10	15	20
Glucose	*K. pneumoniae**		0.00118	0.00488	0.013
	Aeromonas sp.*		0.0033	0.0015	0.00006
	*Klebsiella oxytoca**		0.00011		0.00059
	Citrobacter sp.*	0.00003			0.00004
Acetate	*Klebsiella oxytoca**		0.00014		0.00021
	Citrobacter sp.*	0.00012			0.00021
	*C. testosteroni***		0.00006	0.00009	0.00021

*from Nedwell (1999), **this study

Total nitrate reduced at low temperature, in both batch and continuous cultures, was lower than at high temperature (Table 1) indicating temperature limitation on bacterial nitrate uptake. Low temperature inhibits active transport of substrate and nutrient by reducing the fluidity of the membrane, which then influences transporters molecules in the membrane, presumably because stiffening of the membrane by lowered temperature repress transport protein efficiency (Nedwell 1999). Moir and Wood (2001) suggested that there are two different mechanisms of nitrate transport; (i) nitrate/nitrite antiporter and (ii) nitrate/proton symporter. Protein transporter of nitrate transport is NarK (Zumft 1997; Moir & Wood 2001; Sharma *et al*. 2006). Currently it is reported that there are two different forms of NarK, NarK1, and NarK2 (Moir & Wood 2001; Sharma *et al*. 2006). In *Paracoccus denitrificans* NarK1 is a nitrate/proton symporter and NarK2 is nitrate/nitrite antiporter (Wood *et al*. 2002). Sharma *et al*. (2006) suggested that only the NarK2 protein is required as a nitrate/nitrite transporter by *Pseudomonas aeruginosa* under denitrifying conditions. In the present experiments, this NarK efficiency was probably repressed by low temperature with stiffing of the membrane, so that nitrate uptake was repressed. The bacterial growth is correlated with substrate and nutrient uptake. In nitrate-limiting medium, nitrate is a limiting factor that controls bacterial growth. Therefore, the bacterial growth yield at low temperature was lower than at high temperature (Table 1).

The ability of an organism to scavenge (via its transporters) a substrate is dependent upon its affinity for that substrate. If affinity is described by specific affinity (a_A) the higher the value, the greater is this affinity for this substrate (Nedwell 1999). The specific affinity (a_A) value for nitrate of *C. testosteroni* decreased with decreasing temperature below the growth optimum (Table 2). This trend of decreased affinity for nitrate probably indicates decreasing functional efficiency of NarK as temperature declines. Nedwell (1999) termed that any substrate taken up by some from of active transport will be less available as temperature decreases because microorganism ability to seize the substrate declines at low temperature as a result of "temperature-modulated substrate affinity".

The specific affinity (a_A) value for nitrate of *C. testosteroni* with acetate as a substrate at 10 °C was lower than *Klebsiella oxytoca* with acetate and *K. pneumoniae*, *Aeromonas* sp., *K. oxytoca* with glucose, as well as at 15 °C with *K. pneumoniae* and *Aeromonas* sp. with glucose, and at 20 °C with *K. pneumoniae* and *K. oxytoca* with glucose as a substrate. However there was similar a_A value with *K.*

oxytoca and *Citrobacter* at 20 ºC with acetate as a substrate (Table 3). In steady state of nitrate limiting chemostats, the ability of a bacterial cell to seize nitrate from the environment might be controlled by the affinity of the transport protein (Nedwell 1999). This a_A values reflect the ability of one organism to outcompete the others.

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