Removal of High Concentrations of Nitrate from Industrial Wastewaters by Bacteria

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***Klebsiella oxytoca* isolate 15 was isolated from the grounds of a nitration factory and was found to be tolerant to nitrate at concentrations up to 0.5 to 1 M. Physicochemical parameters for optimal growth conditions for *K. oxytoca* isolate 15 were established. Growth took place when the nitrate concentration in the medium was less than 150 mM, and full nitrate consumption required about 14 g of C per g of N. This strain was able to remove nitrate without accumulating nitrite. The system was scaled up to a 40-liter pilot plant and was operated on-site satisfactorily.**

Nitrate, the most abundant form of nitrogen in the biosphere after atmospheric dinitrogen, is the N source used most widely by living organisms, including higher plants, algae, fungi, and bacteria (5, 19). Assimilatory nitrate utilization involves the reduction of NO_3^- to NO_2^- in a two-electron reaction mediated by nitrate reductase, and further reduction of NO_2^- to NH_3 involves six electrons in a reaction mediated by nitrite reductase (5). Some facultative anaerobes are able to use NO_3^- as an alternative electron acceptor in respiratory chains (21). Despite the wide distribution of organisms able to utilize nitrate and the varied environments where this compound serves multiple purposes, this inorganic form of N is a pollutant; its elimination is of high priority to many environmental protection agencies. Its presence at relatively low concentrations usually does not limit its uptake, as most organisms have active NO_3^- uptake systems (2, 3), and indeed complex microbial communities in biological treatment plants are able to cope with low concentrations of nitrate (<50 mg per liter) in a manner similar to those found in natural environments (3). However, when biological treatment plants receive high NO_3^- concentrations from industrial discharges, cell growth is inhibited because of the futile reduction of NO_3^- to NO_2^- (10). The latter is also a relatively toxic compound for many organisms and is mutagenic in its acid form, HNO_2 (6). At high concentrations, nitrate behaves as a kaotropic agent and is known to remove proteins from the cell membrane (13). Nitrate is also of concern because it causes methemoglobinemia in infants (8).

The elimination of NO_3^- in industrial wastewater streams with a relatively high nitrate content—such as those resulting from the production of nitroaromatic compounds, the synthesis of nitroorganic compounds in pharmaceuticals, nitrogen-containing products in chemical fertilizers, wastewaters from nuclear fuel processing, and the cleaning of tanks in dairy factories—has been considered a difficult task for microorganisms (1, 4, 9, 11, 14, 17, 20). Biotechnological processes for the removal of high NO_3^- concentrations will become possible

only if microorganisms able to preferentially tolerate high NO_3^- concentrations are available and if these microorganisms are able to remove nitrate.

Microbes from the grounds of an explosives factory were relatively tolerant to high concentrations of nitrate: they were able to grow in culture medium containing NO_3^- at concentrations up to 80 mM (16). To isolate these microorganisms and use them to remove NO_3^- from wastewaters with high NO_3^- concentrations, we set up classical enrichment cultures in M8 minimal medium with 20 to 60 mM NO_3^- from wastewaters from the production of dinitroethylene glycol as an N source and 10 to 30 g of glycerol per liter as a C source. Cultures were incubated in a rotary shaker at 200 strokes per min at 30°C in conical flasks. After several growth cycles, strains were selected on solid medium. Thirty-four independent isolates from the enrichments were chosen and grown on minimal medium with 60 mM NO_3^- and 30 g of glycerol per liter, and the generation time was estimated. The isolate that grew the fastest, a gram-negative and oxidase-negative rod called isolate 15, was kept for further analysis. Metabolic tests and analyses of the methyl ester derivatives of total phospholipids identified the strain as probably *Klebsiella oxytoca*. The pattern of phospholipids remained unaltered regardless of the initial NO_3^- concentration used for cell growth. *K. oxytoca* isolate 15 used the following as the sole carbon source in addition to glycerol: glucose, fructose, sucrose, galactose, and lactose. *K. oxytoca* isolate 15 tolerated nalidixic acid (10 µg per ml) and ampicillin (25 µg per ml).

To show that tolerance to NO_3^- was a unique characteristic of this *K. oxytoca* isolate, tolerance was assayed by determining survival after the exposure of cultures to different NO_3^- concentrations. In these assays, *Klebsiella pneumoniae* 50231, *Klebsiella planticola* CECT843, *K. oxytoca* CECT860, *Escherichia coli* ET8000, and *Pseudomonas putida* mt-2 were used as control strains. All these strains grew on minimal medium with NO_3^- as the sole N source.

Tolerance to nitrate in these strains was assayed as follows. Cells were grown on minimal medium with 20 mM NO_3^- to about 10^8 to 10^9 CFU per ml. Then, 0.1-ml samples were transferred to 1-ml solutions with increasing NO_3^- concentrations, ranging from 20 mM to 1 M. After 3 h of incubation,

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TABLE 1. Tolerance of different *Klebsiella* strains to high concentrations of NO_3^- ^a

NO_3^- concn (M)	Survival (% of initial)		
	<i>K. oxytoca</i> isolate 15	<i>K. pneumoniae</i> 50231	<i>K. planticola</i> CECT843
0.02	100	100	100
0.10	100	100	40
0.25	100	66	0.2
0.50	100	6	<0.01
1.00	100	3	<0.01

^a *K. oxytoca*, *K. pneumoniae*, and *K. planticola* cells were grown on M8 minimal medium with 20 mM NO_3^- and 10 g of glycerol per liter for 24 h. Cells were harvested by centrifugation and resuspended to about 10^8 CFU per ml in 20 mM phosphate buffer (pH 6.8) supplemented with the concentration of NO_3^- indicated. After 3 h, the cultures were serially diluted and spread on Luria-Bertani plates to determine the numbers of viable cells (CFU) per milliliter. Survival is expressed as the percentage of the initial number of CFU per milliliter.

serial dilutions were spread on Luria-Bertani plates and the populations of cells surviving the NO_3^- shock were counted. *K. oxytoca* isolate 15 tolerated NO_3^- at concentrations up to 1 without significant loss of cell viability (Table 1); *K. pneumoniae* 50231 did not tolerate concentrations higher than 250 mM (Table 1), and *K. planticola* CECT843, *K. oxytoca* CECT860, *E. coli* ET8000, and *P. putida* mt-2 did not tolerate concentrations higher than 100 mM (see Table 1 for results obtained with *K. planticola*). Incubation of *K. oxytoca* isolate 15 for 24 h in 0.5 or 1 M NO_3^- resulted in the survival of 85 and 40% of the initial load, respectively (not shown).

We also tested whether pregrowth of *K. oxytoca* isolate 15 in higher NO_3^- concentrations, e.g., 80 mM, influenced tolerance to NO_3^- . The results in short- and long-term incubations (data not shown) were similar to those reported above.

The level of NO_3^- tolerance of *K. oxytoca* isolate 15 was at least 5- to 10-fold higher than the level tolerated by similar microorganisms; this makes this strain an excellent candidate for treatment of industrial wastewaters with high NO_3^- loads.

In contrast with the other tested strains, *K. oxytoca* isolate 15 grew on minimal medium supplemented with 100 mM NO_3^- , whereas the other strains did not grow when the initial NO_3^- concentration was higher than 30 to 50 mM.

We then determined whether the initial NO_3^- concentration affected cell growth of *K. oxytoca* isolate 15. Both the lag time before growth resumed after 100-fold dilution and the generation time in the exponential-growth phase of this strain were influenced by the initial nitrate concentration. *K. oxytoca* isolate 15 cells growing exponentially on minimal medium with 20 mM NO_3^- were transferred to the same medium but with increasing NO_3^- concentrations between 30 and 160 mM. The higher the initial NO_3^- concentration was, the longer the lag phase was (between 0 h for 30 mM and 24 h for 160 mM) and the lower the growth rate was once growth resumed (between 3.3 h for 30 mM and 10.2 h for 160 mM) (Table 2). When the initial NO_3^- concentration was lower than 100 mM, isolate 15 consumed more than 99% of NO_3^- without significant accumulation of nitrite (Table 2). The yield of these cultures relative to the C source (glycerol) was about 0.1 to 0.13 g of cell mass per g of glycerol consumed (Table 2). When the NO_3^- concentration was increased to 160 mM, a significant proportion of NO_3^- (about 30%) was consumed without accumulation of NO_2^- . At concentrations higher than 160 mM, no significant consumption of nitrate was observed (data not shown).

The enzymes of the NO_3^- assimilatory pathway in this strain

were detected when the strain was grown on NO_3^- -containing culture medium but not when ammonium was used as the sole N source. This suggested that the nitrate assimilatory pathway enzymes were inducible. The level of nitrate reductase, the first enzyme of the NO_3^- assimilation pathway, was determined in Triton X-100-permeabilized whole cells and was found to be as high (on the order of $1,200 \pm 200$ mU per μg of cell protein) as that in cells growing exponentially at different NO_3^- concentrations. In agreement with the nitrate reductase activity, the rate of NO_3^- consumption by *K. oxytoca* isolate 15 at different concentrations of NO_3^- (2 to 100 mM) was in the range of 0.7 ± 0.1 g of NO_3^- per g of cell protein per h.

The mechanisms available for *K. oxytoca* isolate 15 cells to adapt to the presence of high concentrations of NO_3^- are unknown, but one way to reduce the actual NO_3^- concentration in the cell is to transport nitrate out of the cell by an active efflux system. Efflux systems in bacteria have been described for tolerance to hydrophobic antibiotics (12) and more recently to organic solvents (7, 15).

To establish the optimal operating conditions for on-site treatment of wastewaters, the system was scaled up under aerobic conditions for *K. oxytoca* isolate 15. Air was supplied at a rate of 1 liter per liter of culture medium per min. Agitation (rotational system) was varied between 200 and 1,000 rpm, temperature was varied between 15 and 40°C, and pH was varied between 5.0 and 9.0. In these assays, we used minimal medium with 20 to 80 mM NO_3^- from diluted factory wastewater and 10 to 40 g of glycerol per liter. The optimal operational conditions, defined as shorter lag, faster growth, and full consumption of the C and N sources, were found to be as follows: agitation rate, ≥ 400 rpm; temperature, $27.5^\circ\text{C} \pm 2.5^\circ\text{C}$; and pH, 7.0 ± 0.5 . Figure 1 shows the performance of *K. oxytoca* isolate 15 under optimal conditions in a batch assay with an initial NO_3^- concentration of 80 mM. After an initial lag of about 6 h concomitant with cell growth, NO_3^- and glycerol were consumed until their levels fell below our detection limits (i.e., to $<100 \mu\text{M}$ for NO_3^- and to <0.1 g of glycerol per liter). During the exponential-growth phase, the rate of NO_3^- uptake was estimated to be 0.7 ± 0.1 g of NO_3^-

TABLE 2. Effect of initial NO_3^- concentration on growth of *K. oxytoca* isolate 15^a

NO_3^- concn (mM)		NO_2^- concn (mM)	Glycerol concn (g/liter)	Y_{gly}	Lag (h)	Generation time (h)	Time required for NO_3^- removal
I	F						
30	0.1	0	0	0.12	0	3.3	32
40	0.2	0.01	0.17	0.13	2	3.5	48
80	0.1	0.10	0.10	0.10	6	4.1	68
100	0.8	0.11	0.04	0.10	20	6.2	76
160	110	0.02	3.20		24	10.2	>96

^a *K. oxytoca* isolate 15 was grown on M8 minimal medium with 20 mM NO_3^- until the late exponential phase and then diluted 100-fold in the same M8 culture medium supplemented with one of the initial concentrations of NO_3^- indicated. The amount of C supplied was 10 g per liter for each 20 mM fraction of NO_3^- . The initial (I) and final (F) concentrations of NO_3^- and the final concentrations of NO_2^- and glycerol were determined at the times (in hours) indicated in the rightmost column. These chemicals were analyzed after the cells had been removed by centrifugation ($5,000 \times g$ for 15 min). Nitrate was measured with a specific nitrate electrode and a potentiometer (micropH 2002; Crison). Nitrite was measured by the diazotization method of Snell and Snell (18). Glycerol was measured enzymatically with a kit (Boehringer Mannheim catalog no. 148270). The yield of the culture (Y_{gly}) is given as grams of cell biomass per gram of glycerol. Biomass was measured as dry weight by filtration through a Whatman 1822 915 filter. Generation time refers to the doubling time during exponential growth of the culture. Lag refers to the time required before exponential growth started.

FIG. 1. Removal of NO_3^- in a 2-liter fermentor by *K. oxytoca* isolate 15. The culture medium was factory wastewater diluted to an initial concentration of about 80 mM NO_3^- and supplemented with 40 g of glycerol per liter. Operating conditions were as follows: air, 1 liter per liter of culture per min; pH, 7.0 ± 0.5 ; agitation, 600 rpm; temperature, 30°C . Concentrations of nitrate ($\circ$), nitrite ($\bullet$), and glycerol ($\square$) and growth ($\triangle$), determined as the optical density of the culture at 660 nm (OD660), were measured at the indicated times.

per g of cell protein per h, while glycerol was consumed at a rate of 3.35 ± 0.8 g per g of cell protein per h. The yield of *K. oxytoca* isolate 15 was maximal at temperatures between 25 and 30°C ; at 20°C , the yield was 60% of the maximum. Consistent with the lower yield was that 10 to 20% of the initial NO_3^- was not consumed.

The system was further scaled up to a 40-liter pilot plant and operated as a continuous system on-site with wastewaters produced in the manufacture of dinitroethylene glycol in an explosives factory. The system performed such that in the overflow, the level of nitrate was less than 0.5 mM, the level of nitrite was negligible, and the C content was less than 50 mg per liter. These results suggest that the system can operate satisfactorily for long periods.

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