Removal of Nitrates in Wastewaters

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The adverse environmental impacts associated with a very high nitrate concentration is undesirable due to their extremely toxicity to most aquatic species and human, include also a strongly promotion of eutrophication. Different methods are developed to eliminate this water pollution, so the use of the denitrifying bacteria, is the ideal solution thanks to its low cost and low energy consumption. In this study, we performed the isolation of bacteria from activated sludge. The best denitrifying bacteria selected was incubated in a bioreactor containing a synthetic wastewater rich in nitrate ions. The Griess test (NR 1 + NR 2) and zinc powder were proved the denitrification capacity of this strain, which had the ability of the complete reduction until the last stage passing through nitrite to atmospheric nitrogen, which gave also a reduction percentage of 75% with a significant growth rate. Finally the bacterium was tested both in microscope and in biochemical gallery.

Keywords: denitrification, bacteria, nitrate, nitrogen oxides

Nitrate fertilizers are often used in agriculture with ammonia, but its presence in large quantities leads to take out industrial, domestic discharges water and disturbs the geo biochemical equilibrium. The main effect of nitrate on the environment is devastating to aquatic ecosystems by growing of undesirable plant on the water area or called also eutrophication phenomena, which correspond to the toxicity of aquatic organisms by depletion of oxygen dissolved in the medium [1,2].

The nitrate removal from wastewater is commonly performed by the action of microorganisms according to different way: nitrate assimilation and nitrate dissimilation. The latter has particularly of interest to us and it is called also “nitrate respiration”. In this process, the nitrate play in aerobic condition, the role of oxygen [3]. Therefore, the NO3 are used as electron acceptors [2] which produce NO and / or N2O or N2. In effect, these nitrogen oxides are extremely toxic for the environment; so, N2O is a greenhouse gas and NO is a catalyst for the production of tropospheric ozone which can be transformed into nitric acid, a component of acid rain, furthermore; NO is a strong ligand for metal ions, thereby inhibiting all the enzymes of the respiratory chain of denitrification, because they all have metal ions in their active site, so the denitrification should be complete until the obtaining of atmospheric nitrogen by the following reaction (in the presence of organic substrate as a carbon sources) [4,5],

\[
\text{NO}_3^- + \text{organic substrate} \xrightarrow{\text{microorganisms}} \text{microorganisms} \rightarrow \text{N}_2 + \text{CO}_2 + \text{H}_2\text{O} \quad (1)
\]

The bacteria responsible of this reaction are heterotrophic anaerobic or facultative aerobic, as there exists autotrophic bacteria which can reduce nitrate to atmospheric nitrogen [1].

In this work, denitrifying bacteria were isolated from wastewaters containers containing nitrate at high concentrations. After that, the isolated bacteria were subjected to double purifications in a Petri dishes and tubes, and then they were undergone to nitrate reductase test to specify the bacteria that contains this enzyme. The best denitrifying bacterium selected was incubated in minimal medium rich in nitrate to determine its growth kinetic as a function of time and the rate of the nitrate reduction. Finally this strain was identified by microscope and biochemical tests.

Experimental part

Sampling rate

The reactions of denitrification are often appeared in the process of wastewaters treatment and in the area rich in nitrate ions (the agricultural land) where the bacteria responsible of these operations will grow significantly [5].

Samples of activated sludge were taken from urban wastewater of municipal wastewater plant of Boumerdes (north of Algeria), and stored in polyethylene bottle at 4°C for further analyses.

Media composition

Solid nutrient medium, pH 6.8; peptone 5 g/L, yeast extract 3 g/L, agar 15 g/L, NaCl 5 g/L.

Nitrated bouillon, pH 7: yeast extract 2 g/L, KNO3 5g/L

Minimal denitrification medium, pH 7.4: C6H12O6 2g/L, Na2HPO4 1.35g/L, KH2PO4 0.7g/L, MgSO4.7H2O 0.1 g/L

All solutions were prepared using distilled water, autoclaved before use and we work in aseptic condition to avoid any contaminations. The pH of the solutions was adjusted to a neutral using NaOH 1N and H2SO4 1N.

Purification in the solid medium, using serial dilutions technique for the preparation of the seeding solutions

One milliliter of aerobic activated sludge was added into 9 mL of saline water (0.9% of NaCl). After complete mixing, the solution was diluted to 10, 5, 3.3, 2.5% (V/V) (V volume of the activated sludge solution in V' volume of saline water), and distributed onto Petri dishes containing solid nutrient medium. After incubation at 37°C for around 48 h, colonies from all media were identified, isolated separately in new Petri dishes and stored at 4-8°C for further study.

Nitrate reductase test, selection of denitrifying bacteria

This test involves to an inoculating of the pure strains in tubes contains 5mL of nitrated bouillon, they were

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incubated in an oven at 37°C for 24-48 h. The addition of Griess reagent (NR1+ NR2 : sulfanilic acid + alpha naphtilamine), gives a red color to prove the presence of nitrates, and the addition of zinc powder in the negative tubes proves the presence of nitrates in the solution giving a red color, this test is qualitative, it allows to select the species containing the enzyme nitrate reductase [6, 7]. The reaction mechanism was reported by Griess in 1879. Under acidic conditions, nitrite reacts with the amino group of sulfanilic acid to form the diazonium cation, which couples to α-naphthylamine in para-position to form the azo dye, as the following mechanism (fig.1) [8].

Results and discussions

Inoculation, isolation and purification

In this work, we developed a new denitrifying bacterium isolated from an activated sludge. Different bacterial colonies were formed in the surface of the solid media. The former colonies were isolated on Petri dishes as shown in the figure 2 , and the in the inclined tubes. We found a range of different pure strains which conserved at low temperature for further analysis.

Test of nitrate reductase

The present test allows to selection the strains which had an efficiency of nitrate reduction to nitrite and that to last stage (obtained of atmospheric nitrogen). According to our results, practically most of the bacteria tested possess the enzyme nitrate reductase. After the time of incubation, we marked turbidity (bacterial growth) in all tubes. The addition of Griess reagent (indicator of nitrite) and zinc powder (an indicator of nitrate), allows specifying the most important bacteria. The results are summarized in the table1.

The nitrate reductase test allows a primary selection of the denitrifying bacteria which proved that the S4 could completely reduce the nitrate of the medium (nitrated broth) to atmospheric nitrogen.

Gram staining

The Gram colorations, was obtained by conventional Hucker Gram staining protocol. This method seems less useful for estimating the type of Gram, it consisted to prepare a suspended cells in a sterile physiological water 1h before staining. A smear of cells was then heat-fixed on microscope slides and subsequently stained for 1 min in crystal violet solution, 1 min in iodine solution, washed for 30 s in 96% ethanol and, finally 2 min in fuchsine solution [9].

Evolution of bacterial growth in a minimum medium as a function of time

Cell growth was monitored by measuring of the optical density at a wavelength of 600 nm every 2 h, during 24-48 h of incubation (T 30 °C, pH 7, α120 rpm, dissolved oxygen 0%) in a minimal denitrification medium.

<table>
<thead>
<tr>
<th>Dilutions (% v/v)</th>
<th>Strains codes</th>
<th>Griess test</th>
<th>Zinc powder</th>
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Table 1

RESULTS OF THE NITRATE REDUCTASE TEST
bacterial biomass was allowed us to trace the curve presented in figure 4. The dosage of residual nitrate and produced nitrite are shown in figure 5. During 48 h of incubation, the curve of the growth kinetic of S4 (fig. 4), shows clearly the different bacterial growth phases. The S4 added to fresh media typically go through four distinct phases of growth. During the first 18 h the bacterium was adapted to the new environment (lag phase, not shown in the curve because it was very short). It grows rapidly where cell numbers increase at ever increasing rates with time this phase is called logarithmic or exponential phase. After that, it came a steady-state equilibrium between 20-30 h, it was a stationary phase, where the rate of division of S4 was exactly balanced by the rate of the cell death that due to a loss of limiting nutrients or a buildup of toxins (fermentative products). That was followed by a die-off of cell or a decline phase at time superior of 30 h by accumulations of these toxins in the medium.

The S4 was used the nitrate ions for respiration instead the oxygen molecule, so the dosage of nitrates were allowed a diminution of its concentration by 10 mg compared with the control tube (fig. 5) without going through nitrites using the glucose as a source of organic carbon, so the SB is a facultative anaerobic heterotrophic denitrificant bacteria.

Biochemical tests

Strain S4 had Gram-negative cells with short rods, the colonies formed were white, mobile, circular, and low-convex. The bacterium had smooth colonies with spreading on nutritive agar plates after 48 hincubation at 30°C. Where as, the tests catalase and cytochrome oxidase was negative. Also, the strain S4 was able to reduce nitrite and nitrate.

Nevertheless, they were positive for the utilization of sodium acetate and glucose; but, β-Galactosidase activity was not detected. Starch and urea are degraded too. Additionally, indole and hydrogen sulfide were not produced.

We further found that our strain S4 had different properties which were important: (1) Bacterial colonies developing on the minimal medium or rich medium so it was undemanding. When the major of its nutrients were present in the medium like the organic carbon and nitrate as source of nitrogen and some salts it can effectively develop, they always maintain a mono-layer of cells at the periphery. (2) The S4 had two respiratory properties, aerobic in the presence of oxygen and anaerobic in absence of this molecular. Thus at the anoxic conditions the S4 could perform the respiration by the nitrate inside the oxygen. While, the nitrate could take the role of the electron acceptor instead the oxygen molecule and the bacterium reduce it on nitrite, and then on atmospheric nitrogen. Consequently, this bacterium (S4) may be used as biomass for the treatment of wastewater rich in nitrate.

Our strain S4 could perform the heterotrophic denitrification on anaerobically, like most types of denitrifying bacteria according to several published works, these bacteria have a significant reduction rate. They used nitrate as electron acceptor, as Pseudomonassp which perform the reaction at a rate up to 99% [10]. Indeed various organic carbon sources may be used such as succinate, glycerol and glucose. The mechanism of incorporation of acetate or any another organic carbon is presented in figure 7 [11-13]. However, in the presence of certain carbon substrate bacteria cannot effectively remove nitrates, such as methanol.

Otherwise, there are some autotrophic which can perform the denitrification, such as Thiobacillus denitrificans, this species can use sulfur compounds as electron donor instead of carbon, but it has a low rate reduction compared with heterotrophic processes [1].

Growth of denitrifying strains may be threatened by various parameters such as temperature, pH and the concentration of carbon substrate and even by the by-products (nitrogen oxides) of the reactions when they accumulate in the environment.

Conclusions
The feasibility of denitrification by the strain developed in this study, which called S4, has been demonstrated for a synthetic wastewater, with a reduction rate of 100% for nitrated broth media during short incubation time (less than 48 h) and 75% for the minimal medium rich in nitrate. This cocci can reduce nitrate to nitrogen atmospheric gas under this conventional fixed conditions; at a temperature of 30 to 37°C as a maximal incubation temperature, neutral pH, 120 RPM of rotation speed and at 0% of dissolved oxygen. However, further studies are necessary to validate this set of parameters as well as the application of this new strain in the real wastewater and we expect that S4 will be stably dominant microorganisms in an open system. In a wastewater treatment using this microorganism, the culture requires a single tank and has a very high nitrate removal rate, without formation of any undesirable intermediate products (nitrogen oxides).

References

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