Asian Journal of Chemistry; Vol. 28, No. 2 (2016), 463-466

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19472

Comparison of Biological Nitrate Reduction Effectiveness of Two Strains Isolated from Activated Sludge

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Received: 11 July 2015; *Accepted*: 23 September 2015; *Published online*: 3 November 2015; AJC-17630

Denitrifications reactions are carried out by denitrifying bacteria, which transform the nitrates ions to nitrite and then to atmospheric nitrogen. In this work, two strains B and C were isolated from an activated sludge and incubated separately in a bioreactor containing a synthetic medium rich in nitrates ions. The Griess test and zinc powder have proved the difference between denitrification capacity of the two selected strains. Therefore, strain B was able to reduce nitrate to nitrite as final product of the reduction. Nevertheless, the strain C 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, in synthetic minimal medium. Finally, both bacteria, B and C were identified and tested on microscope.

Keywords: Nitrate, Denitrification, Denitrifying bacteria, Nitrate reductase, Bioreactor.

INTRODUCTION

The adverse environmental impacts associated with a very high nitrate concentration is undesirable owing to its extremely toxicity to most aquatic species and human, include also a strongly promotion of eutrophication [1,2]. Different methods were developed to eliminate this water pollution. So, the biological methods are the ideal solution due to its low cost and low energy consumption [2,3].

The heterotrophic denetrification applied in the wastewater treatment are performed anaerobically where the nitrate ions are the terminal electron acceptor instead of the oxygen by heterotrophic bacteria like *Thiosphaerapantotropha, Pseudomonas, Micrococcus, Dénitrobacillus, Spirillum*, *Achromobacter* and *Alcaligenes* [4]. Some species could transform nitrate to nitrite and other could complete the reduction until obtain the atmospheric nitrogen, passing by the following transformations series:

$$NO_3^- \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The enzyme responsible of these reactions is the nitrate reductase, which is specific for the denitrifying bacteria. It includes molybdenum, flavin adenine nucleotide and pyridine nucleotide. Whereas, the enzyme contains two combined entities; the first one transfer electrons provided by the oxidation of the organic substrate of pyridine nucleotide to the flavin and the other entity transfer these electrons of the reduced flavin to the nitrate ion with molybdenum intermediate [4,5].

This paper investigated a comparison between a performance and the denitrification efficiency of two species isolated from an activated sludge and we also determined its macro and microscopically aspect as a primary characterization of its types and Gram staining.

EXPERIMENTAL

Two strains called B and C were collected from an activated sludge obtained from the wastewater treatment plants of Boumerdes, Algeria. Each strain was enriched in a minimum medium nitrate-rich and then it was purified in inclined solid medium and conserved at a low temperature (4 °C).

Minimum medium nitrate-rich composition (g/L), pH 7.4: [CH_3COONa 1 or glucose 2], KNO_3 1, $NaNO_3$ 1, Na_2HPO_4 1.35, KH_2PO_4 0.7, $MgSO_4 \cdot 7H_2O$ 0.1 [4].

Broth nitrated (g/L), pH 7, (KNO_3 5, yeast extract 2). The strains B and C were incubated in 5 mL of the broth nitrated at 35 °C for 24 to 48 h.

General procedure: Qualitative tests were demonstrated the reduction capacity and the presence of nitrate reductase enzyme for each bacterium. Inoculums of each bacteria were incubated under the same operating conditions, at 35 °C, pH 7.4, shaking rotator of 120 rpm, in a bioreactor equipped with

a pH regulator (maintain a neutral pH during the time of the reaction). Every 4 h, samples were taken for the measurement of different parameter (optical density, nitrate and nitrite concentrations) during the 48 h of incubation.

Detection method: The test of nitrate reductase involves two different steps. The first one consists of Greiss test's which contains two reagents NR1 and NR2: sulfanilic acid + α-naphtilamine, these reagents reacts with nitrite and give thereby a red colour. The second step of this test consists of the adding of the zinc powder in the negative tubes, which give a red colour in combination with nitrate. So, if there is no colour changing in the tube, the medium don't contain ions of nitrate or nitrite. The mechanism of these reactions was presented in the Fig. 1.

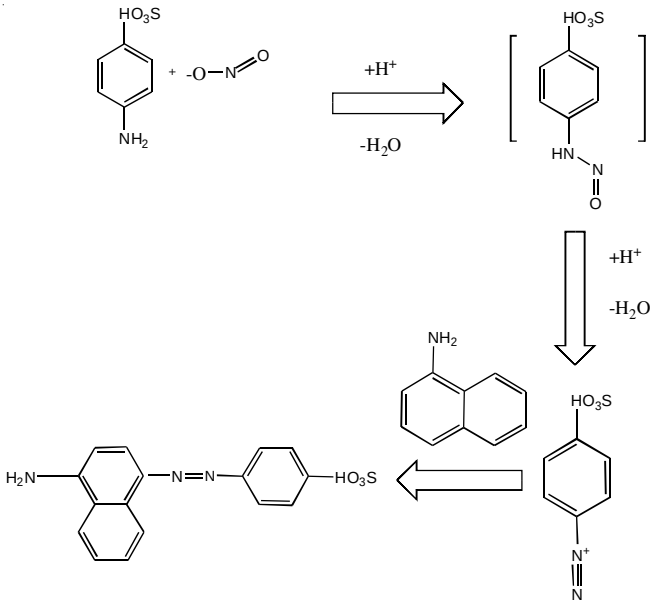

Fig. 1. Mechanism of the Griess reaction [6]

The concentrations of nitrate and nitrite [7] were determined by spectrophotometric methods. The biomass concentration was measured by the determination of the optical density of the medium using Thermo Scientific GENESYS 10S UV-visible spectrophotometer at a wave number of 600 nm. Each strain was tested microscopically.

RESULTS AND DISCUSSION

Isolation and characterization of strains B and C: Seeding of 1 mL of the enriched inoculums in solid medium was conducted of the occurrence of several colonies, which were different in their forms and colours. The purification of this bacteria, in dishes plates and in inclined tubes allowed to lead a wide variety of the bacteria. Thus, the nitrate reductase test in the pure strains was allowed us to select solely the denitrifying bacteria (data not shown).

Both selected bacteria B and C have the ability to perform the denitrification reaction until the stage of atmospheric nitrogen.

Therefore, the Gram staining test's allows to prove both the wall cell types and the purity of each selected bacteria, as shown in the Fig. 2.

The results suggested that the strain B is *Cocci* in its form, positive Gram which it means that, the bacterium had a thick cellular wall of peptidoglycan. Nevertheless, the strain C is

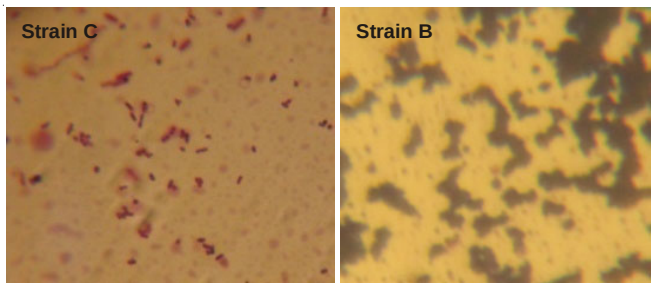

Fig. 2. Gram staining of strains B and C

Diplococci (like digit 8) and it has a negative Gram. Moreover, both strains B and C are facultative aerobic and anaerobic, because they grew on the surface of solid medium and even both bacteria could perform the denitrification reaction under anaerobic conditions.

Thereby, both selected bacteria have the similar growth conditions. The optimization of the parameters that affect the denitrification process, as well as the growth kinetic of both bacteria B and C, allowed to fix them at 30 °C; pH 7 and glucose 2 g/L.

Therefore, the optimal temperature, pH and carbon concentration of growth are the same for a lot of denitrifying bacteria according several researchers [8-10], like the species *Alcaligines* and *Paraccocus denitrificans* [11-13]. Additionally, some parameters could affect the denitrification process, such as dissolved salts concentrations including sodium chloride, nitrogenous substrates concentration and the concentration of produced metabolites [14-18].

Culturing of the strain B: Under optimal growth conditions of the desired strains, the strain B was incubated in a closed bioreactor containing minimum medium rich in nitrate. The dosage of residual nitrate and produced nitrite concentrations are shown in Fig. 3a. Also, the measurement of the bacterial biomass evolution during 48 h of incubation was shown in the Fig. 3b.

The strain B could be grow under anaerobic conditions, using nitrate as a final electron acceptor, this incorporation was provided by nitrate reductase enzyme's, the mechanism of denitrification was described in several studies [19-21]. It consists in oxidation of a carbon substrate on anaerobic conditions. The molecular oxygen was considered as the higher sources of energy compared with the nitrate ions, which could replace the oxygen under anaerobic conditions and take the role of the final electron acceptor. Electron were transferred *via* a phosphorylation chain (ATP formation) according to the mechanism presented in Fig. 4

The curve in the Fig. 3b demonstrate clearly all phases of the bacterium growth, beginning by the lag phase, followed by an exponential growth phase between 0-20 h, after that a stationary phase was coming at 20 h. Finally, a decline phase was appeared at above > 38 h.

Meanwhile, strain B used nitrate ions for its respiration, instead of oxygen molecule. Thus, the dosage of nitrates shown a diminution with 10 mg/L compared with the control tube without going through nitrites, using glucose as a source of organic carbon. Consequently, the results suggested that the strain B is a facultative anaerobic bacterium which can perform the heterotrophic denetrification, without production of toxic products.

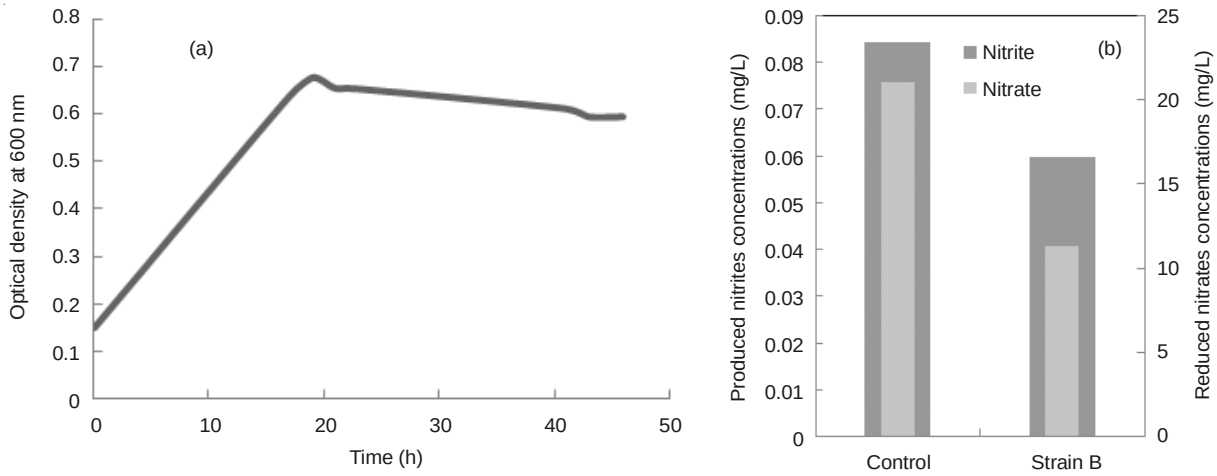

Fig. 3. Evolution of the growth kinetic of strain B during 48 h of incubation (a) and the concentrations of residual nitrates and produced nitrites after the denitification process by strain B (b)

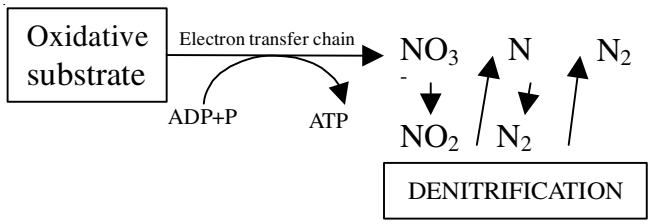

Fig. 4. Mechanism of denitrification reaction

Culturing of strain C: Under the same operating conditions as before, the strain C was incubated in the same volume of the minimal medium in the bioreactor. After 48 h, the strain C has given the results presented in the following two graphs (Fig. 5a and 5b).

Strain C could grow in anaerobic conditions using nitrate as the final electron acceptor instead of oxygen, which was explained by the decrease of the concentration of residual nitrates of 2.5 mg and in parallel the increase of produced nitrite of 0.08 mg. Therefore, a many amount of reduced nitrate was transformed to nitrites by strain C. The energy released by this reduction was used as energy source of its growth (Fig. 4).

Comparison between the performance of strains B and C: The growth evolution as a function of time of both strains B and C was presented in the Fig. 6a. Thus, the Fig. 6b shows the residual concentrations of desired ions after 48 h of incubation of both strains B and C.

According to the growth kinetics of both strains B and C, the results suggested that was adapted to the medium and attain a maximum growth of 0.65 which was better than that marked by the strain C, which did not exceed 0.23 as a maximum value of growth. This result is explained by the difference of the nitrate reduction power of the two bacteria, as shown Fig. 6b, the difference is significant. The strain B reduced about 10 mg of nitrate without production of nitrites, but strain C reduced 2.5 mg of nitrate with production of 0.08 mg of nitrites during the same period of incubation. So this results allowed that the performance of strain B was more important compared with strain C.

According to the literature, the denitrification was performed at anaerobic conditions with a high nitrate reduction rate [22]. It was acted the nitrite as an electron donors. So, different carbon sources could be achieved, such as succinate, glycerol and glucose.

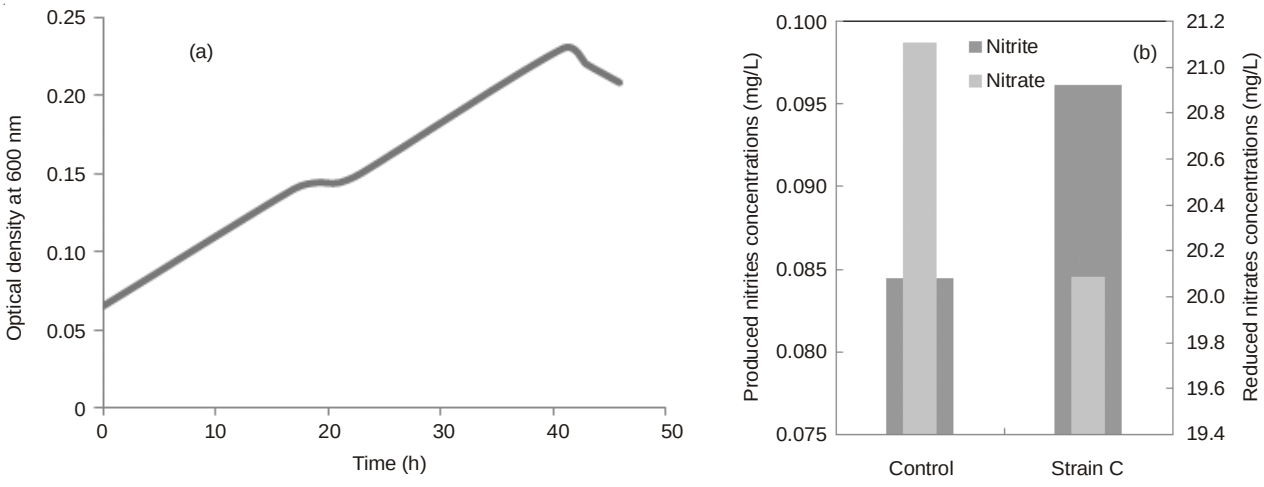

Fig. 5. Evolution of the growth kinetic of strain C during 48 h of incubation (a) and concentrations of residual nitrates and produced nitrites after the denitrification process by strain C (b)

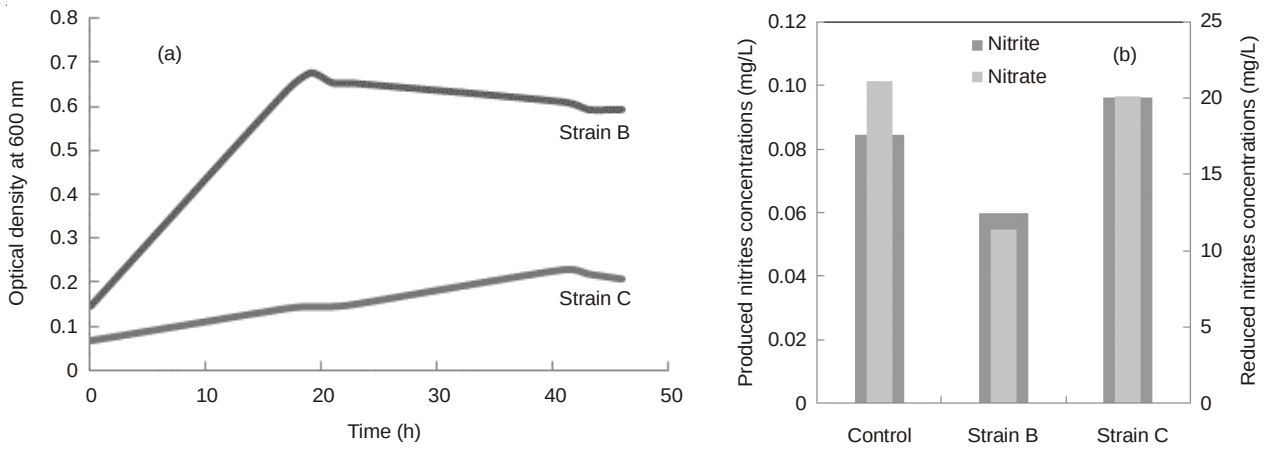

Fig. 6. Comparison between the growth kinetics of both strains B and C during the 48 h of incubation and the concentrations of residual nitrates and produced nitrites after the denitrification process by both strains B and C (a) and (b)

However, some species could not effectively remove the NO_3-N, in the presence of some organic carbon sources such as acetate and methanol [12]. Yet, there was autotrophic denitrification like *Thiobacillus* denitrificans which used the sulfur compound as electron donor instead of carbon, but it had a lower removal rate compared with heterotrophic processes [5].

Conclusion

The feasibility of the denitrification by strain B developed was demonstrated for a synthetic wastewater. A comparison of our strain with the other isolated strains of the same sludge, suggested that the strain B had a higher rate of reduction. Both strain B and strain C, have gave a nitrate reduction rate of 100 % of nitrate reduction in the qualitative analysis with broth nitrated. While, in a minimum medium, strain B have presented a better nitrates degradation performance without production of toxic by-products (oxides of nitrogen) compared with that performed by strain C. This *Cocci* could reduce nitrate to atmospheric nitrogen in conventional operating conditions of 35°C; pH 7, 120 rpm and dissolved oxygen concentration of 0 %. Nevertheless, further works were needed to validate these parameters and to view the possibility of application of the strain B in the treatment of wastewaters rich in nitrates and nitrites ions.

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