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Original Research Article

Biological Denitrification Using Pure Cultures Isolated from Wastewater Treatment Plant of Khenchela (Eastern Algeria)

Oumaima Naili[1,2], Messaoud Benounis[1,*], Abdelhak Darbouche[3] and Asma Kheddouma[3,4]

[1]Laboratory of Sensors, Instrumentations and Process (LCIP), Department of Chemical Engineering, Institute of Science and Technology, University of Khenchela, ALGERIA

[2]Laboratory of biology, University of Setif, ALGERIA

[3] Institute of Biology, Department of Microbiology, University of Khecnhela, ALGERIA

[4]Laboratory ESPA, University of Batna, ALGERIA

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ABSTRACT

The purpose of this study was to investigate biological dentirification of wastewater by pure cultures isolated from activated sludge of wastewater treatment plant of Khenchela (Eastern Algeria). Experiments were performed in batch reactor under anaerobic conditions. Three pure strains were isolated and identified as *Enterobacter cloacae, Pseudomonas luteola* and *Aeromonas hydrophila* Three carbon sources were used in this work; glucose, methanol and lactose. High denitrification was obtained at 2000 ppm for different carbon sources. Kinetic of bacterial growth and denitrification were studied. We show that bacterial growth rate directly influences nitrate removal from the medium and the use of glucose as carbon source provide high denitrification rate than methanol and lactose. These strains isolated from activated sludge will be used to develop new biosensors applied to the detection and determination of nitrate in real water.

Keywords: Denitrification, Wastewater, Batch culture, Microbial process, Nitrates, Activated sludge

Henşle (Doğu Cezayir) Su Arıtma Tesisi'nden İzole Edilen Saf Kültürlerin Biyolojik Denitrifikasyonu

ÖZET

Bu çalışmanın amacı; Henşle (Doğu Cezayir)'nin atık su tesislerinden elde edilen çamur atıklarının biyolojik denitrifikasyonunun araştırılmasıdır. Denemeler anerobik koşullar altında yığınlı reaktörlerde gerçekleştirilmiştir. Üç saf kültür ırk olan *Enterobacter cloacae, Pseudomonas luteola* and *Aeromonas hydrophila* izole edilmiş ve tanımlanmıştır. Bu çalışmada üç karbon kaynağı kullanılmıştır, bunlar; glukoz, metanol ve laktoz'dur. Yüksek denitrifikasyon değişişk karbon kaynakları için 2000 ppm'de elde edilmiştir. Denitrifikasyon ve bakterinin kinetiği de bu deneme de çalışılmıştır. Sonuçlar göstermiştir ki; bakteriyel gelişim oranı direk olarak ortamdan nitratın uzaklaştırılmış ile ilişkilendirilmiştir ve karbon kaynağı olarak glukozun kullanılması metanol ve laktoza göre daha yüksek denitrifikasyon sağlamıştır. Bu atık çamurdan elde edilen bakteri ırkları gerçek su kaynaklarında nitratın tanımlanmasında kullanılacak olan yeni biyo sensörlerin geliştirilmesinde kullanılabilecektir.

Anahtar Kelimeler: Denitrifikasyon, Atık su, Mikrobiyal proses, Nitrat, Aktif çamur

INTRODUCTION

The removal of nitrate is essential for water contaminated with nitrate before being utilized since a large amount of nitrate in drinking water often causes a disease called methemoglobinemia and other health disorders such as hypertension , increased infant mortality , goiter , stomach cancer , thyroid disorder , cytogenetic defects and birth defects (Nag-Choul and Dong-Ju 2007).

Biological removal of nitrate is widely used in the treatment of domestic and complex industrial wastewaters (Delanghe et al. 1994, Lemmer et al. 1997, Sozen and Orhon 1999, Kesseru et al. 2003, Dong and Tollner 2003).

Due to their important roles in wastewater treatment processes, denitrifying bacteria in engineered biological nitrogen removal (BNR) systems are of particular interest to wastewater engineers and microbiologists as a model microbial community (Schlegel, 1992; Ekama, 1999; Lu, 2014). Biological filter capable of simultaneous nitrification and denitrification for Aquatic Habitat at the International Space Station (ISS) was

* Corresponding author: benounis@yahoo.fr

constructed (Uemoto and al, 2014). Bhuvanesh and al (2013) study denitrification of wastewater using a hybrid anoxic reactor (HAR), which uses self-immobilized microbial granules under fluidized condition.
Many authors have studied the effects of carbon sources on nitrate removal from various environmental sites for biological processes. Two natural organic substances; liquorice (*Glycyrrhiza glabra*) and giant reed (*Arundo donax*) were investigated as a carbon source in the biological denitrification of drinking water (Ovez et al, 2006). Rashid and Schaefer (1988) reported that among various carbon sources, glucose and cellulose induced a very high degree of nitrate removal in a soil under anaerobic condition. According to Gomez et al. (2000), ethanol was the suitable carbon source in comparison with sucrose and methanol for nitrate elimination from contaminated ground water. Evaluation of simultaneous nitrification and denitrification under controlled conditions by an aerobic denitrifier culture was reported by Zhang and al (2015).

The aim of the present paper was to investigate the applicability of the pure cultures isolated from activated sludge of wastewater treatment plant of Khenchela (Eastern Algeria) for denitrification processes using different carbon sources.

MATERIALS AND METHODS

Sampling

Activated sludge was collected from wastewater treatment plants of Khenchela. Samples were collected from drying beds under anaerobic conditions, in sterile bottles and had been transported on ice to the laboratory for microbiological analysis.

Isolation of bacteria

Microbial populations of activated sludge were determined by serial dilution of samples in saline solution before inoculation onto Petri dishes of sterile nutrient agar. Then, cultures were incubated in an anaerobic candle-jar placed in a stirred bain-marie (GFL1083) during 24 h at 30 ° C.
Single colonies of bacteria on the nutrient agar were then sub-cultured on the same medium until pure isolates were obtained (Sharifi-Yazid et al. 2001)**.** After, pure strains were conserved on glycerol and stored at -20°C until further tests.

Identification of isolates

Observation of morphology and biochemical test

After obtaining pure cultures, the following tests were performed for identification purposes: Gram’s staining, motility, catalase test, oxydase test, ß galactosidase test, nitrate reductase, urease test, Citrate test, indole production, carbohydrate fermentation, mixed acid fermentations.

Identification of bacteria by API system

Pure isolates were identified using API 20E and API 20NE systems, according to the manufacturer's instructions (BioMérieux).

Batch cultures

Pure cultures were enriched in 100 mL of synthetic medium (Table 1) for 24hbefore inoculation into batch cultures. This medium was used to promote the growth of heterotrophic denitrifying bacteria.
Batch cultures were performed in 500 mL bottles containing 300 mL of nutrient medium and supplemented separately with three different concentrations (500 ppm, 1000 ppm and 2000 ppm) of glucose, lactose and methanol as carbon sources. Inoculums pure enriched cultures (2.5%) were added to bottles and incubated in anaerobic conditions for 48 h at 30°C in stirred bain-marie (Rhee et al. 1997).

Table 1. Composition of the synthetic medium at pH 7**.**

Composition	**Concentration**
KH_2PO_4	1 $g.L^{-1}$
K_2HPO_4	1 $g.L^{-1}$
KNO_3	1 $g.L^{-1}$
NaCl	1 $g.L^{-1}$
$MgSO_4$	0.2 $g.L^{-1}$
$CaCl_2$	0.02 $g.L^{-1}$
Trace elements	1 mL

Trace elements: 6.76 ml HCl (37%), 1.5$g.L^{-1}FeCl_2.4H_2O$, 0.06 $g.L^{-1}H_3BO_3$, 0.1 $g.L^{-1}MnCl_2.4H_2O$, 0.12 $g.L^{-1}$ $CoCl_2.6H_2O$, 0.07 $g.L^{-1}$ $ZnCl_2$, 0.025 $g.L^{-1}$ $NiCl_2.6H_2O$, 0.015 $g.L^{-1}CuCl_2.2H_2O$, 0.025 $g.L^{-1}$ $NaMoO_4.2H_2O$ and 5.2 $g.L^{-1}$EDTA.

Biomass growth

The optical density of suspensions was monitored every two hours using UV-Vis spectrophotometer (SHIMADZU) at 600 nm as indirect biomass measurement.

Determination of nitrates rate

10.5 ml of liquid sample was withdrawn from the batch cultures using syringes and centrifuged for 10 min at 7000 rpm (MLW centrifuge T 52.1) to remove cells from supernatant. Nitrate concentrations were measured by colorimetric method according to sodium salicylate test (Rodier 2009).

RESULTS

Morphological and biochemical characterization

The serial dilution of activated sludge samples from drying beds of wastewater treatment plant on nutrient agar gave different colonies. Three strains, S1, S2 and S3, were selected on the basis of morphology and nitrate reducing test. Gram staining showed that all isolates were Gram negative bacilli. The biochemical characterization of these strains is represented in Table 2.

Table 2. Morphological and biochemical characterization of isolates.

Test	S1	S2	S3
Type of respiration	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
catalase	+	+	+
oxydase	-	+	-
B-galactosidase			
Nitrate reductase	+	+	+
Urease	+	+	+
Citrate	-	+	+
ONPG	+	-	+
Indole	-	-	+

(+) Positive results; (-) Negative results.

Identification of bacteria by API system

Strains isolated were identified by API system in our previous work (Kheddouma et al, 2013). The 7-digit profile number and resulting strains are showed in Table 3.

Table 3: Identification of strains by API system.

	S1	S2	S3
API system	Api20 E	Api20 NE	Api20 NE
Numerical profile	3305573	1547747	5576755
Strain Name	*Enterobacter cloacae*	*Pseudomonas luteola*	*Aeromonas hydrophila*

Bacterial growth of cultures

A preliminary study of bacterial growth and removal nitrate kinetic in the presence of three different concentrations of carbon sources, 500ppm, 1000ppm and 2000ppm, shows that higher cell growth and denitrification rate were obtained at 2000 ppm compared to the other concentrations (Kheddouma et al, 2012).This concentration was used to inoculate the batch cultures. All experiments were made in duplicate and statistical errors are not significant

Bacterial growth using Enterobacter cloacae

The absence of latency phase (Fig.1) indicated that bacterial cells are well adapted to environmental conditions after enrichment. A significant bacterial growth was observed during the first 25hand then bacteria enter in stationary phase. These results showed that *Enterobacter cloacae* has the ability to use the three types of substrates. This can be explained by the fact that in addition to the enzymes involved in glycolysis act, this strain has also the methanol dehydrogenase and β-galactosidase activities.

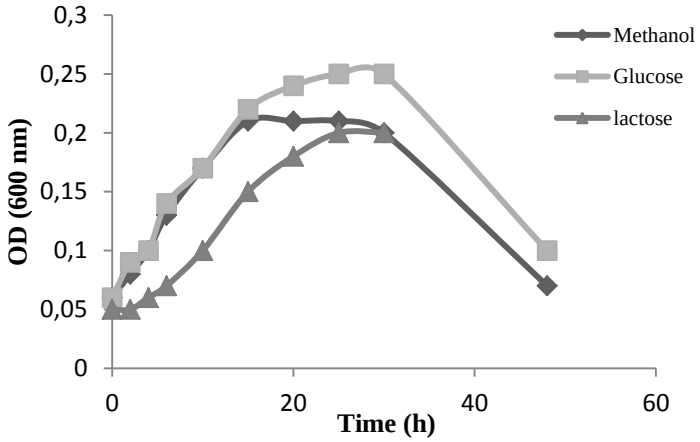

Figure 1. Kinetic of bacterial growth using *Enterobacter cloacae* at 600 nm.

Bacterial growth using Pseudomonas luteola

From Figure 2, it can be seen that no cell growth occurred when the lactose was used as carbon source which lead to the absence of specific enzymes for lactose degradation *(*β-galactosidase).However, in the presence of methanol as carbon source, bacterial growth is similar to that obtained with glucose. The high number of bacterial cells capable to degrading glucose and methanol is represented by high optical density values.

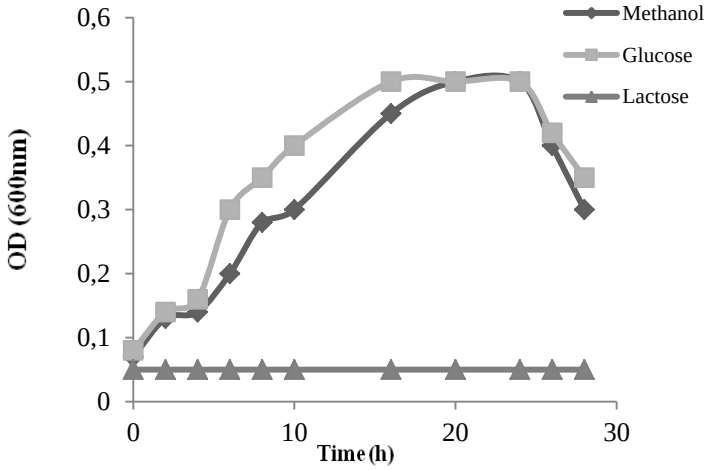

Figure 2. Kinetic of bacterial growth using *Pseudomonas luteola* at 600 nm.

Bacterial growth using Aeromonas hydrophila

A higher growth rate of *Aeromonas hydrophila* was observed during the first 24 h when methanol was used as substrate (Fig. 3).Then, this strain enters in stationary phase after 32 h of incubation. Cell growth was observed too when methanol and lactose were used as substrates.

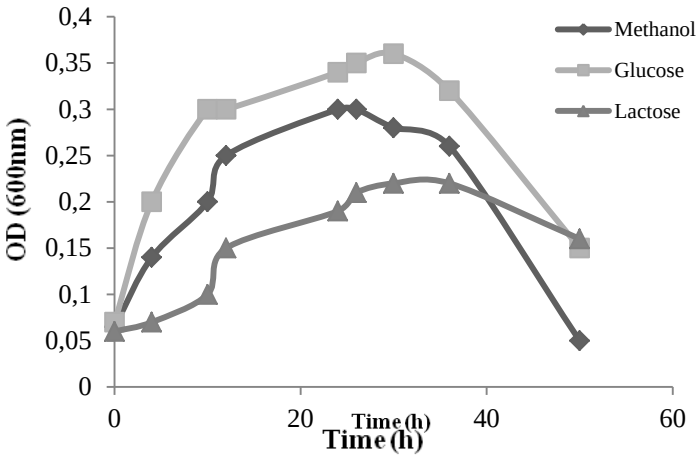

Figure 3. Kinetic of bacterial growth using *Aeromonas hydrophila* at 600 nm.

Nitrate removal from batch cultures

Nitrate removal using Enterobacter cloacae

In figure 4, we show that the rate of denitrification with glucose was higher compared to the two other carbon sources and complete nitrate reduction was observed after 32 h.

For culture with lactose, the reduction of nitrate at a concentration of 20 $mg.l^{-1}$ was reached after 40 h of incubation.

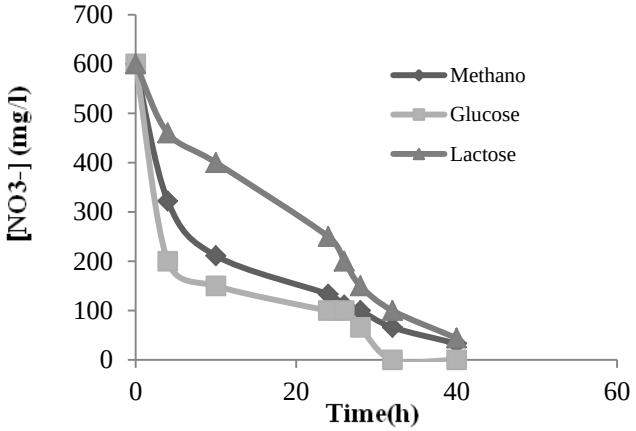

Figure 4. Kinetic of nitrate removal using *Enterobacter cloacae.*

Nitrate removal using Pseudomonas luteola

Figure 5 shows that the use of glucose and methanol as carbon sources nitrate was reduced faster for *Pseudomonas luteola* compared to *Enterobacter cloacae*. No reduction with lactose was observed which lead to the absence of bacterial growth.

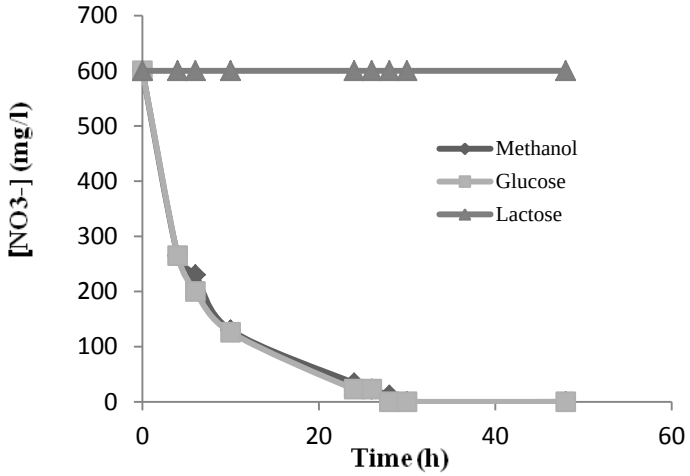

Figure 5. Kinetic of nitrate removal using *Pseudomonas luteola.*

Nitrate removal using Aeromonas hydrophila

In figure 6, total nitrate reduction was achieved after 45 h and 50 h when glucose and methanol was used, respectively. The use of lactose reduced the nitrate slowly; a concentration of20 $mg.l^{-1}$ was reached for NO_3^- after 50 h of incubation.

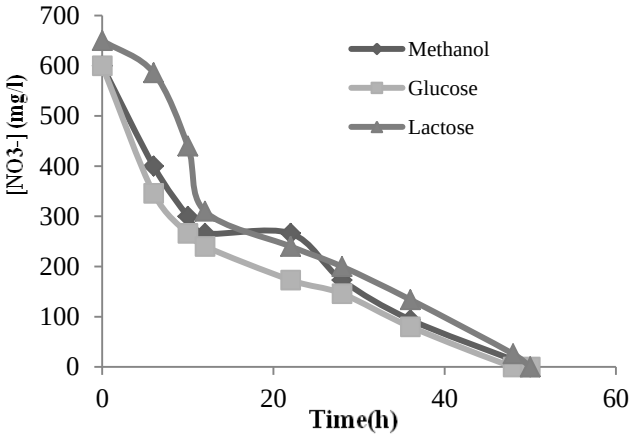

Figure 6. Kinetic of nitrate removal using *Aeromonas hydrophila.*

DISCUSSION

- In the current study, many species were isolated and checked for the nitrate degradation capacities. Among them, three strains were selected and identified as *Enterobacter cloacae*, *Pseudomonas luteola* and *Aeromonas hydrophila.*
- Batch cultures are carried out in anaerobic conditions and inoculated with pure isolated cultures to study nitrate removal from activated sludge samples.
- The use of different carbon sources has shown that simple sugars such as glucose are easily as similable and degradable by bacteria, which will increase the bacterial growth and provide a good reduction of nitrate.
- *Pseudomonas luteola* strain gives a good denitrification compared to the two other strains *Aeromonas hydrophila* and *Enterobacter cloacae*.In the case of glucose as carbon source, we show that nitrate was completely removed after 30 h, 50 h and 52 h using *Pseudomonas luteola*, *Aeromonas hydrophila* and *Enterobacter cloacae*, respectively. However, this process required more time with methanol.
- When lactose was used as carbon source by *Pseudomonas luteola* denitrification was not observed which can be explained by the absence of the enzymes responsible for lactose degradation.

- Bacterial growth rate directly influences nitrate removal from the medium; when bacterial growth increases the nitrates will be reduced faster.
- Finally, the research reported in this paper demonstrated that biological treatment is a technically feasible option for treating wastewaters containing high concentrations of nitrate and can be used to develop new biosensors applied to the detection and monitoring of nitrate in real water.

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