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REMOVAL OF NITRATES FROM BRINE USING *HALOFERAX MEDITERRANEI* ARCHEON

The aim of the study was to optimize a saline brine denitrification by halophilic microorganisms *Haloferax mediterranei*. To define the optimum parameters of the process, the Latin square method was used (Statistica 6.0). The highest specific nitrate reduction rate was observed at a temperature of 37 °C and pH 7.0. There was no significant effect of NaCl on nitrate ion reduction at the concentration range from 2.5 to 3.5 M. During brine denitrification in a batch bioreactor the rates of specific nitrate reduction and nitrate reduction reached 100.5 mg $N–NO_3^-$ per 1 gram of dry mass during 24 h and 135 mg $N–NO_3/dm^3 \cdot day$, respectively.

1. INTRODUCTION

Intensification of agricultural production and continuous industrial development have contributed to an increase in the nitrate content in drinking water. In some regions of Poland, nitrate content has considerably exceeded the permissible levels of 50 mg per one dm^3 [1]. This is particularly evident in rural areas, where in private wells the concentration of nitrate nitrogen is often over twenty times above the permissible level. This situation poses a serious threat to the health of people using polluted water. Therefore, it is now necessary to develop a technology which effectively reduces nitrate concentration in drinking water.

A relatively simple and cheap method, which facilitates effective water denitrification, is an ion-exchange process [2], [3]. The ion-exchange process has mainly been applied to utilize cooling water in heat and power generating plants, but also may be

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used to remove microcontaminants from drinking water. This process is based on chloride ion exchange, in which these ions bound by functional groups of resins are replaced by nitrate ions.

As a result of ion exchange the resin column is saturated with nitrate ions and requires regeneration. For this purpose, primarily natrium chloride (50–150 g/dm^3) or, more rarely, bicarbonate solutions are used. As a by-product of these reactions concentrated saline brine is produced, which contains nitrate ions released from the ion-exchange column, as well as chloride, sulfate and bicarbonate ions. This brine can be considered to be dangerous and aggressive pollutant of the environment. The utilization of saline brine by conventional biological methods using pure bacterial cultures or sewage sludge is impossible because of low water activity inhibiting bacterial growth. In this context, the application of halophilic microorganisms capable of reducing nitrate ions to nitrogen gas may be proposed as a very attractive and practical solution [4]–[6].

The aim of the study was to optimize the denitrification of saline brine by *Haloferax mediterranei*. In the experiment, temperature, pH and sodium chloride concentration were the experimental variables.

2. MATERIALS AND METHODS

2.1. MICROORGANISMS

The halophilic microorganisms *Haloferax mediterranei* (ATCC 33500) were used to denitrify saline brine. *Haloferax mediterranei* can tolerate high NaCl concentration in the environment and may use nitrates under anaerobic conditions as final electron acceptors [7].

The strain of *H. mediterranei* grew on agar slants at a temperature of 37 °C for 5 days. Then the bacteria were transferred to Erlenmeyer flasks, each containing 100 cm^3 of a liquid medium A (g/dm^3) whose composition was as follows: 7.5 g of yeast extract, 2 g of Casamino acids, 20 g of $MgCl_2 \cdot 6H_2O$, 2 g of KCl, 0.1 g of NaBr, 3 g of sodium citrate and distilled water. The cell suspension collected from three slants was used to inoculate 100 cm^3 of liquid medium. After 72 hours 20 cm^3 of bacterial culture was centrifuged at 3600 *g* for 10 min and the biomass obtained was used to inoculate 200 cm^3 of brine. The initial concentration of biomass was 0.06 g of DM/dm^3.

The composition of brine used in the experiment was as follows (medium B): 5 g of yeast extract, 2.0 g of Casamino acids, 20 g of $MgCl_2 \cdot H_2O$, 2.0 g of KCl, 0.1 g of $CaCl_2$ and distilled water to 1 dm^3. The initial nitrate concentrations of 100 and 1000 mg $N\text{–}NO_3^-$/dm^3 were obtained by dissolving the appropriate amounts of sodium nitrate.

2.2. CONDITIONS OF BATCH CULTURE

Bacterial cultures were grown in 300-cm^3 glass flasks whose content was not shaken. Each flask contained 200 cm^3 of saline brine (medium B) which was protected against oxygen with rubber stoppers. In the denitrification process, the experimental variables were in the following ranges: pH 5, 6, 7, 8, and 9, temperature of 20, 30, 37, 45, and 55 °C, and NaCl concentration of 2.5, 2.75, 3.0, 3.25, and 3.5 M. The experiment was conducted over 7 days. The changes in the concentration of biomass (DM) and nitrates in a liquid medium were evaluated.

To test the denitrification process under optimal conditions and to remove nitrates from brines (on a larger scale) bacteria were cultured in a bioreactor, BioFlo III type by the New Brunswick Scientific Company (USA), with the operating capacity of 5 dm^3, equipped with an electrode for pH measurement (Ingold) and a Rushton-type turbine used as an agitator. First the bioreactor was filled with 5 dm^3 of medium B and then inoculated with the bacterial suspension and the initial biomass concentration was 0.06 ± 0.01 g DM/dm^3. Conditions in bioreactor, i.e. a temperature of 37 °C and pH 7.0, were controlled automatically, and the agitation rate was kept at 100 rpm. The concentration of biomass (DM) and the changes in nitrate and nitrite concentrations in liquid medium were evaluated and the activities of nitrate and nitrite reductase were measured.

2.3. ION EXCHANGE

In the research, we made use of the ion-exchange resin Ionac SR-7 (Sybron Chemicals Inc., USA). The testing station consisted of a glass column packed with 30 cm^3 of resin. Water was introduced to the column from the top by a peristaltic pump. The volume of 1 dm^3 of water contained 133 mg of NO_3^- (30 mg of N–NO_3^-), 100 mg of SO_4^{2-}, 40 mg of Cl^- and 120 mg of HCO_3^-. Water pH was 7.4, and its flow rate in the experiment with 30 cm^3 of resin in the above mentioned apparatus reached 25 V/V_{bed}. The process was carried out until the resin exchange capacity had neared exhaustion. Resins were regenerated using 18% solutions of NaCl.

2.4. ANALYTICAL METHODS

Anions analysis

Anions contained in the collected samples of drinking water were determined with High Performance Liquid Chromatography (HPLC). Measurements were taken using a Merck-Hitachi liquid chromatograph equipped with a UV detector. A Polysphere IC AN-1 (Merck) column with a pre-column was used. Furthermore, the concentrations of nitrates and nitrites in brine were determined spectrophotometrically; the former

determination is based on the reaction with brucine [8], and the latter – on the reaction with sulfanilic acid and 1-naphthylamine [8].

Activity of nitrate reductase

The activity of nitrate reductase was determined on the basis of the measurement of nitrites formed from nitrates during the enzymatic reaction. The reaction was carried out at pH 7.2, incubating the samples for 15 min at a temperature of 37 °C. The amount of nitrites formed in the reaction mixture equivalent to the amount of reduced nitrates (in micromoles) was determined spectrophotometrically with 1-naphthylamine [9].

The sample (400 cm^3) was collected from the culture carried out in the bioreactor and centrifuged at 4000 *g*. The biomass obtained was suspended in 0.1 M phosphate buffer (pH 7.2), containing 20% NaCl, and then centrifuged again. The operation was repeated twice and the biomass was condensed to the volume of 20 cm^3. Then the cells were being disrupted for 30 minutes with a ball grinder. Cell disruption was conducted in a Zellmuhle (Germany) ball mill, using glass balls, 0.3 mm in diameter. After the cell disruption and balls separation, the biomass was centrifuged at 5000 *g*. Next, using a test tube, a total volume of 3 cm^3 of the reaction mixture was prepared. It consisted of 1 cm^3 of KNO_3 (100 mM), 0.5 cm^3 of Tris-HCI (100 mM,), 0.5 cm^3 of methylviologen (120 μM), 1 cm^3 of NaCl (1M) and 50 μl of crude extract. The reaction was initiated by the addition of 0.1 cm^3 of sodium hydrogen sulfate (0.5 M). Thereafter the samples were incubated for 15 min in a water bath at a temperature of 37 °C. Afterwards 0.1 cm^3 of formaldehyde was added to each sample in order to terminate the reaction. The content of nitrites in samples was determined based on their reaction with the sulfanilic acid and 1-naphthylamine. Absorbance was measured at 520 nm wavelength. The results were given as the amount of N–N $O_2^- \cdot h^{-1} \cdot g^{-1}$ protein [9].

Activity of nitrite reductase

The activity of nitrite reductase was determined on the basis of the amount of nitrites used to produce nitrogen oxides during the process of denitrification. The reaction was carried out at pH 7.2, incubating the samples for 15 min in a water bath at a temperature of 37 °C. The quantity of nitrites was measured with spectrophotometer at 520 nm wavelength.

The reaction mixture and the incubation time were identical as those in the method used for the determination of nitrate reductase activity, but $N–NO_3^-$ was replaced by $N–NO_2^-$ obtained by diluting 0.01 g of $NaNO_2$ in 100 cm^3 of degassed distilled water. The results were given as the amount of $N–NO_2^- \cdot h^{-1} \cdot g^{-1}$ protein [9].

Proteins

Soluble protein was determined by the Bradford method [10].

Dry matter (DM)

Dry matter was determined by the drier method [8].

3. RESULTS AND DISCUSSION

Microbial denitrification of brines

The aim of the studies was to asses the effect of culture parameters on the rate of brine denitrification by *Haloferax mediterranei* archeon. As the nitrate conversion to gaseous nitrogen is a biological process, critical parameters, affecting bacterial growth, thus their capacity to remove nitrates, are as follows: temperature, pH and NaCl concentration (osmotic potential of solution).

Table 1

Experimental treatments based on the Latin square design affecting the specific nitrate reduction rate by *H. mediterranei* archaeon

Culture	Parameters of culture		
	Temperature (^{o}C)	pH	NaCl (M)
1	20	5	2.5
2	20	6	2.75
3	20	7	3.0
4	20	8	3.25
5	20	9	3.5
6	30	5	2.75
7	30	6	3.0
8	30	7	3.25
9	30	8	3.5
10	30	9	2.5
11	37	5	3.0
12	37	6	3.25
13	37	7	3.5
14	37	8	2.5
15	37	9	2.75
16	45	5	3.25
17	45	6	3.5
18	45	7	2.5
19	45	8	2.75
20	45	9	3.0
21	55	5	3.5
22	55	6	2.5
23	55	7	2.75
24	55	8	3.0
25	55	9	3.25

Investigations were carried out according to the Latin square design. In order to determine the effect of three input values, each acting on five levels, the experiments can be carried out according to the 5×5×5 factorial design yielding the total of 125 experiments. If the number of input values are greater than two and there are no interactions between input values or these values are negligible, it is possible to design a series of experiments on the basis of Latin squares. In this way, it is possible to reduce the number of experiments to 25 and, therefore, to obtain unbiased estimators of the main effects ($\propto = 0.05$). The "Experimental Design" module of the Statistica 6.0 package generates automatically the design of the experiments on the basis of the Latin square design (table 1).

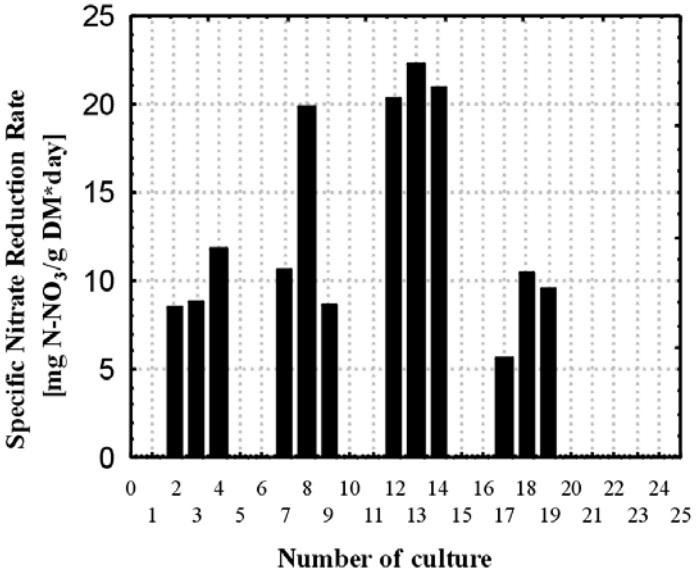

Fig. 1. Specific rate of nitrate reduction by *H. mediterranei* archaeon at the examined values of temperature, pH and NaCl concentration in batch cultures

Figure 1 presents specific nitrate reduction rate for individual culture variants. The statistical analysis of the results obtained showed that the denitrification process essentially depended on the temperature and pH of saline brine (table 2). However, the effect of pH ($p < 0.0087$) was stronger than the effect of temperature ($p < 0.0248$). The maximum specific nitrate reduction rate was obtained for pH 7. At pH 6 and 8 the reduction of nitrate ions was lower than that at pH 7, but the process still progressed efficiently. For extreme values of pH 5 and 9 bacterial growth was not observed.

Denitrification of saline brine was most efficient at a temperature of 37 °C. A high specific nitrate reduction rate was obtained also at a temperature of 30 °C. Specific nitrate reduction rates at the temperatures of 20 and 45 °C were considerably lower

and did not differ significantly. At a temperature of 55 °C no microbial growth was observed.

Table 2

Analysis of variance for the factors (temperature, pH, NaCl) affecting the specific rate of nitrate reduction by *H. mediterranei* archaeon. SS – sum square, MS – mean square, F – Fisher's statistics, *p* – level of propablity, *df* – degree of freedom

Factor	SS	*df*	MS	*F*	*p*
Temperature	93817	4	23461	3.38	0.0248
pH	155779	4	38944	5.62	0.0087
NaCl	5914	4	1478	0.21	0.9259
	83090	12	6924		

The effects of sodium chloride on bacterial growth and specific nitrate reduction rate were different (figure 2). In the range of NaCl concentrations investigated, the effect of salt on denitrification yield was negligible ($p < 0.9259$). This was due to a great adaptation capability of halophilic microorganisms to changeable salinity of the culture medium and a high osmotic pressure. In this process, intracellular biosynthesis of amino acids and modification of ionic forces in the cytoplasm are responsible for an increase in intracellular pressure and a positive pressure gradient in relation to the environment.

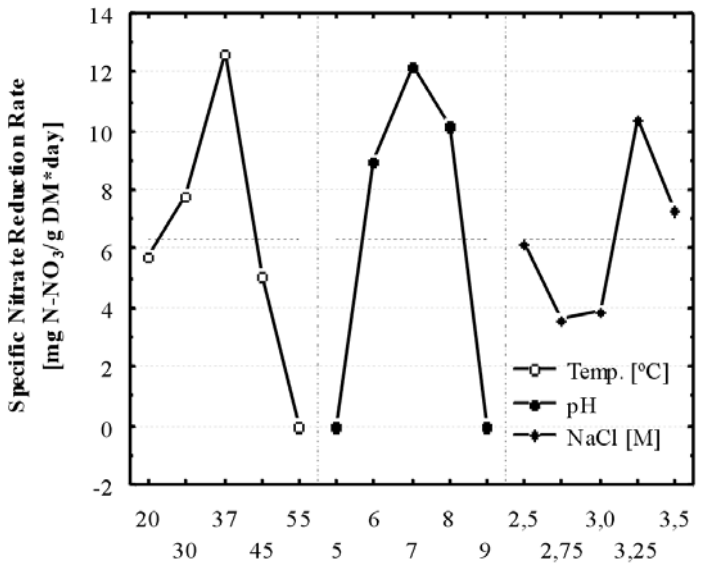

Fig. 2. Effect of temperature, pH and NaCl concentration on the specific rate of nitrate reduction by *H. mediterranei* archaeon in batch cultures

In the next step of the study, periodic cultures of microorganisms were grown under optimum conditions, i.e. 37 °C, pH 7 and NaCl concentration of 3.5 M (figure 3), in a batch bioreactor. The kinetics of nitrate removal was linear. On the 6th day of the process nitrate nitrogen concentration was 190 mg of $N–NO_3^-·dm^{-3}$. The gradual accumulation of nitrites to 0.3–0.9 mg $N–NO_2^-/dm^{-3}$ was observed in the culture medium. When cultured, bacteria increased their density from the initial 0.06 g DM/dm^{-3} to 1.12 g DM/dm^{-3}. As a result, the specific nitrate reduction rate reached 100.5 mg of $N–NO_3^-/g^{-1}$ of dry mass during 24 h and was higher than in previous experiments. Instead, the rate of nitrate reduction was low and amounted to 135 mg $N–NO_3^-/dm^{-3}$ in 24 h, which was due to the low density of cell biomass in the medium. The results obtained were in agreement with previous studies [11], [12] performed with halophilic microorganisms capable of aerobic denitrification of brine.

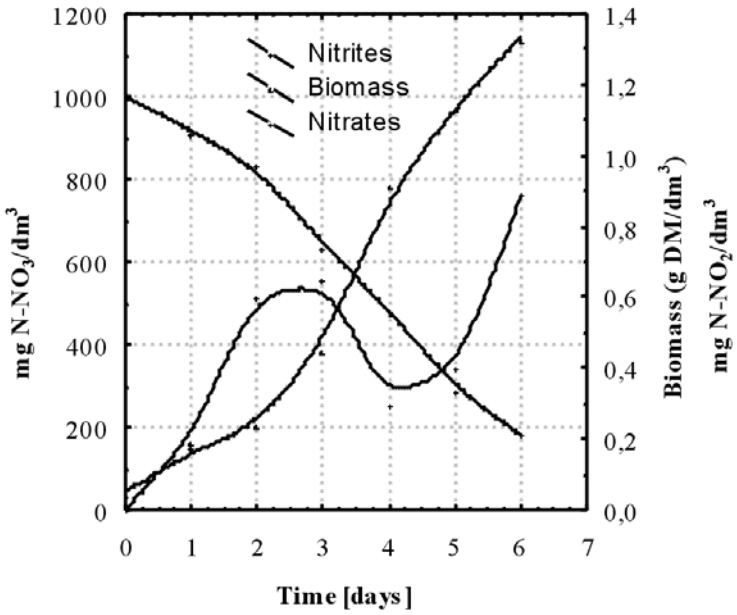

Fig. 3. Brine dentrification by *H. mediterranei* archaeon under optimal environmental conditions in a batch culture conducted in bioreactor

The activities of nitrate and nitrite reductases were determined in a stationary culture carried out in bioreactor (figure 4). In all cases, an initial induction of the enzyme was observed until the 3rd or the 4th day, when the highest activity was reached, and maintained until the process completion. Moreover, in all cases nitrate reductase activity was higher than that of nitrite reductase, which may explain nitrite accumulation during the reduction of nitrates in brine [9].

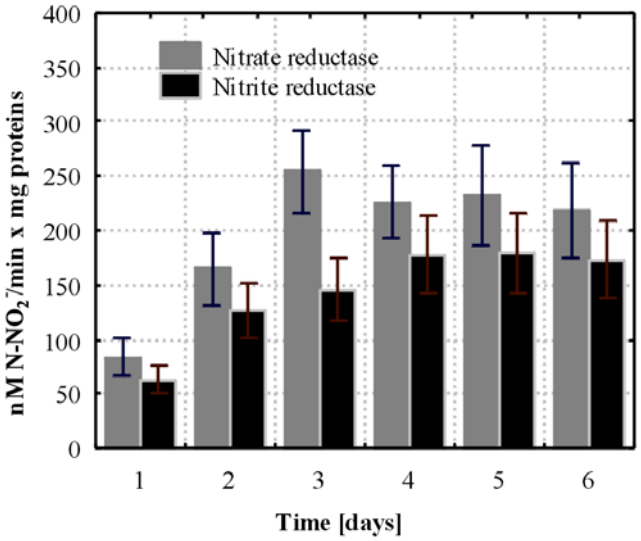

Fig. 4. Activities of nitrate and nitrite reductases of *H. mediterranei* archaeon in batch culture conducted in bioreactor

In the studies on the determination of nitrate reductase activity, it is important to select an appropriate system consisting of an electron donor and an electron acceptor. NORSKOV and MELLOR [13] find that the application of electron donors other than methylviologen, such as curcurmin, bromophenol blue, etc., results in a decerase of nitrate reductase activity to 4–89% of its maximum activity reported for the methylviologen–hydrogensulfate system.

The next step consisted in the denitrification of brine formed as a post-regeneration waste product from the resin IONAC SR-7 (Sybron Chemicals Inc., USA). Moreover, batch culture was carried out. Brine composition was as follows (in 1 dm^3): 500 mg of $N–NO_3^-$, 320 mg of SO_4^{2-}, 30 mg of HCO_3^- and 114.5 g of Cl^-, thus it seemed necessary to supplement brine with nutrients required for an appropriate growth and development of halophilic bacteria, i.e. methanol (C/N = 1.30), phosphates (C/P = 56), 2.0 g of Mg^{+2}/dm^3, 1.0 g of K^+/dm^3, 0.02 g of Ca^{+2}/dm^3, 50 mg of Fe^{2+}/dm^3, 1 mg od Cu^{2+}/dm^3, 5 mg of Mo^{6+}/dm^3 and 750 mg of yeast extract/dm^3.

Microbial batch culture was carried out under optimal conditions, i.e. at 37 °C and pH 7 (figure 5). In the first two days of the process, the microorganisms adapted to new environmental conditions. In that time, they removed from brine only 5% of nitrate nitrogen. After the 2nd day the process gained in dynamics, and the changes typical of a decrease in nitrate concentration were linear. A complete removal of nitrates from brine was recorded on the 8th day of the process. The adaptation effect was also proved by the increment of biomass, whose concentration increased after the

3rd day of the process, reaching on the 8th day the value of 0.78 g DM·dm^{-3}. A gradual nitrite accumulation remained on the level of 2–4 mg N–NO_2^-/dm^3. As a result, specific nitrate reduction rate reached 80 mg N–NO_3/g of DM per day, while nitrate reduction rate was low, amounting to 62.5 mg N–NO_3/dm^3 per day. The latter can be explained by a low biomass concentration of the culture.

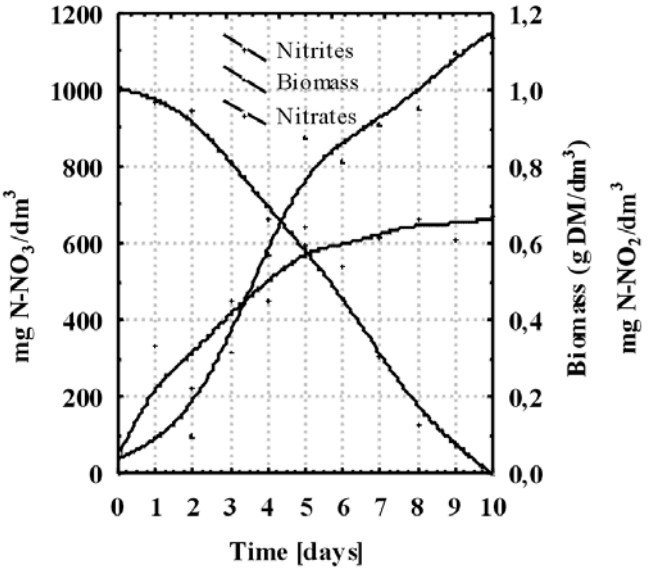

Fig. 5. Denitrification of brine obtained after regeneration of ion-exchange resin using *H. mediterranei*

HOEK and KLAPWIJK [14] used the ion-exchange columns (resin Duolite A 165) with an i.d. of 1.9 cm and the height of 40 cm. The columns after ion exchange were regenerated and as a result the solution of a medium saline concentration was prepared, where microbiological denitrification was conducted. Therefore, the efficiency of denitrification in batch reactor (SBR) after 8 hours exceeded 95%, and after 20 hours it increased to 99% [14].

The other example is a pilot plant installation consisting of three ion-exchange columns connected to denitrification bioreactor (USBR). At the rate flow of 11 m^3· h^{-1} and the initial nitrate concentration of 19.2 mg of N–NO_3^-·dm^{-3} the denitrification efficiency was 90% [5]. Heat and power generating plant produces wastewater, which is very harmful to the environment due to high concentration of organic anions.

For the treatment of these kinds of wastewaters the reactor with fluidized bed was used. The wastewaters after cleaning contained 30 mg of N–NO_3^-·dm^{-3} and 550 mg·dm^{-3} of acetic acid, which was treated as a carbon source. This wastewater comprises also

13 g Cl^- ·dm^{-3}. At the flow rate of 25 $dm^3 \cdot h^{-1}$ the rate of nitrate reduction was 65 mg $N–NO_3^-$·dm^{-3} [15].

In order to remove nitrates form sea water CATALAN-SAKAIRI et al. [16] made use of the denitrification bacteria from the species *Hyphanierobium*. These microorganisms were immobilized on a cellulose material. Saline medium consisted of: KNO_3 (280–560 $g \cdot m^{-3}$), KH_2PO_4, $CuSO_4 \cdot 5H_2O$, some trace elements and methanol as a source of carbon. After treatment the saline comprised 0.7 mg $N–NO_3^-$·dm^{-3} [16].

In a sequencing batch reactor (SBR), CLIFFORD and LIU [2] carried out a biological denitrification of 0.5 N solution of sodium chloride spent regenerant containing up to 835 mg NO_3^- /dm^{-3} and observed that a complete denitrification of this spent brine was achieved in 20 h at a methanol to nitrate-nitrogen ratio of 2.2 [4].

4. CONCLUSIONS

It can be assumed that *H. mediterranei* microorganisms are useful in removing nitrate ions from the brine of high osmotic pressure. The optimum conditions for the growth of *H. mediterranei* archeon exist at a temperature of 37 °C and pH 7. NaCl concentration within the range of 2.5–3.5 M has a negligible effect on denitrification efficiency.

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