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Optimization of Nitrification Process by a Bacterial Consortium in the Submerged Biofiltration System with Ceramic Bead Carrier

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Abstract

Laboratory-scale solid phase submerged system was developed to study the process of ammonium biodegradation. Ceramic beads were found to be an appropriate carrier material for the attachment of thePNN bacterial consortium (*Pseudomonas* sp., *Nitrosomonas* sp., *Nitrobacter* sp.) exhibiting nitrification/denitrification activity. This consortium was previously isolated from a biological activated sludge process at a fish factory wastewater treatment plant. Three organic amendments - molasses, humic acid extract, and malt extract - were used for the ceramic bead pretreatment. Molasses significantly enhanced ($p<0.05$) the process of bacteria attachment onto the ceramic carrier and further ammonium removal from the bulk liquid media. The addition of 0.45% fructose to the column notably enhanced ammonium oxidation, as demonstrated by more rapid formation of nitrites in the medium when compared to the sets without sugars. The results of this study could be incorporated in a larger-scale test of a biofiltration column using wastewater from a fish processing factory.

Keywords: Ammonium biotransformation; Enzyme activity; Ceramic beads; Molasses; Nitrification

Introduction

Biofiltration, an emerging technology using bacteria and fungi [1-5], offers a number of advantages for the treatment of organic and inorganic pollutants in air and water. The packing material used in biofilters is an important factor for establishing high removal efficiency and maintaining good performance over the long term [6]. The characteristics of the filter media have a great impact on the treatment efficiency and, to a great extent, determine construction and operations costs [7,8].

Some of the most frequently used inert packing materials include perlite, porous ceramics, activated carbon, porous lava, polyamide and polypropylene beads, and polyvinyl difluoride cubes has become prevalent [1,9-13]. Although the cost of inert filter beads, in most cases, is higher compared to that of organic counterparts, the former have a range of advantages: chemical and physical inactivity, long lifetime, high performance, more regular shape, uniform air distribution, and reduced number of channeling issues. In addition, inert carriers are also much easier to clean and replace [1,9,14]. Porous ceramic beads have also been shown to be an appropriate packing material for biofilters due to their high gas and liquid film mass transfer coefficients [12].

Studies on the metabolic pathways of autotrophic ammonia-oxidizing bacteria, heterotrophic nitrifying bacteria, anaerobic ammonia-oxidizing bacteria, and ammonia-oxidizing archaea under various engineered ecosystems could provide a more efficient performance of waste treatment processes [15]. As reported by Raby et al. [16], ammonium biodegradation in biotrickling filter with polypropylene spheres and ceramic beads showed a similar efficiency as packing materials. Operating conditions, e.g., flow rate, leachate recirculation, nitrogen load rate, etc., greatly affected the biomass production and its ammonium oxidation activity.

In a previous study [17], we tested six inorganic materials including naturally occurring minerals and rocks, and found that ceramic beads showed significantly higher attachment capacity ($p<0.01$) for the nitrifying consortium of *Pseudomonas* sp., *Nitrosomonas* sp., *Nitrobacter* sp., and *Sarcina* sp. (PNNS). The use of inorganic carriers, however, had to be accompanied by added nutrients that would support the targeted biological processes, including the growth of microorganisms, the formation of biofilm, and biodegradation. The enhancement of these processes is a key factor to a significant increase in biofiltration efficiency.

In this respect, a search for the optimal surface functionalization is considered to be a fundamental tool for development of novel biotechnologies [18].

Commercially available nutrient amendments with complex compositions that are known to stimulate microbial growth include molasses, humic acid extract, and malt extract. Molasses contains about 50% sugar in the form of sucrose, glucose, and fructose, and is rich in mineral elements [19]. Humic substances which are multifunctional, amorphous biopolymers, composed of hundreds of organic constituents that include carbohydrates and condensed aromatic rings substituted by carboxylic, phenolic, and methoxyl groups. Humic compounds have been shown to serve as an ideal ligand for bioinorganic applications [20]. Malt extract contains about 70% carbohydrates, as well as amino acids, β-glucan, and other components, known to stimulate microbial growth [21].

The main goal of this study was to evaluate the effect of different types of ceramic bead pretreatment (with molasses, humic acid

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extract, and malt extract) on the attachment capacity and ammonium biodegradation activity of the PNN bacterial consortium. In addition, the effect of fructose on the process of ammonium biodegradation was tested.

Materials and Methods

Microorganisms and growth conditions

The ammonium-degrading bacterial consortium PNN (*Pseudomonas* sp., *Nitrosomonas* sp., *Nitrobacter* sp.) was previously isolated from the biological activated sludge process of a fish processing wastewater treatment plant and tested in nitrification/denitrification experiments with a two stage biofiltration column [22]. The composition of liquid medium used for PNN cultivation was as follows: 1.0 g/L $(NH_4)2SO_4$; 1.0 g/L K_2HPO_4; 2.0 g/L NaCl; and 0.5 g/L $MgSO_4 \cdot 7H_2O$ (pH = 7.37; redox potential = –31.4 mV).

Pretreatment of ceramic beads with organic amendments

Sterile ceramic beads (Maxit, Estonia) were used as a packing material in the experimental biofiltration system. Three different organic solutions - 30% molasses, 30% malt extract, and 100% humic acid extract – were used for the bead pretreatment. The control was pretreated with distilled water. The pretreatment was performed in duplicate in eight 1 L glass flasks by submerging the ceramic beads in the above solutions for 5 hours. Then the excess liquid was discarded, the beads were dried at 60°C for 24 h and used further as a carrier for the attachment of the PNN consortium.

Bacteria attachment and experiments on ammonium degradation

Biofiltration experiments were performed in 500 mL glass flasks packed with 120 g of dry autoclaved ceramic beads. In order to attach the PNN consortium on the beads, the former was placed in the liquid medium in flasks and incubated over a 7 day period using liquid medium described above, at 22°C. A mean air flow rate of 0.28 L/min was maintained in each flask using air pumps (AC-1500, Resun, China) with 0.45 µm filters. After incubation, the non-attached biomass was removed and the ammonium degradation experiments were conducted using two concentrations of $(NH_4)2SO_4$, i.e., 2.5 g/L and 7.5 g/L, corresponding to 0.53 g/L and 1.6 g/L $N\text{-}NH_4^+$, respectively.

Analytical methods

Auto Kjeldahl Unit K-370 (BÜCHI Labortechnik AG, Germany) was used to determine the content of total nitrogen according to ISO 5983-2:2005. Total carbon was measured using an automatic C/S ELTRA analyzer (ELTRA GmbH, Germany). The concentration of $N\text{-}NH_4^+$ was determined colorimetricaly with Nessler's reagent. Redox potential and pH were measured with a Hanna pH213 pH-meter (Hanna Instruments, USA). Fructose (Penta, Czech Republic) concentration was measured using Waters 600E HPLC system (Waters Corporation, USA). The following setup was used for the analysis: Econosphere Amino Column 250 × 4.6 mm with a diameter of 5 µm and a flow rate of 1.3 mL/min and a mobile phase acetonitrile:water ratio of 70:30. For calibration, carbohydrate standards for glucose, fructose, maltose, and sucrose from Sigma Aldrich were used.

To determine fluorescein diacetate (FDA; Fluka, Switzerland) activity, FDA hydrolysis was measured after 24 h of incubation at +37°C [23]. Fourier transform infrared (FT-IR) absorption spectra were registered on a microplate reader HTS-XT, Vertex 70 (Bruker Optics, Germany) over the range 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. The data were processed using OPUS 6.5 software, correcting the baseline by the rubber band method. Quantitative analysis of carbohydrates, nucleic acids, proteins, and lipids in the biomass was performed as in Grube et al. [24]. Prior to analysis biomass was detached from the ceramic beads by sonication in sterile distilled water for 10 min followed by incubation for 20 min on a rotary shaker (140 rpm).

Structural analysis of ceramic beads and bacterial biomass was performed using scanning electron microscopy (SEM). Samples were fixed in glutaraldehyde solution (5% final concentration in 0.1 M phosphate buffer, pH 7.2) for 20 h. Ceramic beads with immobilized cells were rinsed in distilled water and incubated in a 2% KCl solution for 24 h. Then prepared material was dehydrated in an increasing range of acetone concentrations (40, 60, 80, 90, and 100% 10 min each), and mounted on metal discs. Dried samples were then coated with gold in an Eiko IB-3 ion coater (Eiko Engineering, Japan) and analyzed using the scanning electron microscope Hitachi S-4800 (Hitachi, Japan) at an acceleration voltage of 3.0 kV.

Results and Discussion

Ceramic bead pretreatment

After the 7-day incubation of PNN consortium in the presence of ceramic beads, the bead surface was visualized using SEM (Figure 1). The pore structure of untreated ceramic beads and the surface of beads pretreated with molasses are shown in Figure 1A and Figure 1B, respectively. PNN incubation resulted in the formation of bacterial biofilm on the bead surface (Figures 1B and 1C).

The enzyme, i.e., FDA hydrolysis activity of PNN consortium was found to be the highest on the beads pretreated with molasses ($p<0.05$) (Figure 2). Our previous study [17] demonstrated that molasses had a stimulating effect on the growth of the non-attached consortium in a liquid medium as well as the first phase of the nitrification process.

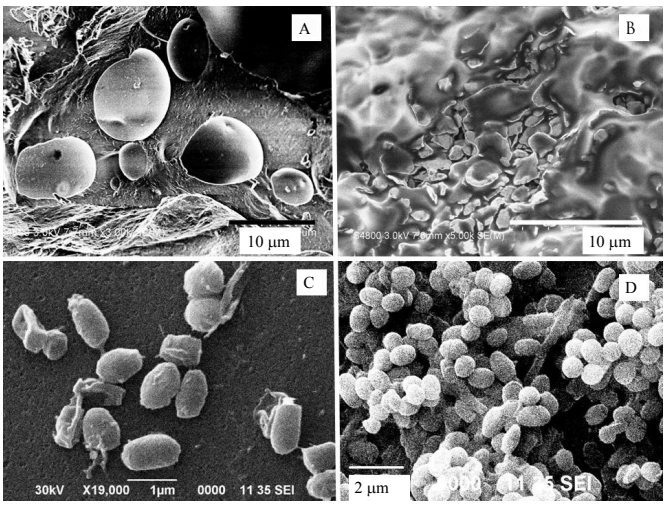

Figure 1: Scanning electron micrographs of the surface of ceramic beads. A – beads without treatment; B – beads pretreated with molasses (by submerging the ceramic beads in 30% molasses during 5 hours, then discarding the excess liquid and drying at 60°C for 24h). C, D - the cells of PNN consortium attached on the bead surface (inoculum of PNN consortium was incubated over a 7 day period in medium in the presence of ceramic beads at 22°C, at air flow rate of 0.28 L/min).

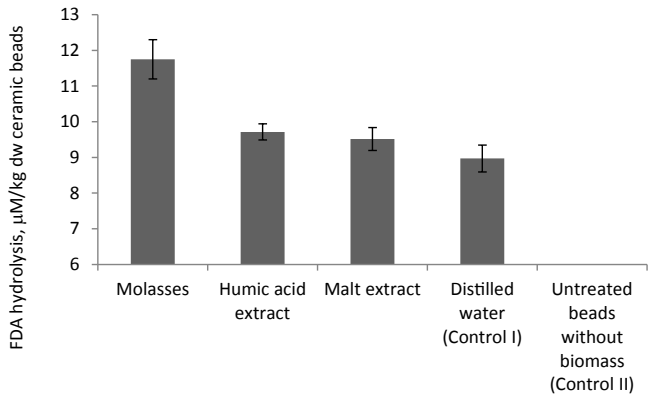

Figure 2: FDA hydrolysis activity of PNN consortium cells attached on the ceramic beads previously treated with different organic amendments or distilled water (Control I). Control II – untreated beads without attached biomass. FDA hydrolysis activity was measured after 24 h of incubation at +37°C. Beads treatment was performed as indicated in Materials and Methods. Error bars represent the standard deviation.

No significant difference ($p>0.05$) in the FDA hydrolysis activity of the attached biomass was observed among the treatments with humic acid extract, malt extract, and distilled water (Figure 2).

The study by Akker et al. [25] evaluated the abundance of ammonia-oxidizing bacteria (*Nitrosomonas* oligotropha-cluster) in a molasses-fed fixed-film activated sludge. It was shown that an excess of molasses could decrease the concentration of ammonium-oxidizing bacteria present in the reactor [25]. In turn, other source of organic carbon, i.e., urea, was shown to stimulate the growth of ammonia-oxidizing communities and the process of nitrification as a whole [26].

In our experiments, *Pseudomonas* spp. could dominate in the PNN consortium when cultivated with molasses. Further studies are necessary to clarify the shift in the consortium community with the addition of organic carbon.

Concentrations of the total nitrogen, carbon and sulfur in the crushed ceramic beads with attached PNN consortium were determined. No considerable changes in the concentrations of N and S were detected in the sets with ceramic beads and attached biomass, among the treatments with organic amendments and distilled water. Total carbon concentration in the beads pretreated with humic acid extract was the highest among the treatments tested (Figure 3A). Untreated ceramic beads without biomass contained considerably lower concentrations of C and S, as compared to the sets with biomass. Nitrogen was not detected in the set with untreated beads (Figure 3A).

Testing the biomass chemical composition, previously detached from ceramic beads, showed that the concentration of carbon in the biomass was similar for all tested samples (256–292 mg/g dw) (Figure 3B). The total nitrogen concentration was the lowest in the biomass detached from the beads pretreated with water (Figure 3B).

Differences in the chemical composition of the bead surface and the attached biomass can be explained by the individual properties of organic amendments used in this study. Concentrations of the total N, C, and S in molasses, humic acid extract, and malt extract are presented in Table 1.

FT-IR spectroscopy was used to study the macromolecular composition of microbial biomass that was attached on the ceramic beads with different types of pretreatment. The concentration of carbohydrates, nucleic acids, proteins, and lipids in the PNN biomass is shown in Figure 4. The concentration of carbohydrates in the biomasses pretreated with molasses or malt extract was higher than that in biomass pretreated with humic acid extract or water. On the other hand, biomass attached on the beads pretreated with molasses or malt extract had lower protein concentration than that pretreated with humic acid or water. Conversely, the highest protein concentration (69.2% dw) was observed in the PNN biomass grown on ceramic beads that were pretreated with humic acid extract. Amount of nucleic acids varied in the range from 8.63 % (pretreatment with humic acid extract) to 16.77 % (pretreatment with malt extract). The highest concentration of lipids in biomass, i.e., 10.1 %, was detected in the set without pre-treatment with organic amendments (Figure 4).

Pretreatment of PNN biomass with molasses and humic acid extract resulted in the most pronounced changes including the highest enzymatic activity and the highest protein concentration of the attached biomass (Figures 2 and 4). In this respect, more detailed

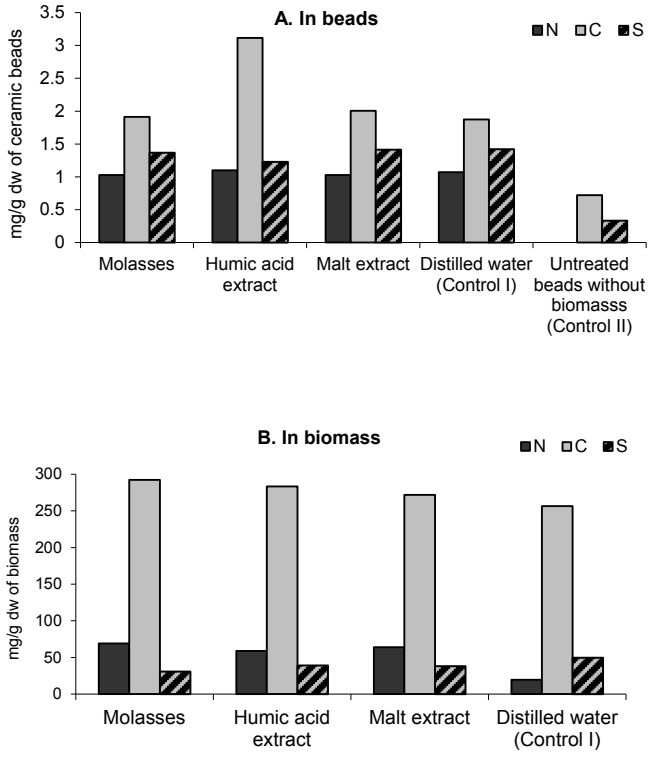

Figure 3: Total nitrogen, carbon, and sulfur concentrations in the crushed ceramic beads with attached PNN consortium (A) and in the biomass of PNN consortium detached from the surface (B). Beads treatment was performed as indicated in Materials and Methods. Control I – treatment of ceramic beads in distilled water; Control II – untreated beads without attached biomass.

Amendment	Total N (mg g^{-1}dw)	Total C (mg g^{-1} dw)	S (mg g^{-1} dw)	DW* (%)
Molasses, 30%	11.9	278.5	1.5	22.7
Humic acid extract, 100%	11.6	417.1	6.0	3.5
Malt extract, 30%	7.9	389.2	0.9	25.4

* - dry weight

Table 1: Total nitrogen, carbon and sulphur concentrations in amendments used for pre-treatment of ceramic beads.

attention was focused on the FT-IR spectra profiles of the samples under these treatments (Figure 5). The spectra of both supernatants were qualitatively similar. Changes of the profiles and absorption band intensities at 1580 cm^{-1}, 1405 cm^{-1}, and 900–1100 cm^{-1} indicated the presence of unconverted carbohydrates from the molasses medium and some components of humic acid extract, as well as, possibly, some extracellular substances produced by the PNN consortium. FT-IR spectrum of biomass showed the overall macromolecular composition of cells that allowed particular components to be identified. The most intensive absorption bands were assigned to carbohydrates (900–1100 cm^{-1} primarily dominated by a sequence of bands due to C-O, C-O-C and C-O-P stretching vibrations), proteins (1500–1700 cm^{-1}, with maximums of Amid I and Amid II, N-H and C=O vibrations, respectively), and lipids (2800–3000 cm^{-1} dominated by the absorption modes of CH_2 and CH_3 groups of fatty acids and aliphatic chains). The shoulder at 1712 cm^{-1} was assigned to C=O stretching of ester and/or carbonyl groups, carbonic acid.

The spectrum of *Pseudomonas* sp. is a typical biosample spectrum where the main absorption bands are assigned to specific functional groups of carbohydrates, proteins and fatty acids [24,27]. Both nitrogen-fixing bacteria *Nitrosomonas* sp., and *Nitrobacter* sp. were grown on the same medium, yet the spectra were different. The strong absorption band with maximum at 1737 cm^{-1} (ester carbonyl stretch) and band at 1450 cm^{-1} (methyl CH3 deformation) in the spectrum of *Nitrosomonas* sp. indicate the accumulated a storage bio-polymer poly –β-hydroxybutyric acid (PHB) [28], while *Nitrobacter* sp. did not accumulate PHB.

Ammonium biotransformation in the submerged biofilter

The rate of ammonium degradation by microorganisms can be influenced by many factors such as the initial ammonium concentration, additional growth factors, and the physiological state of the biomass. We performed experiments to determine how the initial ammonium concentration and the addition of fructose affected the degradation of ammonium in the model system.

In the media without fructose, ammonium degradation by the attached biomass was significantly higher ($p<0.05$) for the beads pretreated with molasses. The stimulating effect of pretreatment with molasses was more pronounced at a lower initial concentration of ammonium sulphate, i.e., 2.5 g/L (Figure 6). As previously documented [29], the activity of ammonium-oxidizing bacteria, in particular *Nitrosomonas* spp., varies with ammonium concentrations.

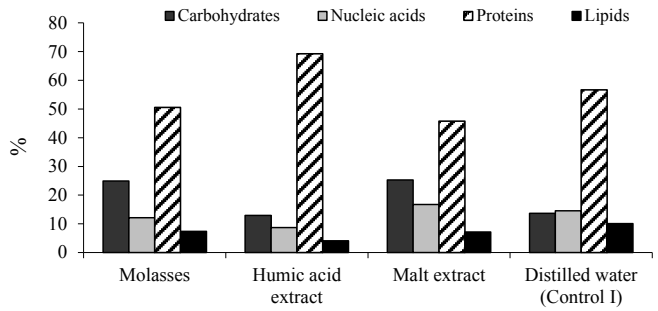

Figure 4: The concentration (% of dry weight) of the principal cell components in the biomass of PNN consortium attached on the ceramic beads pretreated with different organic amendments or distilled water. Beads treatment was performed as indicated in Materials and Methods.

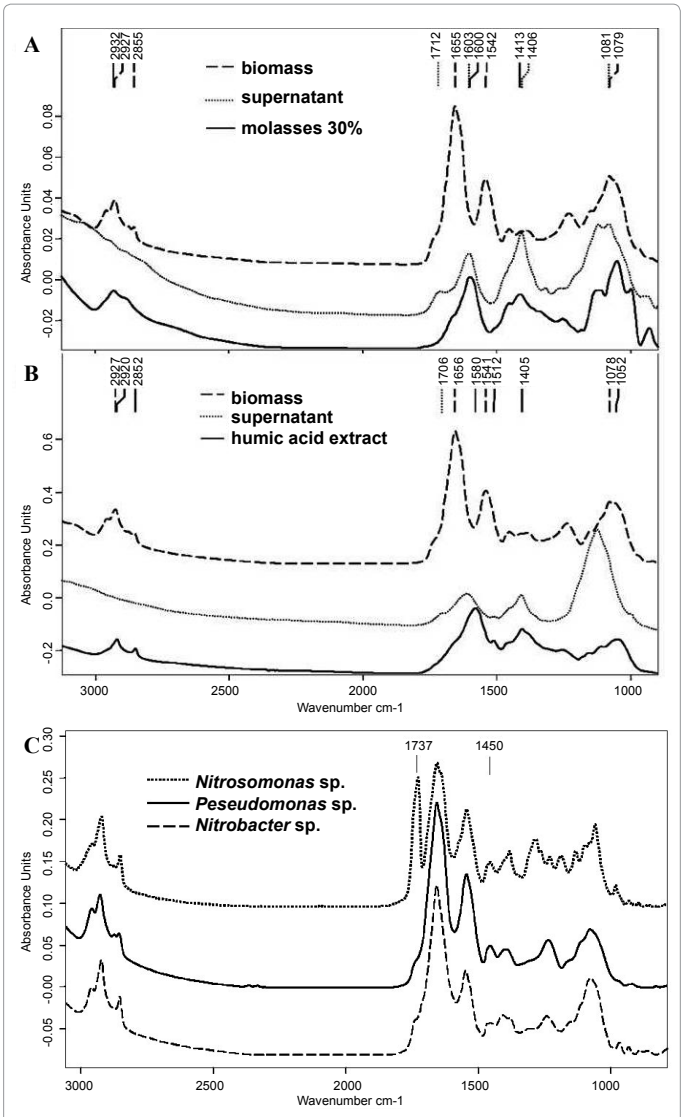

Figure 5: FT-IR spectra of different parts of the system: ceramic beads pretreated with molasses (A), humic acid extract (B), FT-IR spectra of individual PNN consortium strain biomass grown on TGA (C).

The addition of 0.45% fructose to the media enhanced the degradation of ammonium (Figure 6). The role of limited amounts of organic compounds, particularly fructose, as a carbon source to support the growth of *Nitrosomonas* spp. has been reported by Hommes et al. [30] and Schmidt [31]. The ability of *Nitrobacter* spp. to grow heterotrophically has also been documented previously [29,32,33].

In the present experiments, maximum ammonium removal was detected after the first 24 h. Further decrease in ammonium removal rate was limited by the amount of fructose left in media (Figure 7A). The highest consumption of fructose by the PNN consortium was detected in case of the pretreatment with molasses (Figure 7B). Moreover, molasses pretreatment provides the consortium with additional source of fructose. Analysis of the carbohydrates in the molasses revealed that 27% of the total reducing sugars were fructose (results not shown).

The decrease in ammonium removal from the system could be explained by the cyclic growth and detachment of the biofilm in a submerged biofilter. The ammonium removal cycle is affected by

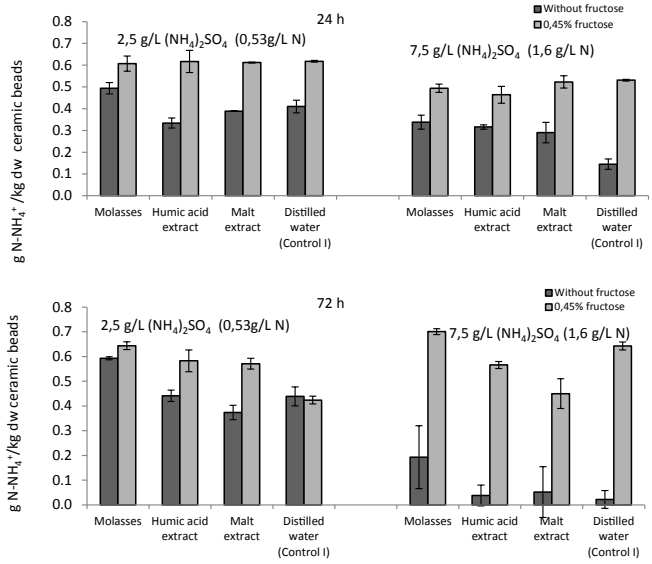

Figure 6: The impact of fructose, initial ammonium concentration, and the type of bead pretreatment on the ammonium nitrogen ($N\text{-}NH_4^+$) concentration in the liquid medium after incubation of the attached PNN consortium for 24 h and 72 h. The initial concentrations of $(NH_4)2SO_4$ were 2.5 g/L and 7.5 g/l, respectively. Error bars represent the standard deviation.

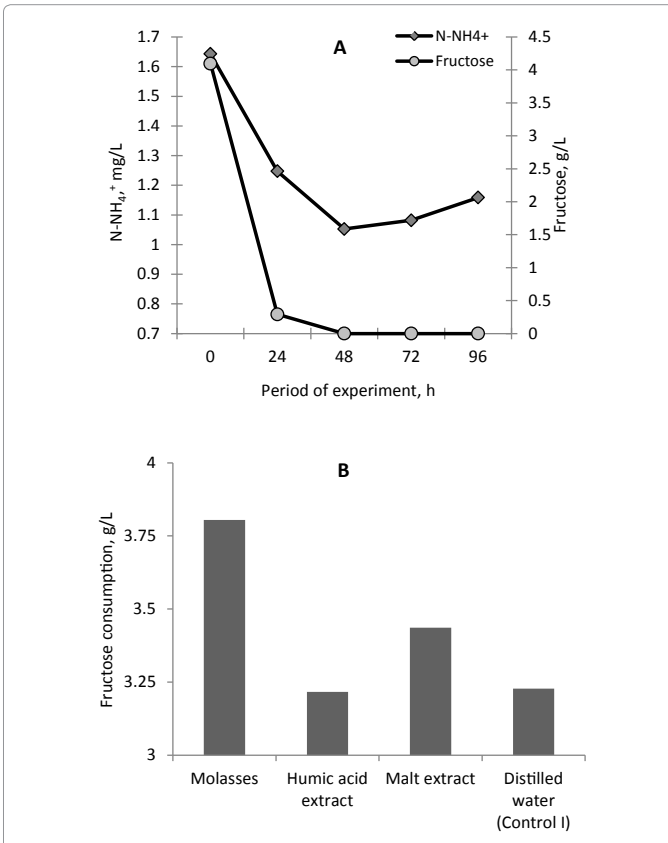

Figure 7: Ammonium degradation and fructose consumption by the attached PNN consortium during 96 h incubation in the liquid medium with 7.5 g/L $(NH_4)2SO_4$. (A) Concentration of fructose and ammonium over time (pretreatment of beads with molasses). (B) The effect of pretreatment type of ceramic beads on the fructose consumption. Beads treatment was performed as indicated in Materials and Methods.

hydraulic conditions, temperature ranges, suspended solids, as well as ammonium concentration [34].

It is important to note that, in present experiments, the addition of fructose notably affected the pH level of the media. A 72 h incubation of the PNN consortium attached on the beads with different pretreatment types resulted in the decrease of pH from 7.37 in the sterile medium (control) to 5.4–5.6 and 6.8–7.1 in the sets with and without fructose, respectively. Redox potential increased from –31.4 mV in the sterile medium to 60.2–71.6 mV and –17 to –5 mV with and without addition of fructose, respectively. Usually the medium for nitrifiers contains $CaCO_3$ in order to maintain an elevated pH level. In the present study, $CaCO_3$ was not added to the medium; therefore, the pH level was more variable and was dependent on the microbial activity. The optimal pH for the growth of nitrifying bacteria varies widely, typically from 7.0 to 9.0; the optimum pH range for *Nitrosomonas* spp. is 7.2 to 8.8 and for *Nitrobacter* spp. is 7.2 to 9.0 [35]. However, autotrophic nitrification at low pH is more widespread than previously thought. The ability of chemolithotrophic bacteria, in particular, *Nitrosomonas* spp., to nitrify at low pH was reported by Tarre and Green [36]. This study was attributed to biofilm and suspended-biomass reactors with pH 4.3 and bulk ammonium concentration of 9.3 mg/L under autotrophic conditions [36].

Conclusions

Ceramic beads are an appropriate carrier for the immobilization of the PNN consortium in the submerged biofiltration system. Molasses pretreatment can significantly ($p<0.05$) stimulate the process of bacterial attachment on the ceramic carrier and the degradation of ammonium ($p<0.05$) in the submerged system, particularly in case of low initial concentrations of ammonium. The addition of 0.45% fructose to the media stimulated the degradation of ammonium. The results obtained in this laboratory-based study need to be further tested in a larger scale biofiltration system using waste water from a fish processing factory.

Acknowledgments

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