Effect of various factors to ammonia biodegradation by two stage biofiltration system

Silvija Strikauska[1], Dzidra Zariņa[2], Olga Mutere[2], Uldis Viesturs[1,2, 3] and Andrejs Bērziņš[2]
[1] *University of Agriculture, Latvia, Jelgava*
[2] *Institute of Microbiology and Biotechnology, University of Latvia, Latvia, Riga*
[3] *Latvian State Institute of Wood Chemistry, Latvia, Riga*

ABSTRACT

An autotrophic ammonia-biodegrading PNNS association was isolated from the biological activated sludge of the fish factory wastewater treatment plant and used in the two-stage biofiltration system with the ammonia load 0.78 g/m^{3}h ensured the total removal efficiency up to 0.69 g/m^{3}h as the result of the denitrification process. Additional investigations were made to study physiological and biochemical properties of individual strains of the PNNS association in order to control their growth under various cultivation conditions with the aim to find out the most optimal conditions for biomass preparation and immobilisation. Individual strains of the association can be revealed and counted because of their different colony morphology using selected medium. Cultivation of individual strains of the PNNS association under aerobic conditions revealed a stimulation effect of $(NH_4)_2SO_4$ in the concentration range of 0.21 – 4.45 g N/l to their growth. Addition of saccharose, glucose, fructose and/or cabbage leaf extract (CLE) in various combinations to agarized medium resulted in the growth stimulation of individual strains of the PNNS association, i.e. *Pseudomonas* sp., *Nitrosomonas* sp., *Nitrobacter* sp. and *Sarcina* sp. The whole association was cultivated in the liquid mineral medium with amendments mentioned above. Stimulation of the growth in the presence of CLE and some reducing sugars was observed. The results obtained in these experiments will be used for further optimisation of the two-stage biofiltration system using the PNNS association.

Abbreviations

CLE	Cabbage Leaf Extract
AM	ammonium containing mineral medium
SA	Saccharose Agar
TGA	Tryptone Glucose Yeast Extract Agar
EMB	Eosin Mehtylene Blue
PNNS association	*Pseudomonas* sp., *Nitrosomonas* sp., *Nitrobacter* sp., *Sarcina* sp.
CFU	colony forming units.

1 INTRODUCTION

Biofiltration is a technology for reducing of odorous emissions, which involves the biochemical capabilities of native or modified biological systems and has some advantages as compared to physical – chemical, burning or mechanical methods. Ammonia is an important component of odorous gases produced in cases of ventilating intensive cattle breeding facilities, manure handling and waste composting (Hong *et al.,* 2005; Kim *et al*., 2007; Mola *et al*., 2004; Pagans *et al*., 2007; Schmidt, 2002).

Changes in microbial community structure during biofiltration play a crucial role in air treatment process as a whole (Sakano *et al*., 1998; Sakano *et al*., 2002; Steele *et al*., 2005). Rapid and available methods for monitoring of the total count and separate groups of the association allow to control and manage the nitrification and denitrification processes. These approaches would be useful also for the study on optimisation of cultivation conditions for preliminary preparation and immobilisation of microbial biomass for further successful biofiltration process. In our previous experiments the 294biofiltration technique for the purification of polluted air was developed (Strikauska *et al*., 1999; Viesturs *et al*., 2002; Viesturs *et al*., 2003). However, it is still unclear, what are the most optimal conditions for biomass pre-cultivation and immobilization. The aim of this work was to study the effect of various amendments, in particular cabbage leaf extract and reducing sugars, to the growth of the PNNS association and its individual strains.

2 MATERIALS AND METHODS

2.1 Microorganisms and cultivation

An autotrophic ammonia-biodegrading association was isolated from the biological activated sludge of the fish factory wastewater treatment plant. The

composition of the liquid medium AM was (in $g.l^{-1}$): $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 1; NaCl, 2.0; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.001; and $CaCO_3$, 10 (Strikauska *et al.*, 1999). pH of the medium was 7.7, Eh -40.7 mV.

Cabbage leaf extract was prepared from white cabbage leafs. 500 g leafs were washed with top water, boiled at 100 °C for 30 minutes, cooled, afterwards a liquid fraction of prepared extract was filtered with 0.45 μm hydrophilic PTFE membrane filter with 1.0 μm APFB glasfiber prefilter (Millipore) and autoclaved at 0.5 atm.

Cultivation of microorganisms in the liquid AM medium was performed in 300ml flasks containing 200 ml liquid medium at +26 °C with periodic agitation in the dark. Samples for analysis were taken after 24h, 72h and 144h of incubation.

For determination of the number of colony forming units on agarized medium, AM medium with 1.6 % agar, Saccharose agar, Tryptone Glucose agar, Eosin Methylene Blue agar (Sifin, Germany) were used. Plates with inoculated samples were incubated at +37°C for 48 h and longer for observing the growth of colonies on the different medium. Biochemical characterisation of strains was performed using API®32E (BioMeriéux, France).

2.2 Analytical methods

The concentration of carbohydrates was determined by HPLC, Agilent 1100 HPLC system was used for the chromatography work and an Agilent Chemstation software for the data analysis. Carbon and sulphur were measured using the C, S analyzator (ELTRA). Total ammonium was determined according to ISO 5983-2:2005. Concentration of NH_3, NO_3^- and NO_2^- were determined colorimetrically with Nessler reagent, salicylic acid, and NitriVer® 3 Reagent, correspondingly. pH and RedOx potential were measured by electrode (Hanna pH213). All chemicals used in these experiments were analytical grade.

3 RESULTS AND DISCUSSION

3.1 Biofiltration in modified solid state fermentation system

Our effort was focused on the development of a biofiltration method ensuring a high ammonia concentration and a limited oxygen environment. Biofiltration process was realized in modified solid-state fermentation system (SSF). The investigations were made at different ammonia concentrations in inlet gas and packing loads. The biodegradation of volatile compounds was investigated in one and two stage systems with inert packing material and chemoautotrophic microorganisms, i.e. the PNNS association. A one-stage biofiltration system with the ammonia load 0.41 g/m^3h ensured the biological elimination capacity 0.33 g/m^3h due to the nitrification processes. A two-stage system with the ammonia load 0.78 g/m^3h ensured increased total removal

efficiency up to 0.69 g/m^{3}h as the result of the denitrification process (Fig. 1). To increase a biodegradation activity of chemoautotrophic microorganisms and biological elimination capacity, the further study was focused on the characterization of microorganisms used for nitrification processes, in order to optimize conditions for culture preparation and immobilization for ammonia biofilter.

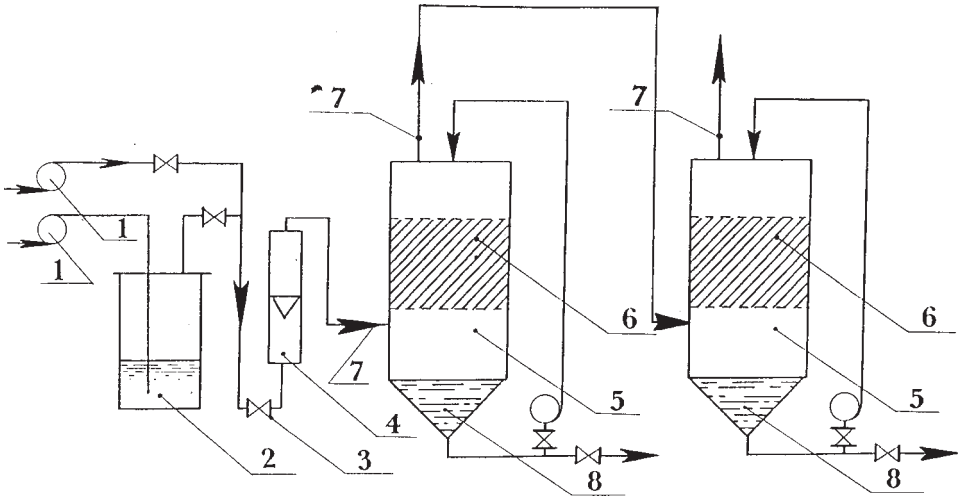

Figure 1. Two-stage biofiltration system: 1 - compressor, 2 - vessel for contaminant under degradation; 3 - valve; 4 - measurement of volumetric flow rate; 5 - gas part of the bioreactor; 6 - biofilter; 7 - sampling points; 8 – submerged.

3.2 Characterisation of the PNNS association by plating method

The colony growth on AM, SA, TGA and EMB medium was compared. Among tested agarized medium, SA and TGA were the most sensitive medium to the specific biochemical properties of individual strains resulted in different colony color, size and shape. Although *Sarcina* sp. was not detected on SA agar, nevertheless this strain was forming the yellow colonies on TGA agar. EMB medium usually is used for cultivation of Gram negative bacteria, however, in our experiments this medium was not appropriate in order to reveal all Gram negative strains of the PNNS association, *i.e. Pseudomonas* sp., *Nitrosomonas* sp. and *Nitrobacter* sp. Growth of microorganisms on the mineral AM medium was slow and therefore, for rapid monitoring, this medium cannot be used. In other cases, e.g. for confirmation of autotrophic growth, this medium is necessary.

3.3 Characterisation of individual strains of the PNNS association by plating method

Cultivation of individual strains of the PNNS association under aerobic conditions revealed a stimulation effect of $(NH_4)_2SO_4$ in the concentration range of 0.21 – 4.45 g N/l to the growth of *Sarcina* sp., *Nitrosomonas* sp., and *Nitrobacter* sp. The effect of CLE and reducing sugars to the growth of isolates was tested on AM medium amended with these compounds in various concentrations and combinations. It is known that the nitrifying bacteria can obtain the carbon for growth from CO_2 and the energy and reductant for growth from the oxidation of NH_3. Therefore these bacteria are considered an obligate chemolithoautotrophs. Recently, however, it was reported that *Nitrosomonas europaea* can utilize limited amounts of certain organic compounds, including amino acids, pyruvate, and acetate, although no organic compound has been reported to support the growth of *N. europaea.* Moreover, it was shown that *N. europaea* can be grown in CO_2-free medium by using fructose and pyruvate as carbon sources and may now be considered a facultative chemolithoorganotroph (Hommes *et al*., 2003). Besides, the growth of *Nitrosomonas* sp. and *Nitrobacter* sp. on glucose was reported (Pan *et al*., 1972). Thus, in our study, the scheme of the experiment provided various combinations of organic compounds, which are supposed to be as carbon source for the growth of individual strains of the PNNS association. After 72h incubation the colonies of isolates were compared by size. The growth of *Sarcina* sp. was stimulated by addition of the mixture of CLE, $(NH_4)_2SO_4$, and saccharose in the concentration-dependent manner. All these compounds added as a single amendment resulted in the growth stimulation in lower extent. In a parallel way, the same compositions of medium with inoculated strains were cultivated under capnophylic conditions to study an effect of CO_2 to autotrophic growth of tested isolates. An enhanced concentration of CO_2 did not reveal any changes in growth rate as compared to the samples cultivated under aerobic conditions. It is known that ammonia oxidizing bacteria obtain usable energy and reductant solely from ammonia and fix carbon autotrophically (Prosser, 1989). Most probably, the effect of CO_2 to colony growth can be visible after the more long incubation.

3.4 Testing of biochemical properties of individual strains of the PNNS association

Strains demonstrated an enhanced growth at enhanced concentrations of ammonia were tested for their biochemical properties, using API test systems ID 32E and API 20 NE. Usually these test systems are used for identification of Gram-negative bacteria. In the PNNS association *Sarcina* sp. is the Gram-positive bacteria. However, the study of tested strains with substrates, provided by API ID 32 E and API 20 NE, was used for biochemical characterisation of microorganisms, not for identification. Results showed that *Sarcina* sp. does not reduce nitrates, in turn *Nitrosomonas* sp.

and *Nitrobacter* sp. reduce nitrates to nitrites. Formation of N_2 was not detected. Urease, α-galactosidase and α-maltosidase was detected only for *Nitrosomonas* sp. among tested strains, in turn, 5 ketogluconate was detected for *Sarcina* sp. and *Nitrobacter* sp. The abilities to hydrolyze urea as a source of ammonia and carbon dioxide and to use the products of ureolysis for modification of the pH in the vicinity of the cell appear to be important ecologically selected traits provided by the urease enzyme (Koper *et al*., 2004). These biochemical properties will be taken into consideration for further study on conditions optimisation for cultivation and immobilisation of the PNNS association.

3.5 Effect of the liquid medium content to the development of the PNNS association

Inoculation of the PNNS association into the liquid AM medium amended by CLE, $(NH_4)_2SO_4$ and reducing sugars resulted in the noticeable changes in biomass growth and physically-chemical data among tested medium combinations. The scheme of the experiment is shown in Table 1.

Table 1.
Addition of various amendments to AM liquid medium, a scheme of the experiment.

Variant of medium	AM medium, ml	$(NH_4)_2SO_4$, 200g/l stock, ml	CLE, ml	Saccharose 20% stock, ml	Glucose 20% stock, ml	Fructose, 20% stock, ml
1	200					
2	195		5			
3	190		10			
4	190	5	5			
5	195			5		
6	195					5
7	185	5	5	5		
8	185	5	5		5	
9	185	5	5			5

The total count of microorganisms determined on TGA medium, varied in dependence on the medium composition. Thus, the higher number of colony forming units was detected in the samples containing 5% CLE (Fig. 2, Table 1, and variant No. 3). Regarding the diversity of microbial community developed in the tested medium compositions, *Sarcina* sp. was detected only in the samples 4, 7 and 9 (Table 1). Colony growth of the tested samples on EMB medium was detected only for the

samples 5 and 6. No colony formation was detected on SA medium. The results shown above indicate to an important role of the medium for the total count determination. Besides, an adequate choice of a cultural medium could provide the valuable information related to the composition of the association, i.e. proportions between single strains of the association. Application of this approach in our further investigations could provide the more detailed information regarding an effect of medium composition to the microbial growth. In particular, it is of great importance to design conditions for cultivation, which provide an efficient equilibrium between single strains of the association for its efficient work in biofiltration system.

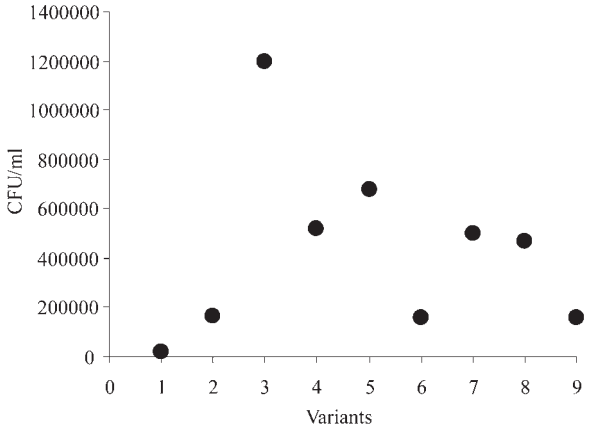

Figure 2. Effect of liquid AM medium content to the total count of microorganisms cultivated during 144 h at +26 °C.

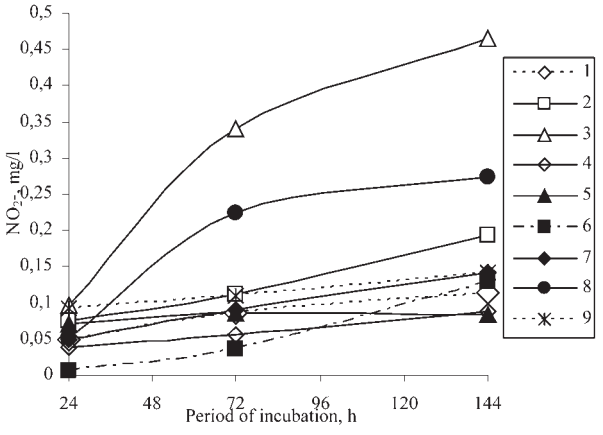

Figure 3. Effect of liquid AM medium content to NO_2^- formation during cultivation of the PNNS association.

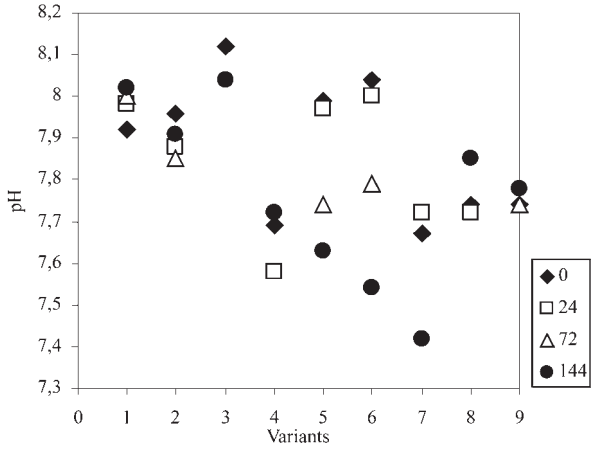

Figure 4. Changes of pH during incubation of the PNNS association (+26 °C, 144 h).

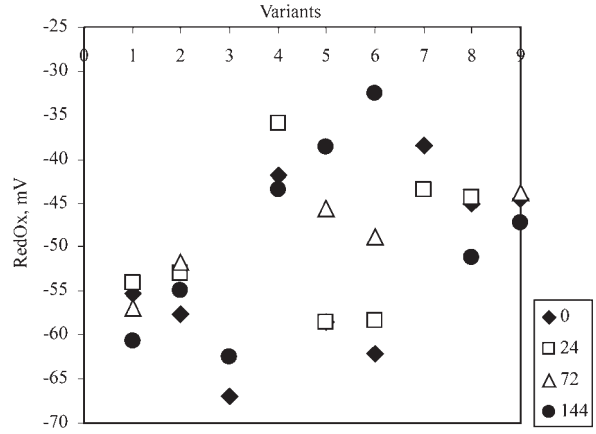

Figure 5. Changes of RedOx potential during incubation of the PNNS association (+26 °C, 144h).

It was shown that formation of NO_2^- by growing biomass was occurred in the presence of ammonia as well as CLE (Fig. 3). The nitrites can be formed as the first stage of nitrification process (for the sample 1), or both, as nitrification and nitrate reduction. It is necessary to note, that the AM medium amended with CLE contains additional amount of carbon and nitrogen, which can be utilized by growing biomass. The analysis of CLE (undiluted) showed that this extract contained the total nitrogen in concentration of 5 g/l, carbon - 13.7 g/l, sulphur – 0.38 g/l. The concentration of saccharose, glucose and fructose was found to be in undiluted CLE 1.29 g/l; 13.62 g/

l and 10.42 g/l, correspondingly. These data should be taken into account in further experiments. According to the data shown in the Figures 3 and 4, pH and RedOx potential were changed upon cultivation. Thus, the medium pH level in the tested samples varied at the beginning of cultivation in the range of 7.67 – 8.12, after 144 h cultivation - 7.42 – 8.04 (Fig. 4 and 5). The simultaneous growth of microorganisms association leads to the noticeable changes of physico-chemical properties of environment.

NO_3^- was not detected during 144h incubation. No noticeable changes in ammonia concentration in the samples were detected during 144h incubation. These results are quite predictable because it is known that an oxidation of nitrites to nitrates by association occurs after a longer period of incubation, i.e. 10-12 days. The second stage of nitrification as well as denitrification is supposed to be investigated in our further experiments.

4 CONCLUSIONS

Assessment of changes in microbial community structure during operation of an ammonia biofilter is of a great importance in the context of process optimisation. The PNNS association used in the two-stage ammonia biofiltration system was studied in the both, fermentation system and batch cultures. Experiments with fermentation system demonstrated a high capacity of the PNNS association in nitrification processes. Batch cultivation showed an important role of various amendments to the growth of microorganisms. In conclusion, further work is needed to understand the relationship between biofilter performance and microbial community dynamics at all stages of the process, i.e. biomass pre-cultivation, immobilisation, adaptation, ammonia oxidation.

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