Nitrification in activated sludge – microbiological insight into nitrogen oxidation process

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Introduction

The removal of biogenic elements from wastewater, especially nitrogen and phosphorus compounds, is a crucial point in its treatment. Sewage directed to an aquatic environment undergoes the natural biochemical processes of self-purification, which consists of dilution, adsorption, sedimentation and the proper purification: biochemical reactions and mineralization. Heterotrophic bacteria and microscopic fungi are responsible for these changes [10]. Figure 1 shows the way in which organic matter is included into detritus food chains.

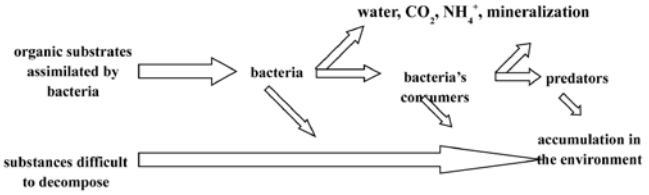

Fig. 1. Biochemical processes and mineralization of sewage along detritus food chains [Fijałkowska et al., 2005, 10]

The chemical methods used in wastewater treatment are relatively expensive and in most cases they are harmful to the environment. In recent years the increase in environmental pollution and the necessity for the removal of anthropogenic waste has led to the introduction of biological methods based on naturally occurring processes [21]. Nevertheless, biological wastewater treatment is faster and more effective than the processes naturally occurring in the environment. Therefore, activated sludge is used.

Activated sludge as an example of complex bacterial biocenosis

Activated sludge is a flocculated mixture of representatives from different microbial groups, such as: [11]:

- bacteria (mainly heterotrophic)
- fungi
- algae
- Protozoa
- Metazoa.

Heterotrophic bacteria are the largest group of activated sludge microorganisms due to the fact that they need a shorter time for reproduction in comparison with autotrophic bacteria and they find plenty of nutrients in the sewage. That is the reason why they represent the first trophic levels in the food chain.

From an ecological point of view, activated sludge is a biocenosis - the highest level of nature's organization. Biocenosis consists of all of the different species populations living in one environment called a biotope. The population is a set of the representatives of one species [Trojan, 1975]. The biotope for activated sludge is a biological reactor. A bioreactor with activated sludge biocenosis is an ecosystem in which the exchange of energy and the flow of matter occurs (Fig. 2).

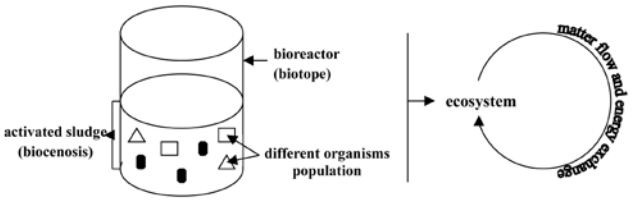

Fig. 2. A bioreactor as an artificial ecosystem

Both lab-scale bioreactors and wastewater treatment plants are at the beginning of wastewater treatment seeded with activated sludge derived from other already operational technological installations. This is not a hard and fast rule because after a while activated sludge will be created naturally. Activated sludge microorganisms can drift into a bioreactor from outside: air, water or sewage flowing into it. The primary biocenosis that was seeded in the bioreactor undergoes modification depending of the type of sewage and the installation being used [10].

There are two mechanisms that have an influence on the composition of activated sludge: selection and adaptation. Selection is based on the elimination of particular species from the system, while other species are allowed to develop. Such factors as: the level of nutrients, the occurrence of electron acceptors, temperature, growth rate, sedimentation and flocculation ability as well as the presence of free-living microorganisms have an influence on changes in the composition of activated sludge. Adaptation-this is the process of adjustment the organism to changing environment conditions, that occurs independently of the selection [11].

Nitrification and its usage in wastewater treatment

Human activity causes an increase in the production of nitrogen-rich wastes. These wastes are harmful to the aquatic environment and that is the reason why they need to be treated effectively [15]. Organic nitrogen compounds directed to wastewater treatment plants (WWTP) undergo ammonification into ammonia. This compound is either built into the bacterial biomass or undergoes nitrification, one of the crucial processes in the nitrogen cycle in the environment (Fig. 3) [13, 14].

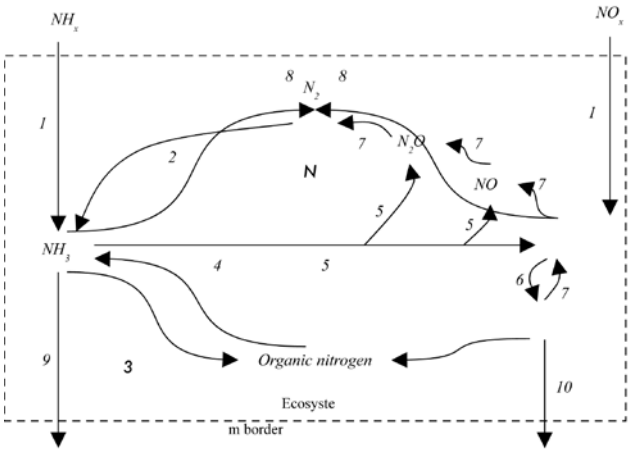

Fig. 3. Nitrogen cycle in the environment; 1 – inflow of atmospheric nitrogen compounds, 2 – nitrogen fixation, 3 – nitrogen assimilation, 4 – ammonification (mineralization), 5 – aerobic ammonia oxidation, 6 – nitrite oxidation, 7 – denitrification, 8 – anaerobic ammonia oxidation (Anammox process), 9 – ammonia evaporation, 10 – nitrate washing out [23]

Ammonia has to be treated properly because it is toxic to aquatic life, it causes eutrophication followed by an increase in the demand for oxygen in water [9].

Nitrification is an aerobic process that consists of two steps of oxidation: ammonia to nitrite and nitrite to nitrate. The first step is called nitritation (equation 1), the second – nitratation (equation 2) [21].

Two nitrification phases are performed by two physiologically and evolutionarily distinct groups of chemolitoautotrophic bacteria that use nitrification as an energy source. The first phase nitrifiers are called **a**mmonia-**o**xidizing **b**acteria (AOB), and the second phase nitrifiers **– n**itrite **o**xidizing **b**acteria (NOB). There is also a group of chemoorganotrophic bacteria that lead to heterotrophic nitrification, but the function of this process is as yet unknown [14, 21].

The autotrophic nitrification scheme is as follows:

$$NH_4^+ + 1{,}5O_2 \xrightarrow{nitritation} NO_2^- + 2H^+ + H_2O \qquad \Delta G^0 = -270\ \text{kJ/mol} \qquad (1)$$

$$NO_2^- + 0{,}5O_2 \xrightarrow{nitratation} NO_3^- \qquad \Delta G^0 = -80\ \text{kJ/mol} \qquad (2)$$

Nitritation is also a two-step process [Bock et al., 1992]. Firstly, ammonia is changed into hydroxylamine (equation 3). This reaction is catalyzed by ammonia monooxygenase (Amo) - a labile membrane protein, consists of few subunits, difficult to isolate and purification.

$$NH_4^+ + O_2 + 2[H] \xrightarrow{Amo} NH_2OH + H_2O \qquad \Delta G^0 = +15{,}4\ \text{kJ/mol} \qquad (3)$$

Subsequently, hydroxylamine is oxidized into nitrite (equation 4). This reaction is catalyzed by a periplasmatic enzyme – hydroxylamine oxidoreductase (Hao) where the donor of the second oxygen atom is water [12]:

$$NH_2OH + H_2O \xrightarrow{Hao} NO_2^- + H^+ + 4[H] \qquad \Delta G^0 = -289{,}3\ \text{kJ/mol} \qquad (4)$$

The factors that have an influence on nitrification are: temperature, pH, aeration, substrate level and the presence of toxic substances in the sewage. However, from a practical point of view, the limitation of nitrification occurs at its first step – ammonia oxidation [11].

Nitrification is performed in WWTP bioreactors that are constantly mixed and aerated and it seems to be a well known biochemical process. But the biodiversity of activated sludge bacteria inside these bioreactors, which contain a wide range of nitrifier species is still unknown to both technologists and biologists. From the point of the efficiency and optimization of wastewater treatment processes such studies of the biodiversity of nitrification are extremely important [25]. Research performed on bacteria leading to technologically significant processes reveals new information that is useful not only in wastewater treatment but also for other branches of biochemistry-based industries.

AOB as a technological and ecological research model

Ammonia oxidation bacteria belong to the chemolitotrophic microorganisms that use ammonia ions as an energy and electron source and carbon dioxide as a carbon source in chemosynthesis [20]. They are gram-negative and obligate aerobic, but Bodelier et al. [4] proved that some species can tolerate a low oxygen level or even anoxic environments. These microorganisms are ubiquitous in the soil, fresh and salty water and in WWTP installations.

Nitrifiers play a major role in the nitrogen cycle. However, from an economical point of view this process can have both a positive and negative influence. AOB produces greenhouse gases [27] and they lower the pH in the environment causing rocks and concrete corrosion [26]. Nevertheless, lower pH can be profitable for the cultivation of some plants [Beiderbeck et al., 1996]. It was also stated that nitrification can initiate the cometabolic removal of recalcitrants such as chlorinated aliphatic hydrocarbons from water or soil [1].

AOB are difficult to cultivate in a laboratory. They grow slowly [22], have a limited range of distinguishable phenotypic features [6] and are sensitive to temperature and pH swings, inhibitors and toxins [13, 17]. Only 25 pure strains of these bacteria are isolated to date [9].

Before the development of molecular tools, *Nitrosomonas* sp. was considered to be a classical AOB research model. It was common knowledge that it was dominant in nitrification installations until it appeared that more than 95% of bacteria playing a crucial role in activated sludge are uncultivable. It is also known than the effectiveness of nitrification is directly connected with the biodiversity of nitrifiers, so molecular approach for bacterial identification is necessary [25].

DNA as the carrier of genetic information

All of the information necessary for cell functioning, growth and multiplication is located in DNA – deoxyribonucleic acid. This molecule is a double-stranded, helically coiled polymer, and consists of monomers called nucleotides. The nucleotide's backbone is build of sugar and phosphate groups joined together with ester bonds. Each nucleotide possesses one of four bases – adenine, thymine, guanine or cytosine. Nucleotides are located in a DNA particle as its genetic code in a way that is characteristic for the organism. Double-stranded DNA is created due to the existence of hydrogen bonds between the bases – a double bond between adenine and thymine and a triple bond between guanine and cytosine. These nucleotides are always coupled and this rule is known in genetics as the base-pairing rule.

The DNA structure can be denatured if the temperature or the level of the denaturing substance is high enough to break the hydrogen bonds. The level of energy necessary for DNA double strand denaturation is called the DNA melting temperature and it depends on the number of hydrogen bonds in the particular DNA fragment. DNA can reassociate into the double helix after denaturation when the temperature or the level of denaturant disappears.

Both the base-pairing rule and differences in the DNA melting temperature are used for performing analysis using molecular biology methods. The most common and most useful technique is **p**olymerase **c**hain **r**eaction (PCR). This method is the basic tool in molecular biology research and a wide group of other techniques rely on it. In short, PCR is artificial DNA replication performed in a test tube using the DNA polymerase as the reaction catalyst. The PCR amplification, the multiplication of DNA into millions of copies, is performed according to the base-pairing rule and because of DNA's ability to denature and reassociate. DNA polymerase is able to amplify a particular fragment of DNA through the application of PCR primers – short DNA fragments, which flank the amplified region on both sides. Primers point at the site of the beginning and the end of the PCR reaction. The scheme of the PCR method is shown in Figure 4.

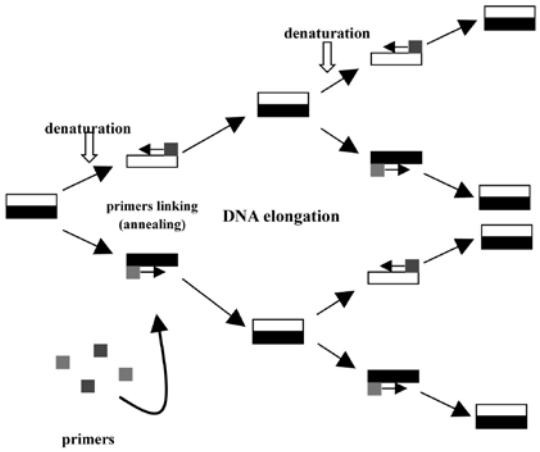

Fig. 4. Polymerase chain reaction (PCR) scheme

Molecular methods as a useful tool for microbiology

Since Koch's times microbial research were performed on pure bacterial strains. It was easy to observe biochemical processes in the test tubes. But due to the science development scientific developments,

microbiologists are unable to obtain more than 95% bacteria as pure cultures because it is impossible for these microbes to exist in an artificial lab environment [18].

In 1985 Pace et al. found an alternative solution for the problems of bacterial cultivation and since then molecular biology tools have begun to dominate in microbial laboratories. Owing to molecular methods it is possible to identify bacteria and the processes they perform in microbial mixtures and this facilitates biochemical research that is so important in modern biotechnology.

Molecular biology techniques are fast, sensitive and their results are repeatable. They can be divided into two groups [7] (Fig. 5):

a) indirect methods (based on PCR amplification),

b) direct methods (without previous amplification).

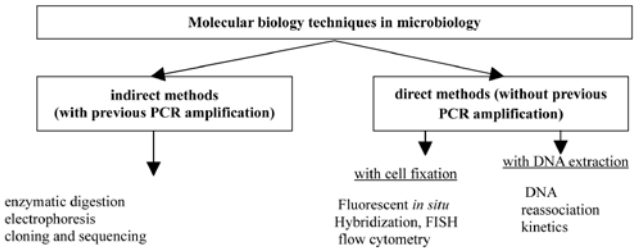

Fig. 5. Molecular biology methods useful in microbial research

How to monitor biochemical reactions using molecular biology tools?

In order to monitor any kind of biochemical process performed by a microorganism at a molecular level it is necessary to find the proper tool. Knowledge of the enzymes and genes that code it is important to understand how microorganisms perform a biochemical reaction and what the effectiveness of the reaction is. As was found, it is not necessary to know the enzyme's coding gene sequence in every type of research. In some cases we can work with universal molecular markers. Such a molecule should be [16]:

- abundant in every cell of the organism being studied
- a relatively large molecule
- functionally stable
- belong to housekeeping genes (genes responsible for basic metabolic reactions)
- possess both conservative as well as variable parts in order to perform identification research and phylogenetic analysis.

Since the 1980s the 16S rRNA coding gene has become a universal marker. The 16S rRNA particle builds a smaller subunit of a prokaryotic ribosome – an organellum responsible for translation (Fig. 6). Some parts of the molecule are group specific and it is possible to analyze a particular bacterial community (e. g. nitrifiers) on the basis of its 16S rRNA coding gene sequence.

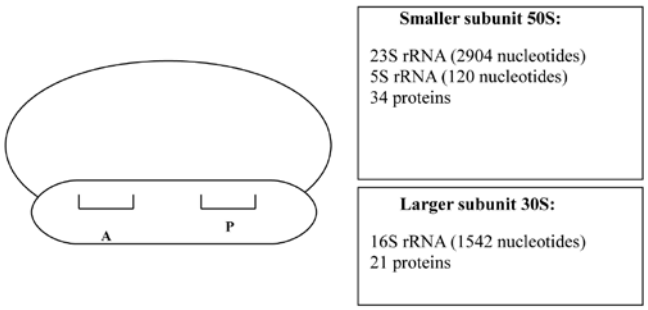

Fig. 6. Prokaryotic ribosome 70S scheme and composition; peptide site (P) and aminoacyl-tRNA site (A) during translation in a smaller subunit [5]

A universal marker is appropriate for monitoring a total community concerning its biodiversity and temporal changes, but for actual monitoring of a biochemical process, it is better to use enzyme coding genes. In the case of nitrification the crucial enzyme is an ammonia monooxygenase (Amo), a labile membrane protein that was mentioned previously [8]. The most important part of this enzyme responsible for ammonia oxidation, is its α subunit, used in the research, due to the fact that this part of the gene sequence is well known. It is also important that this part of the *Amo* gene is structurally stable and that every first phase nitrifier possesses it.

Summary

Modern biotechnology research clearly shows that most fields of science are linked. Biology and chemistry are connected more closely than the others because together they create biochemistry – the chemistry of living organisms. Scientific development has helped researchers in creating useful new tools for biochemistry research. Molecular biology techniques belong to this group. As was described above microbial metabolism can be easily characterized using biochemical language. However, biochemical reactions should be studied bilaterally – chemically and biologically if we want to obtain a clear and full picture of these processes.

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English translation by the Author

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Events IYC'2011

July 2011

6th International Symposium on Macrocyclic and Supramolecular Chemistry (6-ISMSC) - Jul 03 - Jul 07, 2011 - University of Sussex, Brighton, UK

Gold: Faraday Discussion 152 - Jul 04 - Jul 06, 2011 - Cardiff, UK

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