Granulated Mixed Microbial Culture Suggesting Successful Employment of Bioaugmentation in the Treatment of Process Wastewaters

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Original scientific paper
Received: 31. 3. 2001.
Accepted: 1. 6. 2001.

In memoriam Prof. Emeritus Vera Johanides

Present work gives a review of scientific achievements in the employment of bioaugmentation for degradation of xenobiotics from wastewaters of various origins. The authors point out the role of individual microorganisms and of the mixed cultures used as bioaugments for the purpose of bioaugmentation. The study has shown the efficiency of bioaugmentation in wastewater treatment, which led to the development of a new treatment procedure. Being the part of scientific studies on xenobiotics biodegradation, this work presents the activity of the prepared granulated mixed microbial culture in complete degradation of the surfactant (detergents – LAS) and dyes, based on naphthalenesulfonic acids (6-amino-2-naphthalenesulfonic acid), as the selected representatives of sulphonated aromatic substances. Degradation was performed under alternating oxy/anoxy conditions that contributed to oxido-reductive degradation processes of the mentioned substances and, thus, to their complete degradation without generation of degradation intermediates.

Key words:
Bioaugmentation, granulated mixed microbial culture, LAS, 6A2NSA, wastewater treament

Introduction

Biological treatment of municipal wastewater is successful when performed with activated sludge.[1,2] Microbial population of activated sludge is capable of degrading simple chemical structures from municipal wastewater, e. g. carbohydrates, short-chain fatty acids, simple alcohols, proteins and aminoacids.[3] Industrial development has been accompanied by generation of wastewaters. Prior to their release into environment they are mixed with municipal wastewater. This affects quality of wastewater, otherwise suitable for biodegradation with activated sludge. In addition to the simply structured ingredients, this wastewater contains the substances of a very complex chemical structure, sparingly biodegradable and often toxic to microbial population of activated sludge. These compounds belong to xenobiotics.[4,5] In wastewaters xenobiotics sparingly degrade in the presence of microbial population from activated sludge. Their toxic action interferes in particular with biological activity of the sensitive microorganisms that are during bioprocess either washed out from the wastewater treatment system with activated sludge or killed. The result of the so impaired microbiological quality of activated sludge is poor quality of the treated wastewater. This requires reconstruction of the existing systems through the increase of the reactor volume. Hence, by prolonging the adaptation period of sludge to chemical ingredients from wastewater (adaptation), it may be possible to ensure survival of the sensitive organisms and growth of the ones potentially capable of degrading majority of wastewater substances.[6]

At the same time with technical improvements (development) of wastewater biological treatment systems, considerable attention has been paid to microbiological quality of activated sludge and the role of its microorganisms in degradation of xenobiotics.[7] The studies comprised aerobic and anaerobic degradation of xenobiotics in the synthetic medium with the use of the selected microorganisms (SM), originating from the adapted activated sludge,[7] and of genetically engineered ones (GEM[8]). As the result, so selected GEM strains (Figure 1) and SM strains from activated sludge (Figure 2) are used in the existing systems with activated sludge to improve its biological (enzymatic) activity.[9,10]

Genetically engineered microorganisms (GEM) have shown high efficiency in degrading xeno-

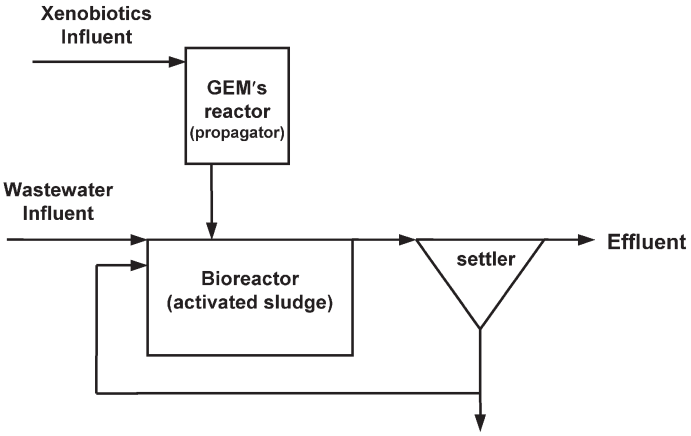

Fig. 1 – *Tretament of wastewater with activated sludge under the addition of the genetically engineered microorganisms (GEM)*[9]

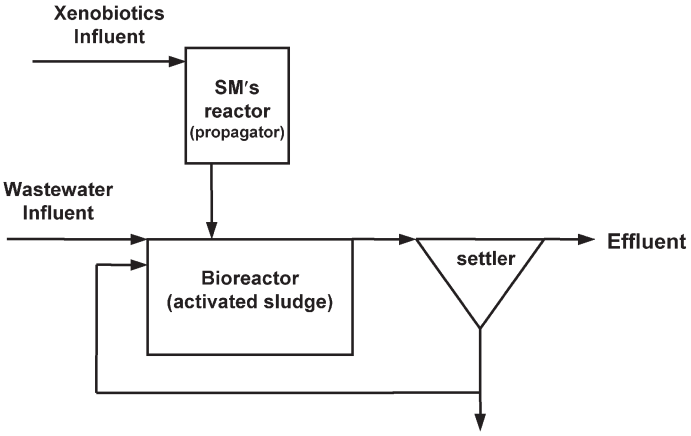

Fig. 2 – *Treatment of wastewater with activated sludge under the addition of the selected microorganisms (SM)*[10]

biotics in the synthetic medium.[8] When added to activated sludge in the biological wastewater treatment systems (Figure 1), due to the action of other substances of wastewater and environmental conditions, they can not survive in microbial population of activated sludge.[9] The selected microorganisms (SM) isolated from activated sludge adapt to a specific xenobiotic in the synthetic medium. When added to the wastewater treatment system with activated sludge (Figure 2), they show the ability to survive with other microorganisms from it, and improve its biological activity in degradation of xenobiotics.[10] Both SM and GEM are being eventually washed out from the system, due to its operating parameters and changed wastewater quality. Such enrichment of activated sludge with microorganisms of known biological properties, aimed at boosting enzymatic activity of sludge to degrade active substances from various wastewaters, is called "bioaugmentation".[11]

In the treatment of wastewater containing xenobiotics, successful bioaugmentation depends on various factors. These are: proper selection of a microorganism (as bioaugment) for the enrichment of activated sludge, and good knowledge of many biochemical-engineering, and microbiological factors required by wastewater treatment process with activated sludge using bioaugmentation.[12]

There are references showing that various studies have been carried out on the efficiency of bioaugmentation in the existing wastewater treatment systems, e. g. treatment of municipal wastewater in sewage lagoons[13] and treatment of process wastewater.[14] Irrespective of partial success of bioaugmentation in the treatment of process wastewater, the method is still under investigation.[15]

Our biodegradation studies of various wastewaters containing different kinds of xenobiotics used mixed microbial cultures containing bacteria, yeast or bacteria and yeast. Fundamental research included biodegradation of xenobiotics in the synthetic medium and in wastewater. Mixed microbial cultures could highly degrade xenobiotics (compounds of a very complex chemical structure) of natural origin or chemically synthesised ones, and to completely mineralise or modify their structure into environmentally friendly forms. In addition to xenobiotics, other wastewater ingredients were degraded, too. Mixed microbial cultures from wastewater showed that lignosulfonates,[16] antibiotics,[17] naphthalenesulfonic acids,[18] thiocyanates,[19] phenol,[20] polyaromatic carbohydrates[20] and formaldehyde,[21,22] underwent degradation as well. Fundamental degradation studies of the substances from various wastewaters with the use of the selected mixed microbial cultures[16–22] have set up the principles of the new wastewater treatment procedure. The procedure is based either on the granulated mixed microbial cultures containing selected microorganisms, rather than active sludge, or on a bioaugment added to activated sludge to improve its enzymatic activity in wastewater treatment systems.[23,24] SM[9] (Figure 1) and GEM[10] (Figure 2) as bioaugments for bioaugmentation are grown on the medium with xenobiotic occurring in the treated wastewater. Mixed microbial cultures as bioaugments in the form of granulated biomass are prepared and grown in wastewater as the substrate (Figure 3)[23].

Operation of the existing systems with activated sludge used to treat wastewater containing xenobiotics, is improved by adding to activated sludge the granulated biomass of the mixed culture of microorganisms as bioaugment (Figure 3). During system operation, due to the increased biological activity, granulated biomass of the mixed culture begins to dominate microorganisms from activated sludge, which are washed out from the system. In the course of the process, quality of the

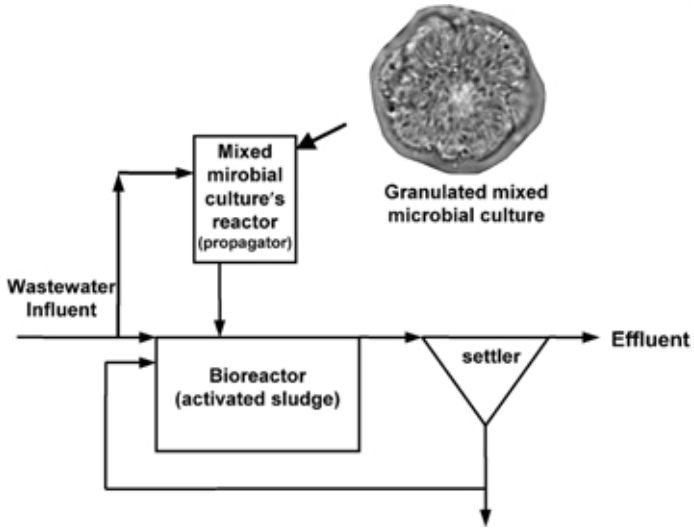

Fig. 3 – *Treatment of wastewater with activated sludge under the addition of the granulated mixed microbial culture*[23]

granules of the mixed microbial culture (alone or with activated sludge) may be affected by significant changes in wastewater quality. The addition of a new biomass (granules) produced in the bioreactor (propagator) maintains the established quality of the granulated mixed culture with respect to the activity to degrade wastewater substances and with respect to the ability to form granules.[23–25]

Physiology of various types of microorganisms (heterotrophic, autotrophic, aerobic and anoxic) and composition of biological treated wastewater allow preparation of mixed microbial cultures. In the treatment system they will be responsible for oxido-reductive biodegradation of various substances with carbon, nitrogen, phosphorus, and sulfur.[25] So prepared mixed microbial cultures in granulated form are capable of biodegradation, nitrification, denitrification, sulphur reduction, and phosphorus accumulation. Degradation studies of various substances of wastewaters using mixed microbial cultures, and the studies of interactions between various microorganisms in the mixed culture, have been performed. They enabled preparation of enzymatically multifunctional mixed microbial culture which can simultaneously perform oxido-(biodegradation of organic substances and nitrification) reductive processes – (denitrification, reduction of sulphates and accumulation of phosphates), relevant to wastewater quality.[25]

There are no literature data about the use of mixed cultures in simultaneous performance of various biological processes. The only thing pointed out is the possibility to use methanotrophes and heterotrophes or methanotrophes and nitrifiers in degradation of sparingly degradable compounds from wastewaters.[26]

This work presents the effect of the granulated mixed microbial culture capable of degrading linear alkylbenzenesulfonates (LAS) and 6-amino-2-naphthalenesulfonic acid (6A2NSA) from wastewater, originated from the production of surfactants i. e. dyes based on naphthalenesulfonic acids.

Material and methods

Granulated mixed microbial culture

Granulated mixed microbial culture contained the selected bacterial strains: four heterotrophic bacteria of *Pseudomonas* spp. genus (LOV 300, LOV 302, LOV 304 and LOV 306), four autotrophic bacteria of *Nitrosomonas* spp. (LOV 500 and LOV 502), *Nitrosococcus* spp. (LOV 101) and *Nitrosospira* spp. (LOV 602) genera (LOV = collection of microorganisms at the Laboratory for biological wastewater treatment, Faculty of Food Technology and Biotechnology, University of Zagreb).

Each strain of heterotrophic bacteria had been kept on agar with the addition of 20 mg L^{-1} LAS i. e. 50 mg L^{-1} 6A2NSA. Autotrophic bacteria had been kept on the following mineral media[27] (g L^{-1}): $(NH_4)_2SO_4$ 2, K_2HPO_4 1, $MgSO_4$ 0.5, $FeSO_4$ 0.4, NaCl 0.4, $CaCO_3$ 1 and $MgCO_3$ 1 at +4 °C.

Mixed microbial culture was prepared of individual heterotrophic and autotrophic microorganisms in almost equal concentration of biomass, grown in solid media (composition equalling that for individual strains) over 48–72 hours at 28 °C. The culture was adapted to LAS i. e. 6A2NSA on the synthetic medium with LAS (up to 40 mg L^{-1}) i.e. 6A2NSA (up to 500 mg L^{-1}). Growth was performed under alternate aerobic (dissolved oxygen concentration 2–4 mg L^{-1}) and anoxic (dissolved oxygen concentration 0.2–0.4 mg L^{-1}) conditions. Sources of nitrogen and phosphorus in the synthetic medium were $(NH_4)_2SO_4$ and KH_2PO_4. LAS was a commercial product (97.5% of active substance) of "Saponia" Osijek, Croatia. 6A2NSA was a chemical substance (98% purity) made by Bayer, Leverkusen, Germany. Growth and adaptation under the above conditions lasted till the activity of the mixed culture was sufficient for oxido-reductive process and formation of the granules (Figures 4 and 5). During growth and adaptation in the synthetic medium with LAS i. e. 6A2NSA, drop in pH below 6.8 had been adjusted with 2 mol L^{-1} NaOH to pH 7.2–7.5.

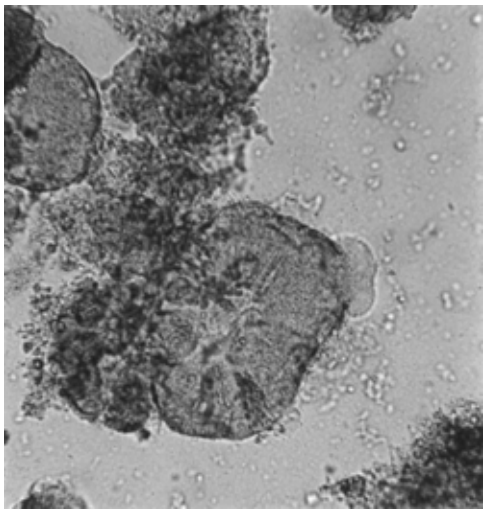

Fig. 4 – *Microscopic view of the granulated mixed microbial culture prepared in the liquid synthetic medium with the addition of LAS (enlarged 16×40)*

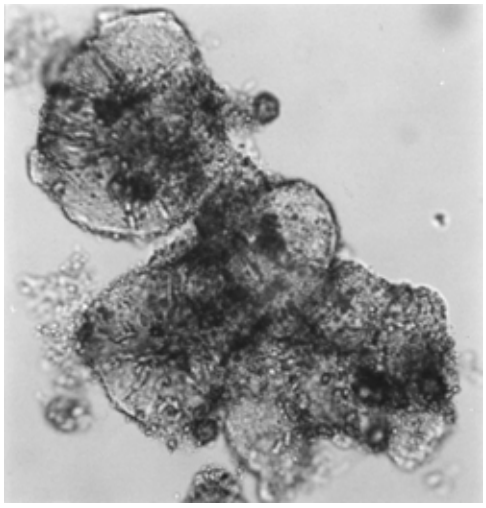

Fig. 5 – *Microscopic view of the granulated mixed microbial culture prepared in the liquid synthetic medium with the addition of 6A2NSA (enlarged 16×40).*

Table 1 – *Chemical composition of wastewater from the production of surfactants (LAS), i. e. dyes based on naphthalenesulfonic acids (6A2NSA).*

Ingredients	Wastewater from production of surfactants (LAS)	Wastewater from production of dyes based on naphthalenesulfonic acids (6A2NSA)
LAS, mg L^{-1}	90	–
6A2NSA, mg L^{-1}	–	500
KPK, mg L^{-1}	1200	1790
N-NH_4, mg L^{-1}	35	22
N-NO_3, mg L^{-1}	10	12
P-PO_4, mg L^{-1}	12	12
pH	8.15	8.45

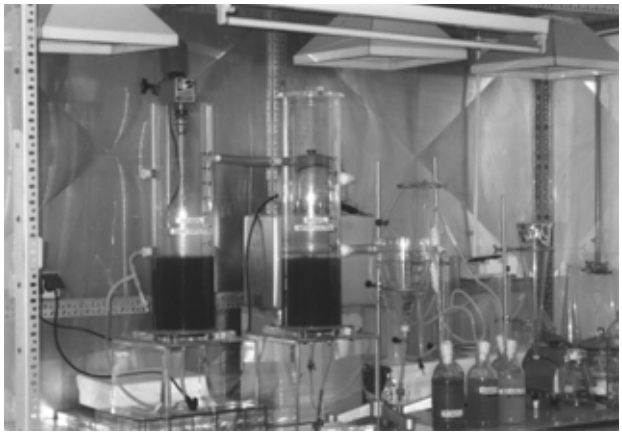

Fig. 6 – *Laboratory model apparatus used for degradation of LAS i. e. 6A2NSA in the synthetic medium and wastewater. The apparatus consists of one bioreactor supplied with aeration system (aerobic bioreactor for aerobic biodegradation and nitrification) and one bioreactor supplied with a blender and aeration system (anoxy/aerobic bioreactor for denitrification and reduction of sulphates and for oxidation).*

Wastewater

Table 1 shows composition of wastewater with LAS i. e. 6A2NSA.

Degradation of LAS i. e. 6A2NSA in the synthetic medium and wastewater was performed under the following conditions: temperature 18–20 °C; concentration of dissolved oxygen 2–4 mg L^{-1} at aerobic step, and 0.2–0.4 mg L^{-1} at anoxic step; biomass concentration 0.8–1 g L^{-1}.

Adaptation experiments with the mixed microbial culture to LAS i. e. 6A2NSA in the synthetic medium were carried out with the laboratory model apparatus (Figure 6), consisting of two bioreactors. Degradation kinetics of the substances from wastewater generated by surfactants production (detergents -LAS), i. e. dyes based on naphthalenesulfonic acids (6A2NSA), had been monitored in one reactor of the laboratory model apparatus.

The experiments were performed on the laboratory model apparatus, with 5 L of culture volume. WTW pH electrodes were used to monitor pH, temperature and concentration of dissolved oxygen.

Analytical procedures

Changes in LAS concentration on the synthetic medium and wastewater were determined by standard APHA[28] methods; structural changes in LAS were recorded by IR spectrophotometer, BOMEM model MB 100 Series MID FTIR supplied with DTGS detector.

Changes in the concentration and structure of 6A2NSA were determined by UNICAM UV spectrophotometer, type Helios Beta and HPLC.[18]

Changes in $N\text{-}NH_4$ and $N\text{-}NO_3$ concentrations were determined by ionic chromatography, chromatograph Dionex DX-100.

Biomass of the mixed microbial culture was determined gravimetrically, by filtering the sample through 0.45 μm filter and drying it over 8–10 hours at 110 °C.

Chemical oxygen demand (COD) of the samples was determined by following the APHA dichromate method.[28]

Results and discussion

The selected mixed microbial culture contained heterotrophic bacteria of *Pseudomonas* spp. genus, capable of degrading lignosulfonates,[16] naphthalenesulfonic acids,[17] thiocyanates,[19] cyclic and aromatic compounds with sulphur[20] as well as autotrophic bacteria of *Nitrosomonas* spp., *Nitrsosococcus* spp. and *Nitrosospira* spp. genera, capable of nitrification of ammonium nitrogen occurring in wastewater and of nitrogen generated by degradation of various nitrogen compounds, e. g. amino-groups of naphthalenesulfonic acids, thiocyanates.[25] Each strain of microorganisms in the mixed culture exhibited versatile enzymatic capability for oxido-reductive processes during degradation of surfactants (detergents-LAS, Figure 7) and 6A2NSA (Figure 8).

The selected mixed microbial culture during preparation of the biomass, as the original inoculate for degradation of LAS in wastewater, was adapted and grown on the medium with LAS level (up to 40 mg L^{-1}) below that in wastewater (Table 1). Therefore, before monitoring degradation kinetics of the substances in the original wastewater from surfactants production (detergents-LAS), mixed microbial culture had been gradually adapted to the substances of wastewater. That was done by degradation experiments of the substances from wastewater in various dilutions with tap water. After 6 consecutive adaptations, degradation kinetics of LAS and of other substances from the original wastewater, had been monitored (Figure 7).

In addition, before monitoring of degradation kinetics of the substances in the original wastewater from dyes production based on naphthalenesulfonic acids (6A2NSA, Figure 8), 7 consecutive adaptations had been made of the mixed microbial culture to wastewater substances.

To ensure alternate oxy/anoxy conditions, required to maintain the activity of the mixed microbial culture for oxido-reductive processes, the experiment with LAS i. e. 6A2NSA degradation was performed in one reactor. The reactor was supplied with the blender to maintain anoxy conditions and with the aeration system to maintain aerobic (oxy)

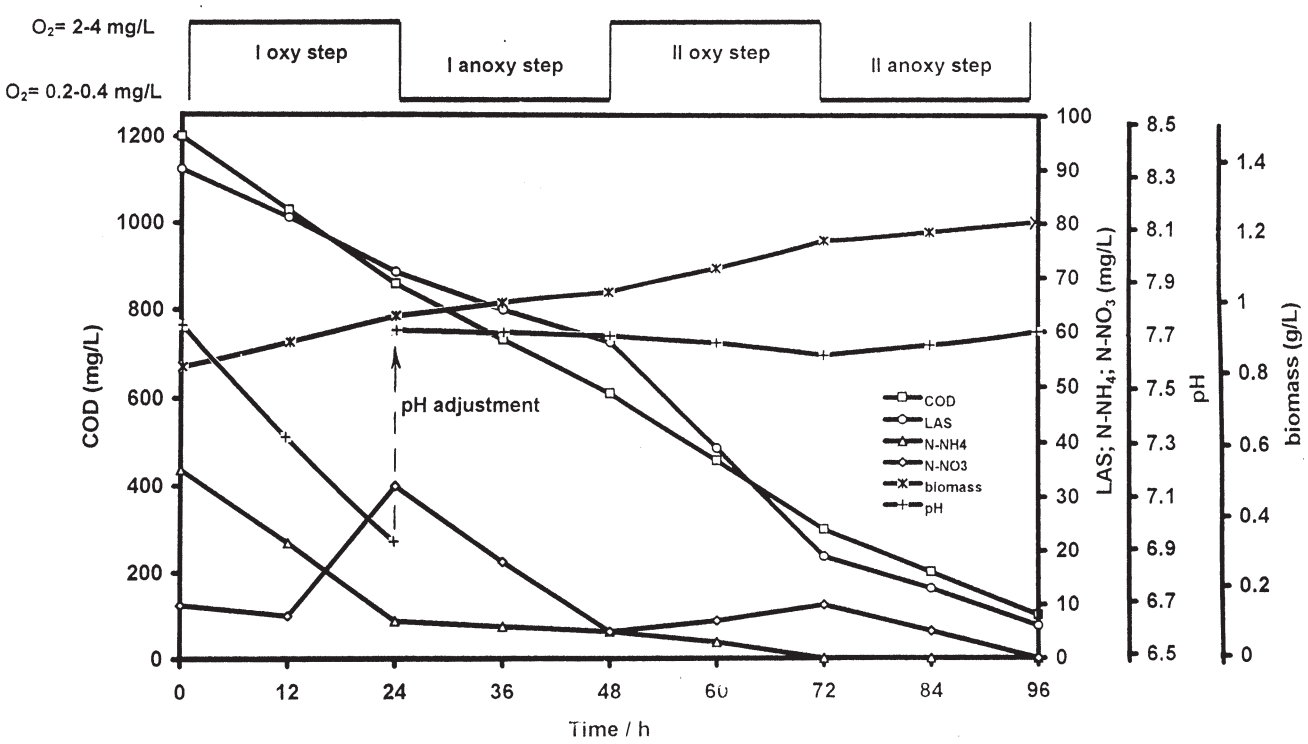

Fig. 7 – *Degradation kinetics of the ingredients of wastewater from surfactants production (detergents-LAS) achieved with the selected mixed microbial culture under alternate oxy/anoxy conditions (aerobic/facultatively anaerobic conditions).*

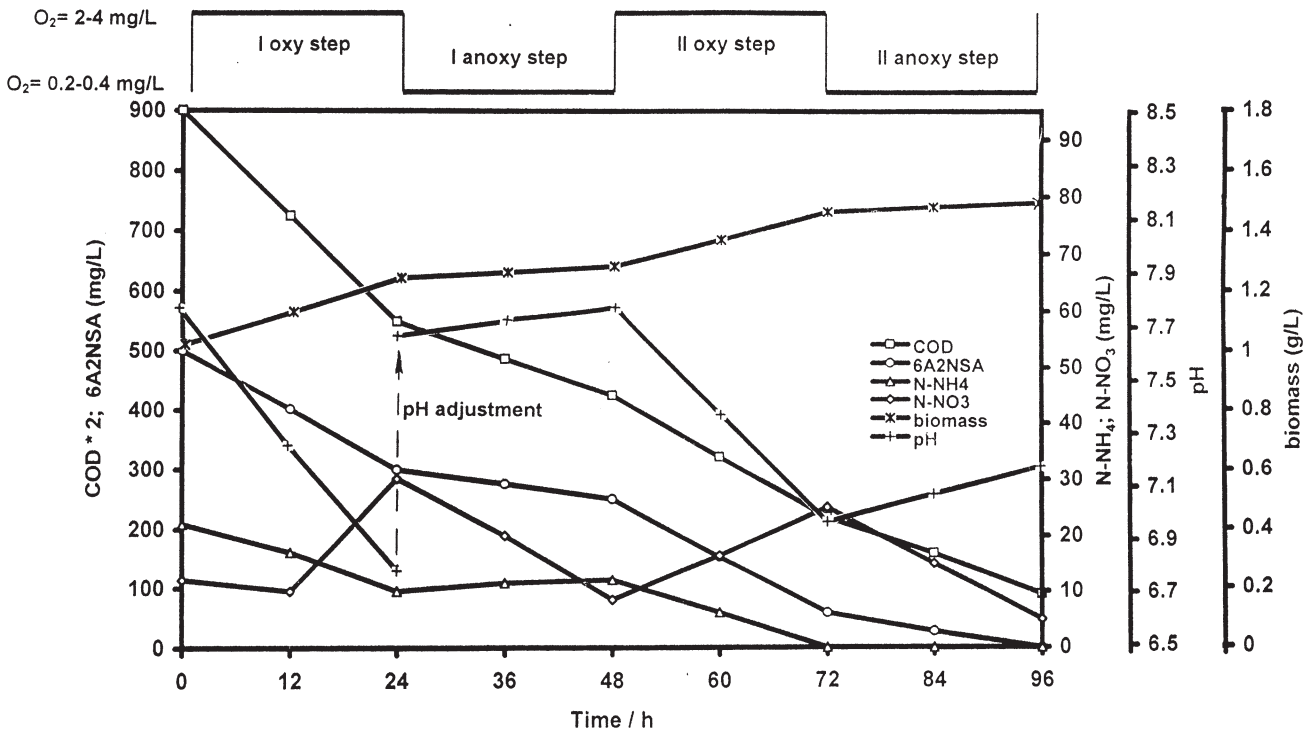

Fig. 8 – *Degradation kinetics of the ingredients of wastewater from dyes production (6A2NSA) achieved with the selected mixed microbial culture under alternate oxy/anoxy conditions (aerobic/facultatively anaerobic conditions).*

conditions. Oxy and anoxy conditions had been alternated in 24-hour intervals each (defined during adaptation of the mixed microbial culture to LAS i. e. 6A2NSA).

Degradation kinetics of the substances in wastewater from surfactants production (detergents – LAS) with the selected mixed microbial culture had been monitored over 96 hours, under alternate oxy and anoxy conditions (Figure 7). During the first aerobic degradation step of wastewater, detergents (LAS) degraded under simultaneous decrease in COD. That was indicative of degradation of other substances and the increase of biomass. At the same time the concentration of ammonium nitrogen went down and nitrate went up. The outcome of oxido-biodegradation of detergents (LAS) and nitrification of ammonium nitrogen that generate sulphate i. e. nitrate, was decreased pH value. To proceed with degradation of the remaining and accumulated substances, it was necessary to keep over 24 hours of the first anoxic degradation step, the initial pH by the addition of 2 mol L^{-1} NaOH. Under anoxic conditions the mixed microbial culture redirected its enzymatic activity to anoxic degradation and reduction of nitrates. Nitrates were reduced from 32 mg L^{-1} to 5 mg L^{-1} under lowered concentrations of organic ingredients, expressed as reduced COD from 860 mg L^{-1} to 610 mg L^{-1}. Decreased concentrations of organic substances, expressed as COD and equalling to about 150 mg L^{-1} of carbon, were enough to reduce nitrate. Under anoxy conditions, the concentrations of detergents were reduced less than under oxy. That might be due to another type of degradation, e. g. hydrolysis of the complex structure detergents (LAS). Growth of biomass was an indicator of the occurring hydrolysis of detergents and of the consequential formation of simple chemical structures. These structures might be potential resources of carbon, essential for the growth of biomass. At the second aerobic step, detergent biodegradation took place simultaneously with oxidation of ammonium nitrogen and generation of nitrate (from 5 mg L^{-1} to 10 mg L^{-1}). These concentrations of nitrates did not have significant impact on changes in pH. Therefore, there was no need for pH adjustment. IR spectrophotometry of the samples showed that the detergents have degraded at the first anoxy, and second oxy steps. Changed absorbancies of various wavelengths revealed that at the first anoxy degradation step of detergents, the concentration of the carboxyl compounds increased (absorbancies at 1 600 – 1 700 cm^{-1}) and that at 1 200–1 040 cm^{-1} absorbancy levels significantly decreased, pointing out to degradation of aromatic sulfonates. IR-spectrophotometry of the second aerobic degradation step revealed consumption of carboxyl compounds and further decrease in aromatic sulfonates absorbancy. At the second anoxy step over next 24 hours, the remaining concentration of organic compounds (detergents and other ingredients of wastewater) expressed as COD (300 mg

L^{-1}), the mixed microbial culture used for reduction of nitrate. Over 96 hours of substances' degradation from surfactants production wastewater (detergents-LAS), performed with the mixed microbial culture, and under alternate oxy-anoxy conditions (in 24-hour intervals each), the detergents degraded from 90 mg L^{-1} to 7 mg L^{-1}; COD dropped from 1 200 mg L^{-1} to 100 mg L^{-1} and all substances containing nitrogen, were entirely removed (Figure 7).

The same mixed microbial culture was used to degrade 6A2NSA from the wastewaters generated by dye production on the basis of naphthalenesulfonic acids (Figure 8). The mixed culture behaved similarly as in degradation of detergents. Whereas in degradation of detergents the source of nitrogen was ammonium nitrogen from wastewater, in degradation of 6A2NSA it was ammonium nitrogen from wastewater, and nitrogen generated by degradation-oxidation of 6A2NSA[18] amino group. At the first oxy step (24 hours) there was significant degradation of 6A2NSA and of other substances expressed as COD value. Biodegradation had been monitored by nitrification of ammonium nitrogen and nitrogen generating from oxidation of amino group. A significant drop in pH, due to accumulation of nitrate and sulphate, required pH adjustment with 2 mol L^{-1} NaOH. The substances resulting from degradation of 6A2NSA and other substances expressed as COD value were used as the source of carbon for biomass synthesis. At the first anoxy step 6A2NSA underwent further degradation, but not that significant as at the first oxy step. There was also corresponding increase in ammonium nitrogen. Simultaneously, there was a reduction in nitrate with the use of carbon from the wastewater substances and the products of 6A2NSA degradation, but without significant changes in pH. At the second oxy step biodegradation of 6A2NSA occurred together with biodegradation of other substances, expressed as COD value accompanied by the growth of biomass. Nitrification of ammonium nitrogen, generated by oxidation of amino group from 6A2NSA went simultaneously, and the accumulated nitrate reduced pH to 7.0. At the second anoxy step over next 24 hours, the remaining concentration of organic substances, expressed as COD value, used the mixed microbial culture for denitrification as the source of carbon.

UV-spectrophotometry in the wavelength range 190–320 nm (Figure 9) showed that degradation of 6A2NSA was accompanied by the changes in the concentrations and structure. Changed concentrations of 6A2NSA were manifested in the reduced absorbancy values at 242 nm (characteristic peak for 6A2NSA). Accumulation of nitrate was manifested in the increased absorbancy at 210 nm. The loss of absorbancy maximum at 242 nm wavelength was the evidence of complete degradation of 6A2NSA (oxidation and nitrification, curve 1, Figure 9). The loss of absorbancy maximum at 210 nm indicated the reduction of nitrate (denitrification, curve 5, Figure 9).

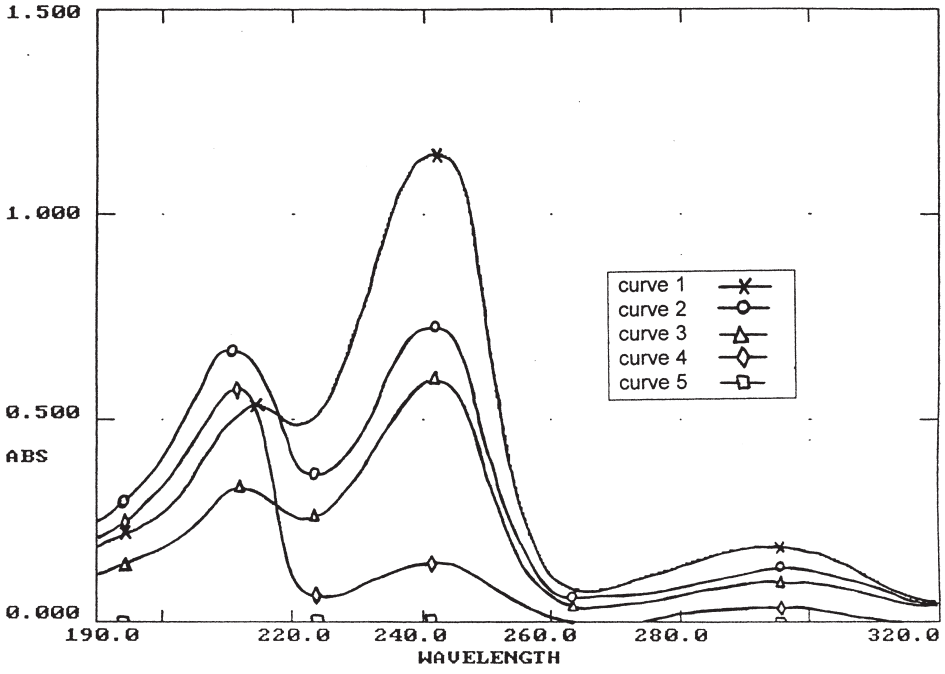

Fig. 9 – *UV absorbancy curves for 6A2NSA achieved during degradation of wastewater with 6A2NSA (absorbancy maximum at 242 nm for 6A2NSA and at 210 nm for N-NO_3). Absorbancy maximum for 6A2NSA before the onset of degradation (curve 1); during oxy degradation step 1 (curve 2); during anoxy degradation step 1 (curve 3); during oxy degradation step 2 (curve 4) and during anoxy degradation step 2 (curve 5).*

Conclusions

The results show that the mixed microbial culture is capable of degrading surfactants (LAS), i. e. of 6A2NSA in wastewater originated from the production of surfactants, i. e. dyes based on naphthalenesulfonic acids. This property is attributed to its versatile enzymatic potentials.

So versatile enzymatic potentials are attributed to heterotrophic and autotrophic bacteria from the mixed microbial culture acting on the principles of co-metabolism.

The evidence of their co-metabolic activity and of the presence of the complete enzymatic system, capable of performing concurrently a whole range of oxido-reducing processes, is degradation of LAS i. e. 6A2NSA being the selected representatives of sulphonated aromatic substances and other wastewater substances without generation of degradation intermediates.

To enable the mixed microbial culture redirect its enzymatic activity for oxido-reducing processes during degradation of wastewater substances, wastewater must, either, already contain reduction inducing compounds (sulphates and nitrate), or have them generated by prior oxidation. These compounds ensure co-metabolic activity between the members of a mixed microbial culture which are necessary for oxido-reducing processes, relevant to the conditions of oxygen supply (oxy/anoxy conditions).

Stability of the members of mixed microbial culture, that may find its place in process wastewater treatment by bioaugmentation, ensures their formation into granules.

ACKNOWLEDGEMENT

The first idea about the importance and the use of mixed microbial cultures in xenobiotics degradation come from late Prof. Emeritus Vera Johanides, who has set the path to new approach in scientific research of microbial ecology.

For scientific achievements in the use of mixed microbial cultures in various microbial processes of wastewater degradation, the authors of this work are greatly indebted to Prof. Vera Johanides.

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