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BIODEGRADATION OF AZO DYE BY ADAPTED MIXED MICROBIAL CULTURES

IVANA GUDELJ, JASNA HRENOVIĆ[1], VICE ŠOLJAN[2], TIBELA LANDEKA DRAGIČEVIĆ[3], ALEKSANDRA ANIĆ VUČINIĆ[4], MARIO ŠILJEG[5], MIRJANA GALANT[6]

Hipalab Ltd., Zagreb, Croatia
[1]Faculty of Science, University of Zagreb, Zagreb, Croatia
[2]Biotech center Pula Ltd., Pula, Croatia
[3]Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia
[4]Geotechnical Faculty, University of Zagreb, Varaždin, Croatia
[5] Energy Institute Hrvoje Požar, Zagreb, Croatia
[6]Usluga Ltd., Pazin, Croatia
e-mail: ivana.gudelj@hipalab.hr

Wastewater effluents from azo dye production and other dye-stuff using industries contain significant amounts of highly resistant azo dyes that require special treatment processes to prevent groundwater contamination. The present study is based on the approach of aerobic followed by anaerobic step for biodegradation and decolorization of azo dye. The main objective of this work was the adaptation, isolation and preparation of mixed microbial culture, from laboratory collection, catabolically able to biodegrade under aerobic conditions bordo azo dye present in mother lye after industrial production of that dye. The anaerobic step needed for biodegradation of azo dye was performed by the use of adapted active anaerobic sludge from a wastewater treatment plant of the sugar industry. The adapted aerobic and anaerobic microbial cultures demonstrated significant biodegradative enzymatic potential and can be further used for development of a continuous aerobic – anaerobic process for the treatment of wastewater from industrial production of azo dye.
Key words: azo dyes, biodegradation, decolorization, microorganisms, wastewater.

Biorazgradnja azo boje pomoću prilagođenih mješovitih mikrobnih kultura. Otpadne vode industrijske proizvodnje azo boja kao i drugih industrija koje se u svojim procesima koriste bojama iziskuju specifične procese obrade u cilju dostatno kvalitetetne obrade istih i prevencije onečišćenja podzemnih voda. Ovaj rad se temelji na anaerobnom, a potom anaerobnom postupku biodegradacije i dekolorizacije azo boje. Suština rada se sastoji u prilagodbi i izolaciji mikroorganizama iz laboratorijske zbirke za pripravu aerobne mješovite mikrobne kulture katabolički sposobne za biodegradaciju bordo azo boje prisutne u otpadnoj vodi nastaloj proizvodnjom te boje. Anaerobni stupanj također potreban za biorazgradnju azo boje proveden je uporabom prilagođenog anaerbnog aktivnog mulja podrijetlom iz uređaja za obradu otpadnih voda šećerane. Prilagođena aerobna i anaerobna mikrobna kultura manifestirale su značajan biodegradacijski enzimatski potencijal, te kao takve mogu biti uporabljene za daljnji razvoj kontinuiranog aerobno-anaerobnog procesa obrade otpadne vode nastale industrijskom proizvodnjom azo boje.
Ključne riječi: azo boje, biorazgradnja, dekolorizacija, mikroorganizmi, otpadna voda.

INTRODUCTION

Synthetic dyes are extensively used in many fields of currently used industries and exhibit considerable structural diversity [1]. Nearly all dyestuffs have been now produced from synthetic compounds [2]. Azo dyes are the most important group of synthetic dyes characterized by nitrogen to nitrogen double bonds [3] bearing aromatic rings, dominate the worldwide market of dyestuffs with a share of about 70 % [4]. Two major sources of release of azo dyes into the environment are the textile and dyestuff manufacturing industries [5]. Approximately 10-15% of azo dyes are released into the environment after industrial manufacturing or usage of them [6]. The majority of these dyes and their degradation intermediates are either toxic [7], mutagenic or carcinogenic [8] and possess serious potential health hazard to all forms of life [9]. The highest rates of toxicity, among all synthetic dyes, were found amongst basic and diazo direct dyes [10]. Dye removal has recently become one of the mayor scientific interests [5]. A wide range of methods has been developed for their removal from wastewaters with intention of decreasing their negative impact on the environment [1].

There are many reports about the use of physiochemical and microbiological methods for dye removal from colored effluents [11-13]. Unfortunately a single universally applicable end-of-pipe treatment solution is impossible because of wide diversity of dyes chemical compositions and the concentration of color in wastewaters, which usually depends on daily and seasonal variations that are dictated by industrial routines of their use or production [14]. The treatment of azo-dye-containing wastewaters still remains and presents a serious ecological and technical challenge [9,15]. Currently used physiochemical techniques for the treatment of colored wastewaters such as: adsorption, precipitation, chemical oxidation, photodegradation or membrane filtration have serious restrictions because high costs caused by intensive energy requirements, formation of hazardous by-products [16], generation of significant amount of sludge, which must be handled [17] and can cause secondary pollution problems [3,10,18].

Microbiological methods are effective in removing dyes from large volumes of effluents [10]. They are generally considered as cost-competitive alternative methods to physiochemical decomposition processes [5] and also environmentally friendly as they can lead to complete mineralization of complex pollutants [9,19]. Systems based on the use of mixed microbial populations are more effective due to sinergistic metabolic activities of microbial community [14, 20, 21]. Azo dyes are generally considered as electron-deficient xenobiotic compounds, generating electron deficiency in the molecule and making the compound less susceptible to microbial attack and very recalcitrant against oxidative biodegradative processes in conventional activated sludge treatment units [11,16, 22-24].

The most often used concept for the removal of azo dyes in biological wastewater treatment systems is based on anaerobic treatment, for the reductive cleavage of the azo linkage, in combination with aerobic treatment, for the degradation of the products from azo dye cleavage, aromatic amines [8, 25-26]. During last twenty years several research papers have been published about combination, sequential or integrated, anaerobic-aerobic bioreactor treatment of azo dye-containing wastewaters. Azo dye reduction in the anaerobic phase of those bioreactor systems is generally efficient, but process often requires long reaction time which can speed up addition of redox

mediators. The consequent removal of aromatic amines under aerobic conditions has been less unequivocal [1,17]. Although analytical data indicate that many of the aromatic amines can be removed from the wastewater, and although the limited amount of available toxicity results all show far-reaching detoxification during aerobic treatment, it is clear that not all aromatic amines can be completely mineralized [17,19,27]. In many cases, the aromatic amines from azo dye reduction become highly reactive in the presence of oxygen. In aerobic bioreactors autooxidation and possibly reaction with compounds from the sludge matrix compete with biodegradation [17] and represent an important part of the persisting chemical oxygen demand in the effluent after biological treatment of azo dye containing wastewaters [27].

A new approach to decolorization and biodegradation of azo dyes from simulated wastewater in aerobic sequencing batch reactor has been also reported [29]. More than two thousand structurally different azo dyes has been industrially produced and used [8]. They cause and manifest various and unpredictable biodegradative behaviour. It is than understandable that our knowledge concerning their behavior in the environment and health hazard connected with their use is still incomplete. It is to be expected that complexity of the complete degradation of synthetic dyes will increase within increasing structural complexity of new synthetic dyes [1].

Scientific knowledge should surely become more effective at determining, isolating and preparing of well characterized microorganisms which maintain exceptional catabolic ability and tolerance in a broad range of chemicals and environmental stress, that are likely to suit specific bioremediation conditions and requirements [29]. Newly isolated, appropriately adapted [1] or genetically engineered microorganisms may accomplish degradation of synthetic molecules, such as azo dyes, which persist under normal environmental conditions [30,31]. It would also be advantageous to find a rule by which it would be possible to determine the most appropriate treatment method according to the dye structure [32].

EXPERIMENTAL

A series of experiments were undertaken to investigate aerobic bioconversion and anaerobic biodegradation of azo dye present in mother lye after industrial production of that dye.

Wastewater – Dyestuff, Reagents, Microbiological Media

Chemical structure of azo dye consisted in mother lye after its production is shown in Figure 1. Microbiological media were obtained from Biolife and other chemicals used were of analytical grade.

Table 1. Chemical composition of untreated wastewater from the production of bordo azo dye used in the experiments (*Total Organic Carbon,**Dissolved Organic Carbon,***Chemical Oxygen Demand,****Total Suspended Solids)
Tablica 1. Kemijska kakvoća neobrađene otpadne vode nastale tijekom industrijske proizvodnje bordo azo boje korištene u pokusima (*ukupni organski ugljik,**otopljeni organski ugljik,***kemijska potrošnja kisika,****ukupne suspendirane čestice)

Parameter	Concentration (mg/L)
TOC*	8 240 mg/L
DOC**	7 200 mg/L
COD***	22 000 mg/L
TSS****	6.46 mg/L

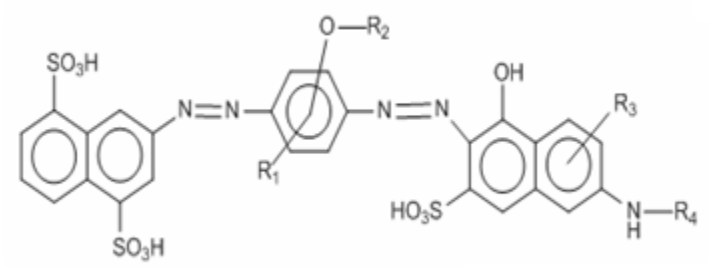

Figure 1. Chemical structure of bordo azo dye
Slika 1. Strukturna formula bordo azo boje

Adaptation, Isolation and Preparation of Aerobic Mixed Microbial Culture

Bacterial and yeast strains used for preparation of aerobic mixed microbial culture, in this research, were taken from collection of microorganisms from the Laboratory of Faculty of Science, Division of Biology, University of Zagreb. Enough long adaptation of bacterial and yeast strains on the aromatic substrates as partially structural analogues of the azo dye and finally on azo dye as a sole carbon source was performed and led to the isolation of degrading microbial strains. 25 bacteria strains were cultivated on plates, during 48 hours, at the temperature of 27^{o}C on: Plate count agar with 1000 mg/L naftalen-sulfonic acid, Technical agar with 500 mg/L naftalen-sulfonic acid and 150 mg/L azo dye, Technical agar with 300 mg/L of azo dye as a sole carbon source. Three yeast strains were cultivated on plates, during 48 hours, at the temperature of 27^{o}C: on Sabourad media, on Sabourad media with addition of 1000 mg/L naftalen-sulfonic acid, on Technical agar with addition of 500 mg/L naftalen-sulfonic acid and 150 mg/L azo dye and finally on Technical agar with 300 mg/L of azo dye as a sole carbon source. Growth and adaptation under previously described

conditions lasted till the enzymatic activity of the strains was sufficient for biodegradation of azo dye and its use as a sole source of carbon.

Microbial colonies that appeared on Technical agar medium, with azo dye as sole source of carbon, were six bacterial and one yeast strain and identified as: *Bacillus circulans, Flavobacterium devorans, Flavobacterium indoltheticum, Pseudomonas cepacia, Pseudomonas desmolitica, Pseudomonas putida* and *Trichosporon penicillatum*.

After identification they were washed gently with sterile water, resuspended into the sterile flask and stored at 4^{o}C in liquid medium for the subsequent aerobic experiments.

Aerobic Biodegradation Experiments

Aerobic biodegradation was studied in experiments with 500-ml Erlenmeyer flasks, filled with 200-ml of basal medium, first under aerobic conditions and concentration of oxygen 0.3-0.5 mg/L and secondary under microaerofilic conditions and concentration of oxygen 0.1-0.2 mg/L.

The initial concentration of biomass was 0.5 g/L. Incubations were carried out on an orbital shaker set at 60-80 rpm and temperature of 27^{o}C. Three concentrations of azo dye were chosen and added in basal media during this study, namely: 100 mg/L, 300 mg/L and 1000 mg/L.

Samples were periodically taken for UV-VIS determination of color biodegradation. The UV-VIS spectral changes served as a toll of confirmation for disappearance of bordo azo dye structure and formation of a metabolite during aerobic and microaerofilic biodegradation treatment.

Adaptation of Anaerobic Sludge

The anaerobic biodegradation of azo dye was performed by the use of adapted anaerobic sludge taken from a wastewater treatment plant of the sugar industry. The adaptation on complex structure of azo dye consisted in wastewater was performed in reaction bottles of 1000 ml where all fittings and closures were sealed with silicone in order to maintain strict anaerobic conditions.

The initial concentration of biomass used was 10 g/L, the temperature was 35^{o}C and pH 7.2-8.4. For the first step of adaptation phenol was used in gradually increasing concentrations from 100 mg/L to 300 mg/L and 500 mg/L. The second step of adaptation was performed under the same conditions, as described before, with gradually increased addition of the equal mixture of naphthalene, phenantrene and antrazene in concentrations of 100 mg/L, 300 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L. Each step of the whole adaptation process, which lasted four weeks, was controlled by the measurement of produced biogas in gradual cylinders until its volume became constant. The adapted anaerobic sludge was used for the subsequent anaerobic experiments in liquid medium.

Anaerobic Biodegradation Experiments

The anaerobic biodegradation of azo dye was performed in bottles of 500 mL where all fittings and closures were sealed with silicone in order to maintain strict anaerobic conditions. 200 mL of previously adapted anaerobic sludge in concentration of 7 g/L was used as inoculum and 150 mL of mother lye was added. The process was

performed at temperature of 35°C, pH 7.2-8.4, and biodegradation was controlled measuring the changes of: UV-VIS spectra, COD value, concentration of N-NH_4 and N-NO_3. The process was stopped at the time when the produced biogas, measured in gradual cylinders, reached its constant volume. Taking in account that the metabolic state of anaerobic microbial community, and addition of simple carbon source to the system are often the rate-limiting factors during anaerobic experiments Na-acetate in concentration of 100 mg/L was added as cosubstrate.

Analytical Methods

Wastewater parameters were analyzed by standard APHA methods [33].The test samples were taken at different time intervals and filtered through 0.45 μm membrane filters. The filtrates were scanned in the range of 190-400 nm and structural changes of azo dye and color intensity were determined on an UV-VIS spectrophotometer Lambda EZ 201 produced by Perkin Elmer. Concentrations of N-NH_4 and N-NO_3 were determined by spectrophotometer DR/2000 produced by Hach Lange GmbH. Microbial biomass was determined gravimetrically, by filtering the sample through 0.45 μm filter and drying it at 110°C.

Variation in chemical oxygen demand concentration during the experiments was determined by spectrophotometer DR/2000 produced by Hach Lange GmbH. Electrodes from WTW GmbH were used for monitoring of pH, temperature and concentration of dissolved oxygen. Bacterial strains were identified on the principles of Bergey's Manual of Systematic Bacteriology using commercially kits API50 and API20NE produced by BioMérieux Ltd. Yeast strains were identified on the basis of morphological growth observation, as there were used only three strains.

RESULTS AND DISCUSSION

Biodegradation of azo dyes occurs under anaerobic, anoxic and aerobic conditions by different groups of bacteria and yeasts, where location of the reactions can be either intracellular or extracellular [9,34].

It is to expect that further development of biotreatment processes will be facilitated by identifying the most effective microorganisms and ways to reduce time that is needed for treatment of contaminated wastewater effluents [8]. The acclimation and sufficient adaptation period is actually a result of the time needed for enzymes to be induced [31].

Most of the available studies are still at the laboratory scale and with synthetic wastewaters [9]. The possibility of aerobic decolorization and biodegradation of azo dye consisted in real industrial effluent followed by anaerobic step was investigated.

Microbial Growth on Plates

Azo dyes are recalcitrant to microbial degradation because they have complex aromatic molecular structure and the strong electron-withdrawing property of the azo groups is thought to protect against attack by oxygenases so that the conventional aerobic

wastewater treatment processes usually can not efficiently decolorize azo dye-contaminated effluents [11]. The first step in the bacterial degradation of azo dyes, in either anaerobic or aerobic conditions, is the reduction of -N=N- bond.

This reduction may involve different mechanisms, such as enzymes, low molecular weight redox mediators, chemical reduction by biogenic reductants like sulfide, or a combination of these, and the location of the reactions can be either intracellular or extracellular [9].

The switch on-off mechanism exists in microorganisms for azoreductaze and aromatic amines degradation [28]. During experiments performed on solid media the microorganisms able to grow on mother lye consisting the azo dye as a sole source of carbon were selected. It is important to note, that no decoloration zones were noticed on plates, which means that biomass grown on the media biodegraded azo dye intracellulary. The results indicate that concentration of 300 mg/L of azo dye as a sole carbon source was adequately chosen and sufficient to induce the enzymatic switch on-off mechanism and did not show the negative effect on microbial transport mechanism.

Aerobic Experiments in Liquid Medium

For aerobic microorganisms to be significant in the reductive process they must be specifically adapted. That adaptation involves long-term aerobic growth in the presence of compounds of gradually increasing chemical structure. In azo dye biodegradation, during adaptation period the microorganisms synthesize an azo reductaze specific for biodegradation of this compound which under controlled conditions can reductively cleave the azo bound in the presence of oxygen [14]. The achieved results of aerobic and microaerophilic tests of this research indicated that there was no significant difference between aerobic and microaerofilic conditions in structural changes of complex compounds of azo dye, which was the reason that for the subsequent experiments in presence of oxygene the microaerophilic conditions were chosen. The results of microaerophilic biotests confirmed that biodegradation of bordo azo dye can be achieved with mixed microbial culture previously adapted on azo dye as a sole source of carbon. During the process biodegradation was monitored by changes of UV-VIS spectra (Figure 2). The peak located between 300 and 350 nm moved to interval of 190-220 nm where belong molecules of lower aromatic structure. Production of free NH_4-N also confirmed that biodegradation occurred and was 13.5 mg/L at the end of process.

During microaerophilic biotests COD and color intensity were not significantly changed. As for the tests biomass concentration of 0.5 g/L was used, it could be expected that the use of potentially higher biomass concentration could contribute to more efficient reduction of the COD value. It is important to emphasize that particular biodegradation process under microaerophilic conditions in continuous aerobic-anaerobic treatment process can be used only as the first and pretreatment step and preparation for the most effective performance of subsequent anaerobic biodegradation.

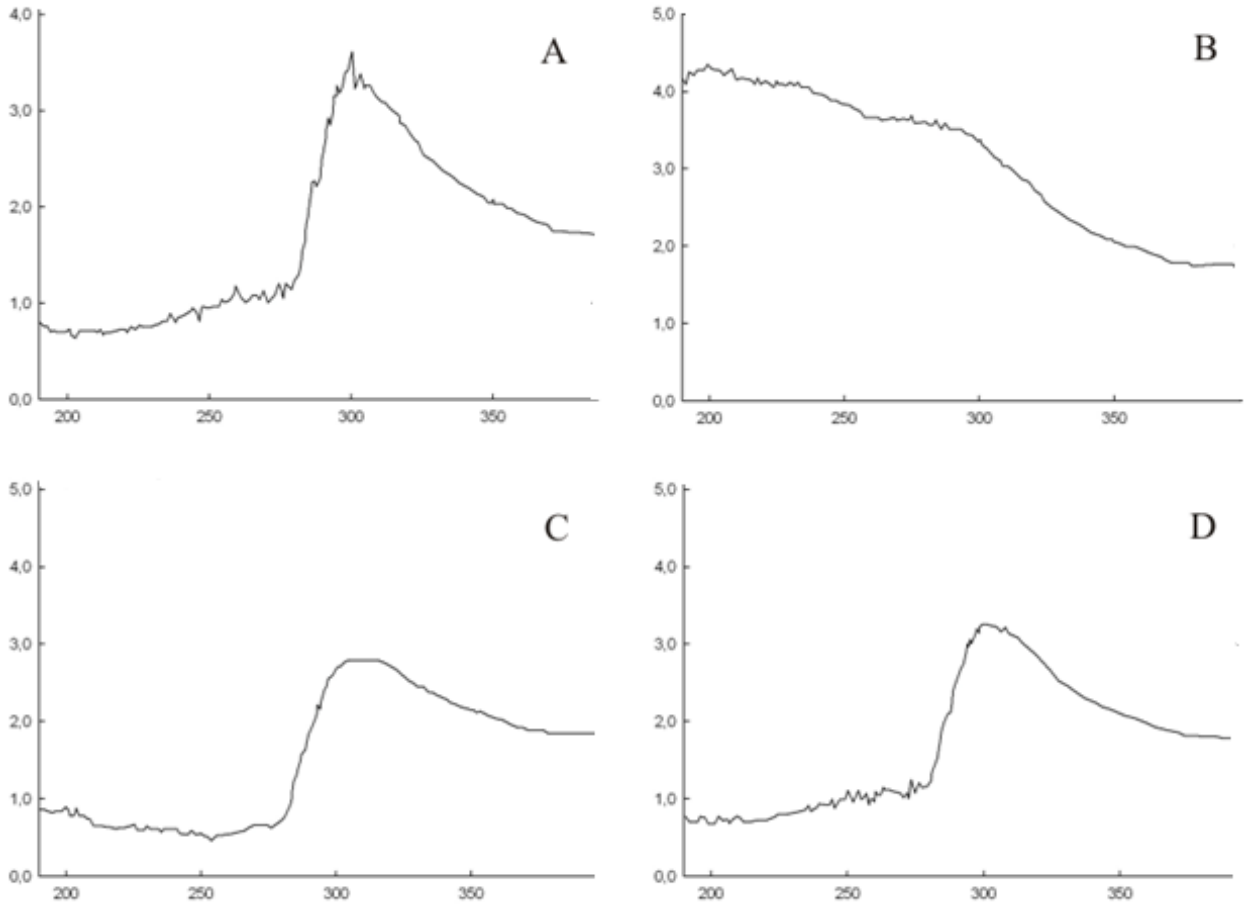

Figure 2. UV-VIS spectra of bordo azo dye biodegradation before treatment-A, after 3 days-B, after 5 days-C and after 7 days-D of treatment process with prepared mixed microbial culture under microaerophilic conditions

Slika 2. UV-VIS spektar biorazgradnje bordo azo boje prije obrade-A, nakon 3 dana-B, nakon 5 dana-C i nakon 7 dana-D obrade pomoću priređene mješovite mikrobne culture u mikroaero-filnim uvjetima

It is less possible that the decrease in biodegradation efficiency (Figures 2C, 2D) occurred because of toxic effect of the dye or because of blockage of active sites of azoreductaze enzymes by dye molecule. It is more likely that it happened by high reactivity of aromatic amines from azo dye reduction in the presence of oxygen. It is further presumed that the decrease in biodegradation efficiency should be possible to overcome within the further optimization of the all vital operational parameters of continuously performed process and surely significant shortness of the process duration – shorter than 3 days where, according to the experimental data, that problem does not exist.

A partial understanding of potential biodegradation pathway for azo dyes has been presumed on the basis UV-VIS spectra changes of microaerofilic experiments Figure 3.

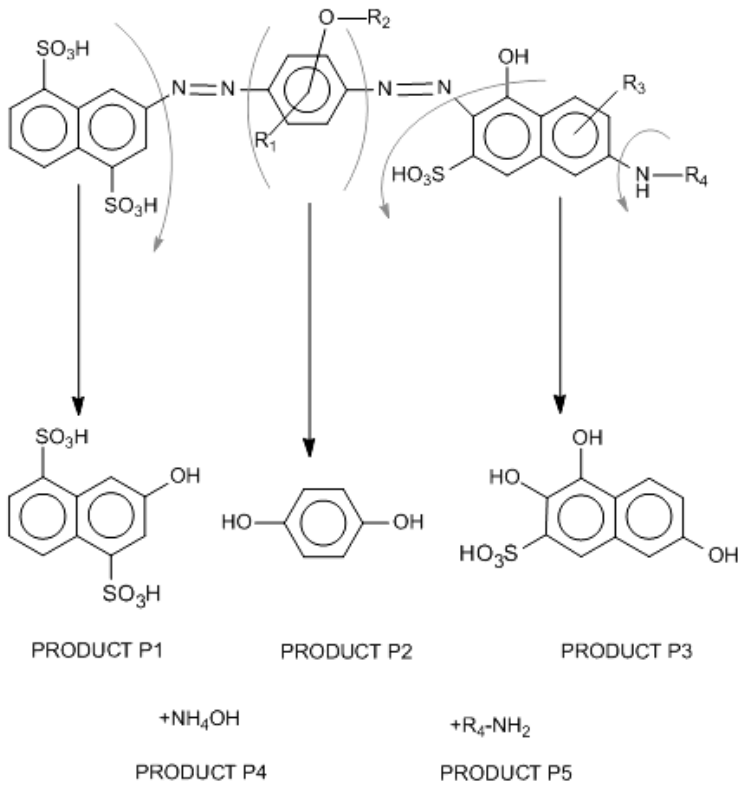

Figure 3. Tentative mechanism for aerobic biodegradation of bordo azo dye with prepared mixed microbial culture and metabolite formation
Slika 3. Prijedlog aerobnog metaboličkog puta biorazradnje bordo azo boje pomoću priređene mješovite mikrobne kulture i nastanak metabolita

Anaerobic Experiments in Liquid Medium

The long retention times often applied in the anaerobic phase of the biodegradation studies indicate that the reduction of many azo dyes is a relatively slow process [17]. The ability of the bacterial cells to reduce dyes must be tested to determine the type of wastewater that can be treated by the particular system. The effect of each of the vitally important factors on the color removal process must be investigated before the biological system can be used to treat industrial wastewater [14]. On the basis of results achieved in anaerobic biodegradation tests, gradually increasing the concentration of azo dye consisted in mother lye, it can be concluded that strong structural changes of azo dye can be achieved with adapted anaerobic sludge (Figure 4). The UV-VIS spectral changes exhibit a shift of the maximum of absorption towards shorter wavelengths upon anaerobic microbial treatment conditions. The color removal efficiency increased with time, indicating that the biomass of adapted anaerobic sludge was sufficiently mature to biodegrade and decolorize wastewater. This is in accordance with the previous findings that a microbial population in a dedicated

treatment system could become adapted to withstand and decolorize increasingly concentrated wastewaters [23].

The anaerobic biotests also showed that in prolonged time bordo azo dye tends to form structures of similar UV-VIS profile as were starting ones, although the fact that COD had been significantly reduced from starting 4623 mg/L to 2211 mg/L. It is important to note that although the complex structures have been detected over a longer period, the return of complex structures, can present further hydroxylation prior complete biodegradation.

That negative effect should also be possible to overcome within the further optimization of the all vital operational parameters during continuous process and surely significant shortness of the process duration.

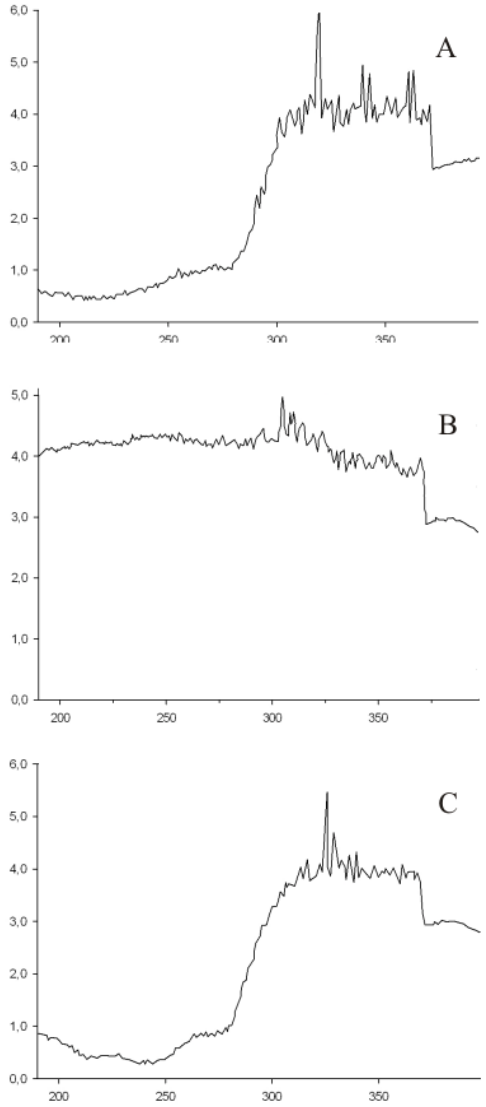

Figure 4. UV-VIS spectra of bordo azo dye biodegradation before treatment-A, after 7 days-B and after 11 days-C of treatment process with adapted sludge under anaerobic conditions
Slika 4. UV-VIS spektar biorazgradnje bordo azo boje prije obrade-A, nakon 7 dana-B i nakon 11 dana-C provedbe procesa pomoću prilagođenog aktivnog mulja u anaerobnim uvjetima

As a main goal of current environmental biotechnology is to establish highly efficient biological processes that use the naturally existing catabolic potential for detoxification of poorly degradable pollutants these results confirm that the use of microbial strains that are well adapted on chemical analogues can be very important toll to enhance bioremediation processes including biodegradation of complex xenobiotic compounds such as azo dyes. On the basis of aerobic and anaerobic experiments the most appropriate bioprocess parameters should be determined and chosen for further implementation of a continuous laboratory combined aerobic-anaerobic process for azo dye biodegradation present in mother lye. Further studies should also include precise analytical determination of presumed aerobic metabolite products.

CONCLUSION

The results show that adapted aerobic mixed microbial culture is capable for ozo dye bioconversion and adapted anaerobic sludge is capable of azo dye biodegradation. That property is attributed to its versatile enzymatic potential, which has been induced by induction of switch on-off mechanism trough adequately performed adaptation process.

Such cultures could be used for the development of a continuous aerobic-anaerobic treatment process, or for the bioaugmentation of exiting azo-dye-containing wastewater treatment units. Two phase aerobic-anaerobic system based on the use of previously well adapted microbial consortia should be useful for further biotechnological exploitation serving as an effective tool for biodegradation of azo dye, leaving much less possibility for persistence of aromatic amines in the effluent than in usually used anaerobic-aerobic systems.

The results of these experiments undoubtedly demonstrate the great microbial enzymatic potential and importance of adequately adapted microorganisms to facilitate biodegradation of recalcitrant xenobiotic compounds such as azo dye.

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