UDC 547.566.33:579.266
ISSN 0352-9193

original scientific paper

Removal of Azo Dye Reactive Red 120 by Mixed Bacterial Culture

Uklanjanje azo-boje »Reactive Red 120« mješovitom bakterijskom kulturom

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Received: May 4, 1994
Accepted: February 28, 1995

Summary

Azo dyes belong to the largest group of marketed colourants. Removal of dyes from effluents is the major environmental problem because certain aromatic compounds carrying HSO_3^- groups resist biodegradation or are incompletely degraded.

In the present study microbial removal of azo dye Reactive Red 120 from aqueous solution was investigated. Mixed bacterial culture previously aerobically grown in the presence of this dye was used in static culture experiments. It was found that some ecological factors affected dye decolourisation. The fastest dye removal was obtained at 28 °C, pH = 6.8 with 0.20 volume fraction of culture inoculum and by addition 0.1 volume fraction of nutrient broth.

Sažetak

Azo boje pripadaju najvećoj skupini bojila prisutnih na tržištu. Uklanjanje bojila iz efluenata velik je problem u zaštiti okoliša jer su određeni aromatski spojevi s HSO_3^- skupinama teško ili nepotpuno razgradljivi.

U radu je ispitana mogućnost uklanjanja azo-boje »Reactive Red 120« s pomoću mješovite bakterijske kulture, prethodno aerobno uzgajane na ovoj boji. Ustanovljeno je da neki ekološki čimbenici utječu na razgradnju bojila. Najbrže uklanjanje bojila postignuto je pri 28 °C, pH = 6,8 s 0,20 volumnog udjela bakterijskog inokuluma i uz dodatak 0,1 volumnog udjela hranjivog bujona.

Introduction

Sulphonated azo compounds are widely used as dyes for textiles, food, drugs and cosmetics *(1,2)*. Azo dyes are compounds containing azo groups (-N=N-) which are linked to sp^2-hybridized carbon atoms. The azo groups are mainly bound to benzene or naphthalene rings, but in some cases they are also attached to aromatic heterocycles. The diazotization of an aromatic or a heteroaromatic primary amine is the first of the two reaction steps by which practically all azo dyes are produced *(3)*.

It is estimated that about 15 % of total world production of colourants is lost in their synthesis and processing. The major environmental problem of colourants, therefore, is the removal of dyes from effluents. Biological treatment, chemical coagulation, adsorption, oxidation or isotope radiation have been applied for dyeing waste water treatment *(4–6)*.

Complete mineralization of azo dye would be an ideal demand for decolourisation of coloured effluents. Unfortunately, however, synthetic dyes are, with very few exceptions, xenobiotics. The natural systems of microorganisms in rivers and lakes do not contain enzymes which are designed by nature to degrade such compounds under aerobic conditions *(7)*. Under anaerobic conditions, such as in the digestion of sewage sludge, dyestuff degradation at least takes place slowly. Nevertheless, there is some evidence that after a long period of adaptation in a chemostat, it is possible to develop cultures of microorganisms which mineralize selected azo dyes *(8–15)*. The most probable mechanism is the reduction of the azo bonds as the first step of dye removal, i.e.:

$$R_1 - N = N - R_2 + 4e^- + 4H^+ \rightarrow R_1 - NH_2 + R_2 - NH_2$$

(R_1, R_2: variously substituted residues).

This primary degradation may either be caused by reduction systems in the cells of facultative or obligate anaerobic microorganisms or in higher organisms *(8)*. Then follows the cleavage of aromatic rings from the azo compound under aerobic or anaerobic conditions *(2)*.

In the present work, decolourisation of azo dye Reactive Red 120, containing azo groups bound to benzene and naphthalene rings, by adapted mixed bacterial culture, under the static culture conditions, was studied.

Materials and Methods

Media

Mineral medium. The medium containing mass concentration expressed in g/L of the following constituents: 2.5 $(NH_4)_2HPO_4$, 1.0 KH_2PO_4, 0.1 $MgSO_4 \times 7H_2O$ and 5.0 NaCl, was prepared and autoclaved at 121 °C. With the addition of the proper mass concentration of dye Reactive Red 120 this mineral medium was used for cultivation of mixed bacterial culture.

Enriched mineral medium. This medium was prepared by adding 0.1 volume fraction of previously dissolved and sterilised nutrient broth (8 g/L) in 1 L mineral medium and was used for the adaptation of mixed culture and in dye transformation assays. By addition of Agar Bios C (20 g/L, Biolife, Milano, Italy), such mineral medium was used for determination of cell counts and for the maintenance of adapted mixed bacterial culture.

Azo Dye

Water soluble diazo dye Reactive Red 120 (Fig. 1) synthesized by the chemical group of the Faculty of Chemical Engineering and Technology, Zagreb, was studied. Stock solutions of dye (γ = 10 g/L and 25 g/L) were prepared by dissolving in distilled water, and added to the mineral medium to give final mass concentrations of 0.01; 0.1; 1.0; 2.0 and 5.0 g/L.

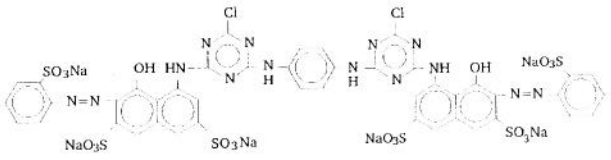

Fig. 1. Structure of azo dye Reactive Red 120
Slika 1. Struktura azo-boje »Reactive Red 120«

Microorganisms and Cultivation Conditions

Mixed culture able to transform Reactive Red 120 was isolated from multispecies bacterial community by mixing filtrate of garden soil, activated sludge (sewage treatment plant, Velika Gorica) and sludge from the leather tanning waste water (Almeria, Zagreb). The culture prepared in this way (20 mL) was transferred to Erlenmeyer flask containing 80 mL mineral medium with final mass concentration of dye γ = 100 mg/L. The culture was incubated at 28 °C and pH = 6.9 in a rotary shaker (200 rpm) during 28 days. Since within that period no significant changes in bacterial number were detected, in the second run the medium was supplemented with 0.25 volume fraction of separately prepared nutrient broth (8 g/L). This stimulated bacterial growth and after 28 h the number of cells increased from 2×10^8 to 7.5×10^9, but no dye decolourisation was observed.

In the next experiment, inoculated mineral medium with addition of the same amount of nutrient broth (φ = 0.25) and dye (γ = 100 mg/L) was transferred to sterilised serum bottles (100 mL) and sealed with rubber membrane caps. The bottles were placed in a thermostat at 28 °C without shaking. In this manner, dye decolourisation started after 24 hours lag-phase and was completed within 120 h. After three repeated cultivations under the same conditions decolourisation time shortened, so that the dye disappeared within 24 h.

Thus adapted mixed culture was streaked out on agar plates and after incubation (28 °C for 48 h), morphology of surface grown colonies was monitored and the cells stained by Gram. Mixed culture was maintained on agar slants containing mineral medium, nutrient broth (φ = 0.1) and dye (γ = 10 mg/L). Viable cell counts were estimated as colony forming units (CFU/mL) on enriched mineral medium agar after decimal dilutions and incubation at 28 °C for 2 days.

Dye Transformation Assays

Bacterial cells from agar slant were resuspended in mineral medium with dye γ = 100 mg/L and incubated in thermostat at 28 °C for 24 hours. Different volume fractions (φ = 0.1; 0.15; 0.20) of prepared inoculum were then transferred to glass tubes (20 mL) and azo dye (γ = 1.0; 2.0 and 5.0 g/L) and nutrient broth (φ = 0.05; 0.1; 0.15; 0.20) were added. The tubes were completely filled with mineral medium and sealed with rubber caps, and incubated in the thermostat as static culture at different temperatures (20 °C, 28 °C and 37 °C) as described by Haug and co-workers (*2*). Immediately after inoculation the concentration of dissolved oxygen in the culture medium was 5.5 mg/L (determined by oxygen meter YSI 54 A, USA). Tubes were sacrificed for every measurement, and only as many tubes were incubated as measurements were planned. Disappearance of azo dye during transformation experiments was monitored spectrophotometrically (spectrophotometer Cecil CE 2292, London, Great Britain) by following the decrease in absorbance at the wave length of the dye absorption maximum (λ = 566 nm). Culture samples were previously centrifugated at 18000 g for 10 min (Beckman J2-21 M/E). Data were converted to a concentration of azo dye (mg/L) using calibration curve. In all experiments triplicate runs were made.

In order to determine the optimal conditions for microbial decolourisation of the dye Reactive Red 120, the effects of temperature, pH-values, volume fractions of bacterial inoculum and nutrient broth on dye transformation were studied.

Results and Discussion

According to morphological characterization of the mixed culture grown on mineral medium with dye addition (Table 1) it was found that this culture contained 10 morphologically different species. Nine of the ten were Gram-negative bacteria and most of them irregularly-shaped coccobacillus or bacillus. Growing under the aerobic condition in mineral medium with the addition of dye Reactive Red 120 (shake culture) this mixed culture did not reveal the capability of dye decolourisation. Although the addition of nutrient broth stimulated bacterial growth, the dye was not removed during 28

days of incubation on a rotary shaker. However, growing in the same medium under the static culture conditions (in sealed tubes) this mixed culture readily decolourised the azo dye. Similar observation had been previously reported by Haug and co-workers, and Yatome and co-workers *(2, 9)*. A control experiment (without culture addition) showed that no decolourisation was observed, indicating that dye decolourisation was the result of microbial activity. Further experiments with single strains isolated from the mixed culture (data not presented) showed that growing under the static conditions none of the strains were able to decolourise the investigated dye separately.

Figures 2 and 3 show the effect of nutrient broth on the kinetics of dye removal at two dye concentrations ($\gamma = 0.1$ and 2.0 g/L). It was found that even 0.1 volume fraction of nutrient broth enhanced the dye decolourisation since approximately 90 % of initial dye concentration was removed in 12 (Fig. 2) and 48 hours (Fig. 3), respectively. For further tests 0.1 volume fraction of nutrient broth was added to mineral medium. The results obtained for a higher dye concentration ($\gamma = 5.0$ g/L) showed a similar pattern of dye decolourisation rate (data not shown). These results are in agreement with

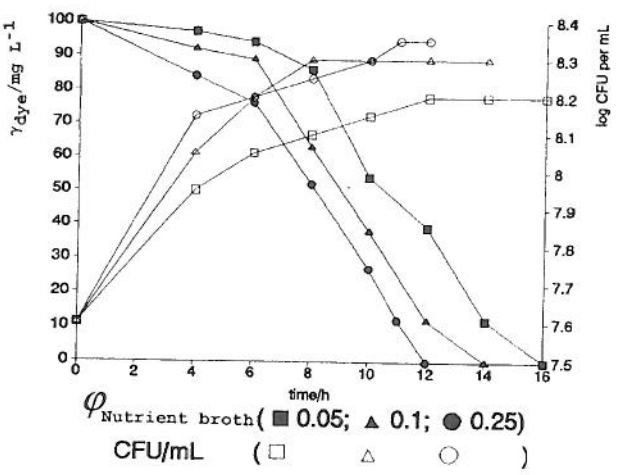

Fig. 2. Transformation of dye ($\gamma = 100$ mg/L) by mixed culture in medium supplemented with different volume fractions of nutrient broth ($\varphi_{inoculum} = 0.2$ at 28 °C and pH = 6.8)

Slika 2. Transformacija bojila ($\gamma = 100$ mg/L) s mješovitom kulturom u podlozi s različitim volumnim udjelima hranjivog bujona ($\varphi_{inokuluma} = 0{,}2$ pri 28 °C i pH = 6,8)

Table 1. Morphology of bacterial colonies present in mixed culture
Tablica 1. Morfologija bakterijskih kolonija prisutnih u mješovitoj kulturi

No. of strains Red.br. soja	Colony appearance Opis kolonije	Colony size / mm Veličina kolonije	Description of the cell morphology Morfološki opis stanice	Gram staining Bojenje po Gramu
1.	flat, circular, undulate, drab plosnata, okrugla, rubovi valoviti, sivosmeđa	7	rods štapići	G-
2.	flat, circular, irregular, rhizoid, flesh coloured plosnata, okrugla, rubovi nepravilni, rizoidna, boje puti	10–15	coccus koki	G-
3.	umbonate, circular, entire, white središte ispupčeno, okrugla, rubovi cjeloviti, bijela	2–4	coccus koki	G+
4.	umbonate, circular, entire, orange-coloured središte ispupčeno, okrugla, rubovi cjeloviti, narančasta	5	long rods dugi štapići	G-
5.	convex, circular, entire, yellow-green konveksna, okrugla, rubovi cjeloviti, žutozelena	4	short rods kratki štapići	G-
6.	umbonate, circular, entire, drab, yellow-green pigment in medium središte ispupčeno, okrugla, rubovi cjeloviti, sivosmeđa, stvara žutozeleni pigment	4	rods štapići	G-
7.	flat, circular, filamentous, flesh-coloured plosnata,okrugla,končasta, boje puti	6	long rods dugi štapići	G-
8.	umbonate, irregular, undulate, flesh coloured središte ispupčeno, nepravilna oblika, valovita, boje puti	6	coccus koki	G-
9.	flat, circular, dentate, cream coloured plosnata, okrugla, pilasta, blijedožute boje	2–4	rods štapići	G-
10.	raised, irregular, rhizoid, colourless ispupčena, nepravilna oblika, razgranata, prozračna	7	rods štapići	G-

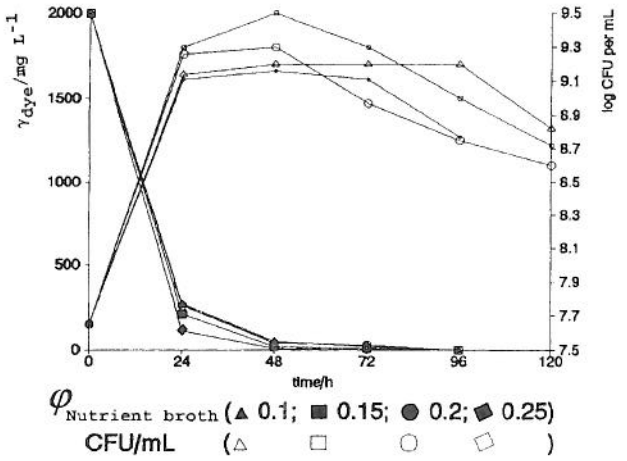

Fig. 3. Transformation of dye ($\gamma = 2000$ mg/L) by mixed culture in medium supplemented with different volume fractions of nutrient broth ($\varphi_{inoculum} = 0.2$ at 28 °C and pH = 6.8)

Slika 3. Transformacija boje ($\gamma = 2000$ mg/L) s mješovitom kulturom u podlozi s različitim volumnim udjelima hranjivog bujona ($\varphi_{inokuluma} = 0,2$ pri 28 °C i pH = 6,8)

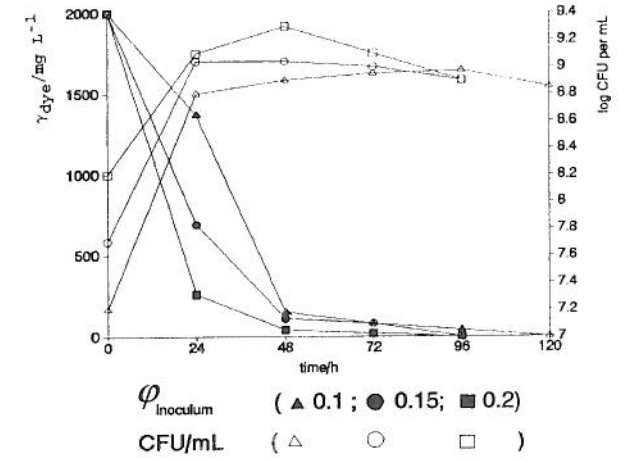

Fig. 4. Influence of different culture inoculum volume fractions on the dye ($\gamma = 2000$ mg/L) removal from medium ($\varphi_{nutrient\ broth} = 0.1$ at 28 °C and pH = 6.8)

Slika 4. Utjecaj različitih volumnih udjela inokuluma ($\gamma = 2000$ mg/L) na uklanjanje boje iz podloge ($\varphi_{bujona} = 0,1$ pri 28 °C i pH = 6,8)

the ones previously published *(2,8)* in which the rate of dye removal by mixed bacterial community was considerably enhanced in the presence of glucose and yeast extract.

A series of experiments conducted with the addition of different volume fractions of mixed culture inoculum showed that $\varphi = 0.2$ was optimal for the dye removal. During the first 24 hours exponential bacterial growth was observed and after that the cell counts were constant over 72 h. Since oxygen depletion in medium was observed after the first 4 hours of incubation and extensive decolourisation was demonstrated after 24 hours (Fig. 4), it seems justified to propose that the reduction of azo bonds of Reactive Red 120 was the result of the microbial activity of facultative anaerobes present in mixed culture.

As shown in Figures 5 and 6, the rate of the dye decolourisation was dependent on the temperature and pH of the medium used for its removal. Applying 2.0 g/L of azo dye in mineral medium the kinetics of decolourisation was not significantly different at 28 °C and 37 °C. However, at 20 °C it was evidently slower (Fig. 5). When lower dye concentrations were used (up to 1.0 g/L) no prolonged time for dye decolouristion was observed at 20 °C (data not shown). In practice, effluents from dye manufacturing plants containing higher dye concentrations (e.g. 7.0 g/L) would be firstly treated by a physico-chemical process such as flocculation *(16)*, and the remaining dye content (0.5-1.0 g/L) by microbial activity.

A significant difference in the kinetics of dye decolourisation could be noted when dye transformation was performed in the buffered mineral medium, pH = 5.4 to 9.2 (Fig. 6). It was evident that in the kinetics of dye decolourisation there was no significant difference in the range between pH = 6.8 and pH = 8.1. At pH = 9.2 dye removal was prolonged and lasted 120 hours and at pH = 5.4 dye removal was not observed at all during the 144 h (data not shown). It was also observed that at pH = 5.4 bacterial growth was poor, which was in agreement with the results reported by Ogawa and co-workers *(10)*.

In summary, according to the literature *(7)* the ability to degrade azo compounds aerobically has been restricted to a few bacterial strains which utilise certain carboxyl-substituted structures. On the other hand, it has been known that various biological systems possess the ability to cleave the azo bonds by reduction under anaerobic conditions *(17)*. Some intestinal bacteria *(Proteus vulgaris, Streptococcus faecalis)* were also found to reduce azo compounds *(18,19)* and utilise them as carbon source and energy. However, it has been also reported *(20,21)* that microorganisms can bring about chemical alterations of sulphonated and chlorinated aromatic or aliphatic compounds without deriving sufficient carbon and energy for growth from these reactions. This fortuitous process has been called co-metabolism or co-oxidation, and is thought to reflect a lack of substrate specificity of some of the microbial transport mechanisms and enzymes. Clearly, for co-metabolism to occur the bacteria must obtain the bulk or all of their carbon and energy from other substrates. In addition, some of the authors *(2,18)* studying the reduction of azo dyes by bacteria noticed the extreme lack of specificity of the azo reductase system.

Although the mechanism of sulphonated azo dye Reactive Red 120 transformation was not the subject of this paper, the results obtained indicate that adapted mixed bacterial culture was able to transform this complex synthetic dye under the static conditions. It appears that the addition of nutrient broth stimulated respiratory activity of cells, thus rapidly depleting the medium of oxygen and enabling decolourisation of azo dye. This also suggests that facultative anaerobes present in mixed culture were responsible for the azo dye transformation. Furthermore, a mutualistic interaction between the members of mixed culture was essential for dye decolourisation.

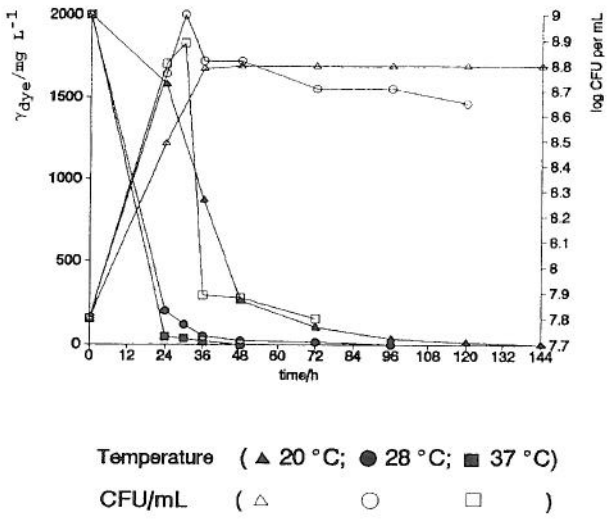

Fig. 5. Dye removal ($\gamma = 2000$ mg/L) from medium by mixed culture at different temperatures and pH = 6.8 ($\varphi_{\text{inoculum}} = 0.15$, $\varphi_{\text{nutrient broth}} = 0.1$)

Slika 5. Uklanjanje boje ($\gamma = 2000$ mg/L) iz podloge mješovitom kulturom pri različitim temperaturama uz pH = 6,8 ($\varphi_{\text{inokuluma}} = 0{,}15$, $\varphi_{\text{hranjivog bujona}} = 0{,}1$)

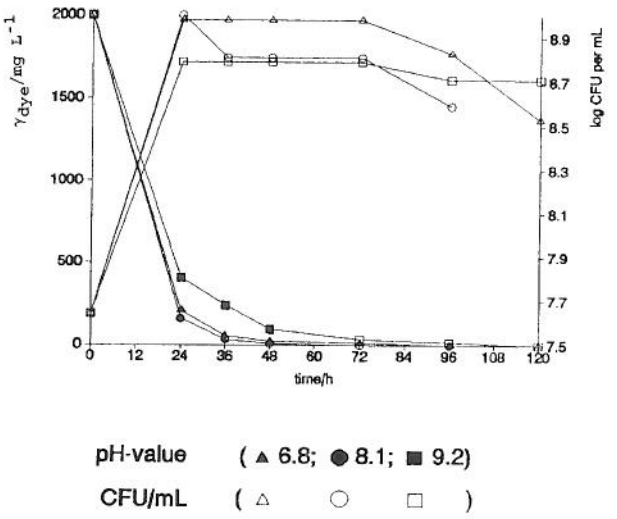

Fig. 6. Dye removal ($\gamma = 2000$ mg/L) from medium by mixed cul ture at different pH and 28 °C ($\varphi_{\text{inoculum}} = 0.15$, $\varphi_{\text{nutrient broth}} = 0.1$)

Slika 6. Uklanjanje boje ($\gamma = 2000$ mg/L) iz podloge mješovitom kulturom pri različitim pH-vrijednostima i 28 °C ($\varphi_{\text{inokuluma}} = 0{,}15$, $\varphi_{\text{hranjivog bujona}} = 0{,}1$)

It is tempting to speculate that relatively fast decolourisation (24–48 hours) of relatively high dye concentrations (up to 2.0 g/L) in comparison with the published data *(8,10)* was due to the mixed culture activity and azo dye structure. According to literature *(9,14)* chemical structure of azo dyes also affects the process of decolourisation. Thus, dye containing naphthalene rings is more rapidly decolourised than dye containing only benzene rings.

Acknowledgment

The work has been supported by the Ministry of Science and Technology, Republic of Croatia (Project No. 2--14-037).

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