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Decolorization of Azo Dyes by *Aspergillus sojae* B-10

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Biodegradation of azo dyes such as Amaranth, Sudan III and Congo-Red by *Aspergillus sojae* B-10 was demonstrated using *Aspergillus sojae* B-10. *Aspergillus sojae* B-10 showed the greatest decolorization ability when it was cultivated in a nitrogen-limited medium containing, azo dyes(10 mg/l), 2.0% glucose, 0.06% sodium nitrate, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$ at pH 5.0 and 37°C for 5 days. Under optimal conditions, Amaranth started being decolorized within 24 hr and was almost complete after decolorization of 4 days incubation. Sudan III was completely decolorized after a cultivation of 5 days. However, Congo-Red was not completely decolorized until 5 days of cultivation.

Dyes are released into the environment in industrial wastewater from two major sources, mainly the textile and the dyestuff industries (17, 10). Compared to other common industrial chemicals, the amount of dyes entering the environment can be toxic or carcinogenic substances (1, 9). In addition, dyes are considered to be a pollution problem because of their potential to cause environmental color change. Among the dyes causing pollution problems, azo dyes are the largest class of dyes with the greatest variety of color (2, 8).

A necessary criterion for the use of azo dyes is that they must be highly stable in light and during washing. They must also be resistant to microbial attacks (10). Therefore, they are not readily degradable and are typically not removed from water by the conventional wastewater treatment system.

Although a certain proportion of azo dyes is eliminated from waste streams by adsorption to sludge, they are not typically degraded under aerobic (8, 11, 13) or anaerobic conditions. Azo dyes are generally considered to be nondegradable under aerobic conditions. Specially *Basidomycetes* sp. can be expected to be effective in degrading these dyes (3, 4, 6, 12, 15).

Recent results in a number of investigations have shown that the ability to degrade such a large array of chemical compounds as the azo dyes is due to the lignin degrading system of *Basidomycetes* sp., which occurs in response to nutrient limiting conditions (5, 7).

In part, the lignin-degrading system consists of a number of peroxidases that are excreted by the fungus under these conditions of nutrient limitation (5, 7). These peroxidases are commonly called as ligninases (18) or lignin peroxidases (19) which have the ability to catalyze the depolymerization of lignin as well as the initial oxidation of a wide variety of other compounds.

The potential biodegradation of azo dyes by some fungal strains, however, demonstrates that it is possible to use carefully adjusted conditions in the culture for the selection of bacteria that decolorize azo dyes. This study investigated the biodegradation of a set of azo dyes through direct involvement of some *fungi*.

MATERIALS AND METHODS

Microorganisms and Medium

Strains of *Aspergillus* used are listed in Table 1. *Aspergillus sojae* B-10 and *Aspergillus sojae* 4253 were obtained from OK Bok Food Co. in Pusan. Korea, *Aspergillus awamorii* B-5 and *Aspergillus inui* A-13 were available in our laboratory. They were stored on an agar slant medium at 10℃. Before the experiment, spores were precultured on an agar slant medium in a test tube at 37℃ for 7 to 10 days.

The liquid medium used for this experiment contained 20 g of glucose, 2 g of $NaNO_3$, 1 g of KH_2PO_4 and 5 g of $MgSO_4 \cdot 7H_2O$ in 1*l* of deionized water(pH 5.0).

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Decolorization of Azo Dyes

500 *ml* Erlenmyer flasks were used to decolorize the azo dyes such as Amaranth, Sudan III and Congo-Red. 100 mg of Amaranth and Congo-Red were dissolved in 1 *ml* and prepared to give a final concentration of 10 mg/*l* of each dye. Sudan III was dissolved as 1 g in 100 *ml* of ethyl alcohol and was prepared to give a final concentration of 10 mg/*l*.

The spore suspension was prepared by adding a 10 *ml* of the liquid medium to the agar slant in a test and shaking it for 30 sec. The cultivation was started by inoculating a spore suspension into a 500 *ml* Erlenmyer flask with spore suspension. The resulting initial spore concentration in the medium was 1×10^9 spores/*l*.

500 *ml* Erlenmyer flasks containing 100 *ml* of each dye medium were shaken at 220 rpm on a rotary shaker with an eccentricity of 2.0 cm for 5 days. The fermentation temperature was 36℃. A 3 *l* jar fermentor (Marubishi Co.) was used in this experiment. The aeration rate was controlled at 1.0 vvm. 2% of the inoculum, which was grown for 42 hr at 36℃ in a 500 *ml* Erlenmyer flask (the seed medium was 100 *ml*), was used for fermentation in a jar fermentor.

Assay of Decolorized Dyes

Aspergillus sojae B-10 was allowed to grow for 6 days as described under the experimental conditions. On the day 6, one of the dyes was added to each culture at concentration of 10 mg/*l*. Dye decolorization was determined spectrophotometically by monitoring the absorbance for 5 days.

After incubation with *Aspergillus sojae* B-10, some of the dye was absorbed by fungal mycelia. In an attempt to solubilize any absorbed dye, the mycelial mat was homogenized in 10 *ml* of methanol in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 5,000×g for 10 min, and the mycelial pellet was suspended in an additional 5 *ml* of methanol, then recentrifuged. The two resulting supernatants were combined.

The absorbance of the supernatant was then determined. The wavelengths, in nanometers, used for the decolorization and absorbance ratio of Amaranth, Sudan III and Congo-Red were A_{520}/A_{332}. A_{500}/A_{351} and A_{496}/A_{351}, respectively. Decolorization(%) was quantified by measuring optical density(OD) at the characteristic wavelength of each dye.

RESULTS AND DISCUSSION

Spectrum of Three Azo Dyes

Fungal degradation, as well as fungal transformation, reduced the intensity of azo dyes in solution, whereby

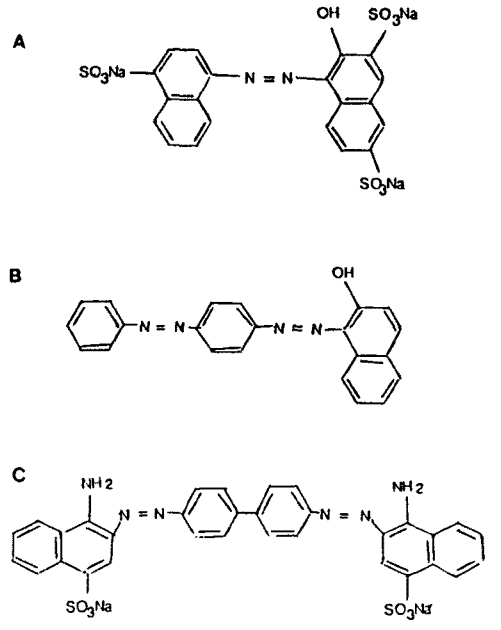

Fig. 1. Chemical structures of azo dyes.
Amaranth(A), Sudan III(B) and Congo-Red(C).

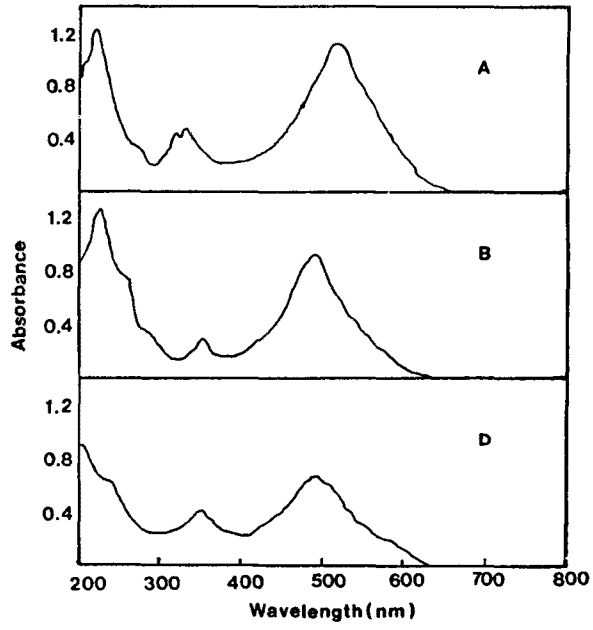

Fig. 2. Spectra of azo dyes.
Amaranth(A), Sudan III(B) and Congo-Red(C). Spectra of the dyes in basal media, pH 5.0 were measured with a spectrophotometer (Shimadzu UV-160A).

it was necessary to measure the soluble dye absorbance at various wavelengths. The chemical structure and visible spectrum of each of the three azo dyes used in this study are shown in Figs. 1 and 2, respectively. The absorbance measured at two wavelengths for dye decolorization decreased significantly, whereas the absorbance ratios (A/A) remained constant. During dye decolorizations, the pH was changed to be 4.3± 0.1 in nitrogen limited cultures. To ensure that decolorization was not simply a result of pH change, the effect of pH on the visible absorption was assayed between pH 3.5 and 5.0. The spectra of Amaranth and Sadan III were unaffected in this pH range. Decolorization of Congo-Red in culture was monitored at wavelengths of $A_{496/351}$ as a matter of convenience, since only a small change in culture pH was observed in the experiment. A small increase of only 0.4 pH unit was observed when dyes were incubated in nitrogen limited cultures.

Therefore, the conclusion was made that decolorization was not be due to pH change.

Screening of Fungi for Decolorization of Azo Dyes

Some fungi have been reported to decolorize melanoidine (14, 16, 20), azo dyes (5, 12) and heterocyclic dyes (12). This experiment was carried out to investigate the ability of the tested fungi for biodegrdation of specific set of target dyes through direct involvement of the fungi.

Azo dye decolorization by all 7 strains was investigated on a basal medium containing 2.0% glucose, 0.5% sodium nitrate, 0.2% potasium diphosphate and 0.1% magnesium sulfate. Samples were taken for 10 days. The level of decolorization of Amaranth, Sudan III and Congo-Red was observed between 5 and 10 days after inoculation. The highest levels of azo dyes decolorization are shown in Table 1.

Table 1. Decolorization of azo dyes by fungi grown on basal media at 37°C for 7 to 10 days.

Fungal strains	Decolorization yield(%)		
	Amaranth	Sudan III	Congo-Red
Aspergillus oryzae IFO 4758	42	40	41
Aspergillus oryzae IFO 6623	58	56	56
Aspergillus Sojae B-10	86	83	82
Aspergillus sojae 4233	40	41	62
Aspergillus awamori B-5	63	65	40
Aspergillus inui A-13	12	13	10
Phanerochaete chrysosporium ATCC 4725	32	30	27
Phanerochaete chrysosporium ATCC 345441	28	23	25

Aspergillus sojae B-10 produced the most mycelia than other tested strains and decolorization of Amaranth, Sudan III and Congo-Red was 86%, 83% and 82%, respectively. Thus, *Aspergillus sojae* B-10 was selected to decolorized for further investigation.

Effect of Carbon Sources

Various sugars and sugar alcohols were examined to determine the most suitable carbon sources for decolorization of three azo dyes. To investigate these, defined media were prepared replacing glucose with a variety of carbon sources. Table 2 shows the results of dyes decolored with various carbon sources. Of the carbon sources tested, 2% maltose, xylose, arabinose, sorbitol, and mannitol supported little or poorly decolorization. Glucose supported the highest levels of azo dyes decolorization in the defined media. Decolorization of Amaranth, Sudan III and Congo-Red was 94.3%, 90.8%, and 91%, respectively. Glucose was found to be the most suitable substrate for dye decolorization. To confirm the effect of glucose concentration on azo dye decolorization, *Aspergillus sojae* B-10 was grown in different media ranging from 1.0% to 4.0% glucose (Table 3). The highest level of decolorization of azo dyes was observed

Table 2. Effect of various carbon sources on the decolorization of azo dyes by of *Aspergillus sojae* B-10.

Carbon sources	Decolorization yield(%)		
	Amaranth	Sudan III	Congo-Red
Glucose	94.3	91.8	91.0
Fructose	86.4	84.0	83.0
Galactose	82.3	83.7	80.0
Maltose	70.3	70.1	67.0
Sucrose	71.4	70.8	78.1
Xylose	51.1	50.4	50.3
Arabinose	54.0	50.0	50.4
Sorbitol	50.3	50.6	48.1
Mannitol	50.1	48.7	47.7

*2% of each carbon source was contained in the basal medium.

Table 3. Effect of glucose concentration on azo dye decolorization by *Aspergillus sojae* B-10.

Glucose(%)	Decolorization yield(%)		
	Amaranth	Sudan III	Congo-Red
1.0	90.8	90.2	88.6
2.0	92.0	92.6	91.4
3.0	88.7	86.2	85.8
4.0	77.3	78.1	78.0

in the medium containing 2.0% glucose, but little differences in the level of decolorization were observed in media containing 1.0%, 3.0% and 4.0% glucose.

Effect of Nitrogen Sources

Various organic or inorganic nitrogen sources, were investigated to determine the most suitable nitrogen source for decolorization. Table 4 shows the effect of various nitrogen sources on decolorization by *Aspergillus sojae* B-10. Organic nitrogen sources were not found to be effective for decolorization and only sodium nitrate among nitrogen sources can decolorize markedly azo dyes than others. *Aspergillus sojae* B-10 enhanced azo dyes decolorizing ability in the presence of an additional nitrogen source, most noticeably sodim nitrate (Table 5). Generally, decolorization of azo dyes was higher when the nitrogen sources were present in addition to sodium nitrate and ammonium tartarate (5, 7). When sodium nitrate was only added to the basal medium without other nitrogen sources, decolorization of dyes occurred after 24 hr. Over 85 to 95% of the initial color of all the azo dyes disappeared within 4 days in the liquid medium containing inorganic nitrogen. To confirm these results, *Aspergillus sojae* B-10 was grown in a basal medium containing the concentrations of 0.02%, 0.04%, 0.06%, 0.08%, 0.2%, 0.4%, 0.6% and 0.8% sodium nitrate. The results shown in Table 5 demonstrate that a nitrogen limited culture grown on 0.06% sodium nitrate was most active for decolorization. The rate of decolorization by nitrogen sufficient culture was slower than that by nitrogen limited culture grown on 0.8% sodium nitrate.

Complete loss of color in culture fluids did not occur for any dyes even after 6 days of incubation while 95% of colorless dye in the culture broth under nitrogen limited conditions was lost within 4 days (4, 5). Decolorization experiments were carried out in culture medium containing different concentration of nitrogen sources, 0.04% to 0.8%. It was observed that 0.06% nitrogen was optimal for decolorization of azo dyes. Some of the dyes appeared to be absorbed to the mycelium, while almost of the dye decolorization from the nitrogen limited cultures occurred within 4 days.

Azo dyes could not be completely extracted from the fungal mycelia in nitrogen limited cultures, even after repeated treatment with solvents. At the end of these extensive extractions, the mycelia remained light brown or pink in color after all extraction attempts.

These phenomena were imagined that *Aspergillus sojae* B-10 released a small amount of enzymes for decolorization in case of cultivation of nitrogen limited culture condition. Bumpus and Brock (4) and Cripps *et al.* (5) reported that crystal violet was decolorized by lignolytic enzymes released from *Phanerochaete chrysosporium* which was incubated under nitrogen limited condition.

Time Courses of the Typical Decolorization by *Aspergillus sojae* B-10.

A typical time course for azo dyes decolorization was shown under optimal culture conditions as established by experiment.

In these experiments, 800 m*l* of culture medium in a 3 *l* fermentor was used for decolorization of three

Table 4. Effect of nitrogen-sources on decolorization of azo dye by *Aspergillus sojae* B-10.

Nitrogen sources	Decolorization(%)		
	Amaranth	Sudan III	Congo-Red
Peptone	48.5	43.6	48.5
Malt extract	46.2	44.0	40.3
Yeast extract	45.0	43.0	39.1
Ammonium tartarate	88.0	87.3	85.0
Sodium nitrate	92.3	92.6	90.2
Ammonium nitrate	86.3	86.4	82.0
Ammonium sulfate	74.0	73.1	70.1

Table 5. Effect of various sodium nitrate concentration on azo dye decolorization by *Aspergillus sojae* B-10.

Sodium nitrite(%)	Decolorization(%)		
	Amaranth	Sudan III	Congo-Red
0.02	92.3	92.5	90.0
0.04	94.0	93.7	90.5
0.06	97.2	97.0	93.0
0.08	94.6	93.1	90.3
0.1	90.1	89.8	86.2
0.2	86.3	85.0	80.7
0.4	82.0	80.4	80.1
0.6	76.3	73.3	72.8
0.8	68,4	66.9	67.0

Table 6. Time course for azo dye decolorization by cultures of *Aspergillus sojae* B-10.

Time course(days)	Decolorization(%)		
	Amaranth	Sudan III	Congo-Red
1	20.2	18.6	18.4
2	38.6	36.3	36.0
3	74.9	72.0	71.4
4	92.0	90.4	88.2
5	97.8	97.4	93.0

azo dyes. Three optimum conditions for decolorization by *Aspergillus sojae* B-10 were observed at pH 5.0, 37℃ and 5 days of incubation in a culture medium containing 2.0% glucouse, 0.06% sodium nitrate, 0.1% potasium pyrophosphate and 0.5% magnesium sulfate. Loss of colors of three azo dyes occurred within 24 hr after incubation. Amaranth was decolorized easier than Sudan III and Congo-Red. Decolorization of Amaranth began within 24 hr and was almost completely decolorized within incubation of 4 days (Table 6). Sudan III was completely decolorized after 5 days cultivation. Nevertheless, Congo-Red was not completely decolorized until 5 days of cultivation, but it was completely decolorized after 8 days of cultivation (data not shown). This experiment indicated that degradation and adsorption fo dyes on fungal mycelia were the important mechanism for the removal of azo dyes from a cultivation of *Aspergillus sojae* B-10.

Further research on the decolorization of azo dyes is required to take practical advantage of treatment of azo dye wastewater by using fungal mycelia.

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