

***Pseudomonas fluorescens* can be used for bioremediation of textile effluent Direct Orange-102**

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Abstract: Dyes present in the effluent of textile industries are recalcitrant molecules difficult to be degraded biologically. In this study we used *Pseudomonas fluorescens* to degrade Direct Orange - 102 dye. *Pseudomonas fluorescens* NCIM 2100 was obtained from National Chemical Laboratory, Pune, India, that was adapted to grow on Direct Orange - 102. The dye was subjected to degradation by the bacterium and its metabolic products were identified by UV, ¹HNMR and IR spectrophotometry. The dye was first broken down into 3, 7- diamino- 4 hydroxy - naphthalene - 2 sulfonic acid sodium salt. This compound is further degraded into 7-amino -3, 4- dihydroxy - naphthalene- 2- sulfonic acid sodium salt or 3- amino-4-7- dihydroxy-naphthalene -2 sulfonic acid sodim salt or 1,3,4,5,6,7,8 - heptahydroxy naphthalene - 2 - sulfonic acid sodium salt. These breakdown compounds were non - toxic in nature. Therefore, *Pseudomonas fluorescens* can be used for bioremediation of textile effluent containing Direct Orange-102 dye.

Resumen: Los colorantes presentes en las aguas residuales de la industria textil son moléculas recalcitrantes difíciles de degradar biológicamente. En este estudio usamos *Pseudomonas fluorescens* para degradar el colorante Anaranjado Directo - 102. Se obtuvieron *Pseudomonas fluorescens* NCIM 2100 del Laboratorio Químico Nacional, Pune, India, que estaban adaptadas al crecimiento en Anaranjado Directo 102. El colorante fue sometido a degradación por la bacteria y sus productos metabólicos fueron identificados por espectrofotometría UV, ¹HNMR e IR. Primero el colorante se degradó en sal sódica de ácido 3,7 - diamino - 4 - hidroxil - naftaleno - 2 - sulfónico. Este compuesto se convierte a su vez en sal sódica de ácido 7 - amino - 3, 4 - dihidroxil - naftaleno - 2 - sulfónico, o sal sódica de ácido 3-amino - 4,7 - dihidroxil - naftaleno - 2 - sulfónico, o sal sódica de ácido 1,3,4,5,6,7,8 - heptahidroxil - naftaleno - 2 - sulfónico. Estos compuestos resultantes de la degradación no fueron tóxicos en la naturaleza. Por lo tanto, se puede usar *Pseudomonas fluorescens* para la biorremediación de las aguas residuales textiles que contengan colorante Anaranjado Directo 102.

Resumo: Os corantes presentes no efluente das indústrias têxteis são de moléculas recalcitrantes difíceis de degradar biologicamente. Neste estudo usou-se a *Pseudomonas fluorescens* para degradar o corante "Direct Orange - 102". A *Pseudomonas fluorescens* NCIM 2100 foi obtida do Laboratório Químico Nacional, Prune, Índia. O corante foi sujeito a degradação pela bactéria e os seus produtos de metabólise forma identificados com recurso a UV, ¹HNMR e Espectrografia de IR. O corante foi primeiramente cindido no sal sódico do ácido 3,7-Diamino-4-hidroxilo-naftaleno-2-sulfónico. Este composto é seguidamente decomposto no sal sódico do ácido 7-amino-4,7-dihidroxil-naftaleno-2-sulfónico ou 3-Amino-4,7-dihidroxil-naftaleno-2-sulfónico ou no sal sódico do ácido 1,3,4,5,6,7,8-Heptahidroxil-naftaleno-2-sulfónico. Estes compostos da cisão não eram tóxicos na natureza. Assim, a *Pseudomonas fluorescens* pode ser usada na bioremediação dos efluentes têxteis contendo "Direct Orange - 102".

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Key words: Bioremediation, Direct Orange - 102, metabolic products, *Pseudomonas fluorescens*, spectrophotometry, textile effluent.

Introduction

Today we are facing a major environmental problem in the form of increasing pollution. Production of textile involves bleaching, mercerizing, carbonizing and dyeing, etc. Polyvinyl, alcohol, gums, PCP, cellulose materials, dyes and other substances are present in textile effluent. Azo and reactive dyes constitute the largest class of dyes used commercially in textile industries for dyeing nylon, polyacrylonitrile modified nylon, wool, silk, cotton, etc. Dyes are recalcitrant molecules difficult to be degraded biologically. Heavy metals as cadmium, chromium, copper, lead, zinc are present in the dyes. Black *et al.* (1980) have demonstrated that aniline dyes could be mutagenic and carcinogenic to biota. These chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom-feeding species of fish (Diachenko 1979). A large number of methods are available to treat the waste-water containing dyes (Senior *et al.* 1976). Presently, most of the processes used for the treatment of dye effluents are chemical processes, which are costly and produce large amount of sludge and are less efficient. Now a days, biological processes are getting more and more attention since they are cost effective, eco-friendly and do not produce large quantities of sludge.

The understanding on microbial degradation and decolorization of a dye is limited. However, results indicate that maximum dyes adopt reductive process of degradation. Various bacteria, fungi, actinomycetes, algae have been reported to degrade different pollutants (Leahy & Colwell 1990; Spain 1995). *Pseudomonas* sp., *Alcaligenes* sp., *Acinetobacter* sp. are a few important bacteria useful in bioremediation of halogenated aromatic compounds (Chaudhary & Chapalamadugu 1991). Biodegradation of nitro aromatic compounds is reported to be possible by *Desulfovibrio* (Preuss & Reieger 1995). *Phanerochaete chrysosporium*, a white rot fungus, is reported for degradation of a number of xenobiotics (Bumpus 1995; Bumpus & Aust 1987; Cripps *et al.* 1990). Some anaerobic bacteria can also bio-degrade dye stuff (Idaka *et al.* 1987; Nigam *et al.* 1996). Bacterial strains have been reported to have initial degradative dehalogenation steps yielding hydroxy benzoic acid, which is subsequently metabolized through meta-ring cleavage (Ko-

bayashi *et al.* 1997; Mark *et al.* 1984). The mechanism of degradation is associated with the presence of NAD (P) which acts as electron donor. Basidiomycetous fungi, *Ganoderma lucidum* and *Phanerochaete chrysosporium* have been found very effective in bioremediation of the textile effluents (Upadhyay 2000). A few reports are available on the biodegradation products or intermediates of different dyes. Yatome *et al.* (1991) reported the degradation of Crystal Violet by *Bacillus subtilis* IFO 13719. Besides Crystal Violet, two other triphenylmethane dyes (Prarosiline & Victoria Blue) were degraded by growing cells of *Bacillus subtilis* IFO 13719. Biodegradation of Acid yellow-9 was recently reported by the authors (Pandey & Upadhyay 2006).

Materials and methods

Culture

The culture of *Pseudomonas fluorescens* NCIM 2100 was obtained from National Chemical Laboratory, Pune, India, as a pure culture.

Culture medium

Pseudomonas fluorescens was grown in King's B medium as described by Chobchuenchom *et al.* (1996). pH of the medium was adjusted to 7.2 with phosphate buffer before sterilization.

Experimental procedure

The experiments were performed using 250 ml flasks as described by Kar *et al.* (1997) in a temperature controlled incubator shaker. The experiments were done in triplicate and the flasks contained 50 ml King's B medium supplemented with the dye 200 mg l⁻¹. Cell suspension of the bacterium containing 10⁶ cell ml⁻¹ was used as inoculum. Temperature of the culture was maintained at 37 °C. The culture was incubated for 4 days on a rotatory shaker at a speed of 200 rev/min. The sealed flasks were oxygenated by pure oxygen for 3 minutes every day.

Analytical methods

Samples were removed after 4 days and were

centrifuged by high speed cooling centrifuge (Remi C-24) at 10000 rev/min for 5 minutes at 4 °C temperature. The bioremediation experiments with cell-free extract before and after degradation of the dye by *Pseudomonas fluorescens* were performed spectrophotometrically.

UV spectrophotometry

The UV and visible spectra of the samples were measured in ethanol with a Perkin Elmer double beam UV-Vis Spectrophotometer 117. Quartz cells (1 cm square) having 1.0 cm path length were used for the determination. Hydrogen discharge tungsten filament lamp was used as a source of light and maximum absorbance was recorded.

¹HNMR spectroscopy

The samples were subjected to ¹HNMR spectroscopy. Proton NMR spectra of the samples were run at Bruker AC 200 M Hz in D₂O using TMS as an interval standard 0.5 ml aliquot of the samples and 0.5 ml of solvent were taken in 5 ml O.D glass tube for the determination.

IR spectroscopy

The IR spectra of samples were obtained by placing solid compound in Nujol between salt plates (NaCl, 0.1 mm cell), without a spacer, in one of the beams of a double beam of a Perkin Elmer-783 Spectrophotometer Nernst glower, molded rod containing a mixture of zirconium oxide, yttrium oxide and erbium oxide that was heated to around 1500 °C by electrical means, which was used as a light sources of IR irradiation. Grating was used to obtain monochromatic light. The stretching and bending position of bands were observed and compared with reference compounds. Band position was presented in wave number unit (cm⁻¹ reciprocal centimeters). Band intensities were expressed as transmittance (T). The functional groups present were counted according to band positions (Dyer 1991).

Results

UV Spectroscopy of Direct Orange - 102 before degradation showed maximum absorbance at 246.9 and 275.1 nm (Fig. 1). After degradation the maximum absorbance were obtained at 247 nm and 276.7 nm. The peaks were completely from Direct Orange - 102 proving that Direct Orange - 102 changed to other compound (Fig. 2).

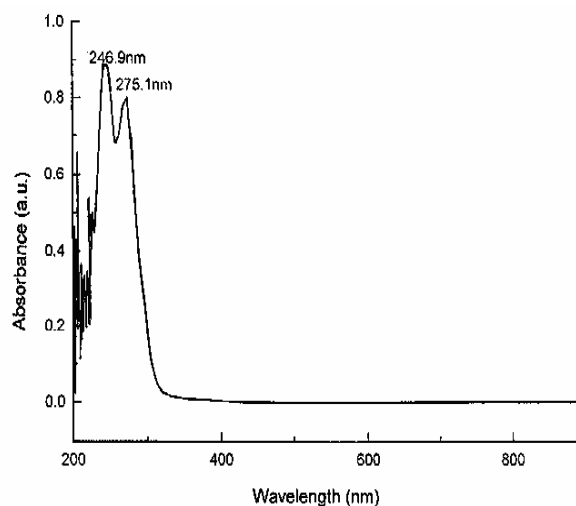


Fig. 1. UV spectrum of Direct Orange - 102 before degradation by *Pseudomonas fluorescens*.

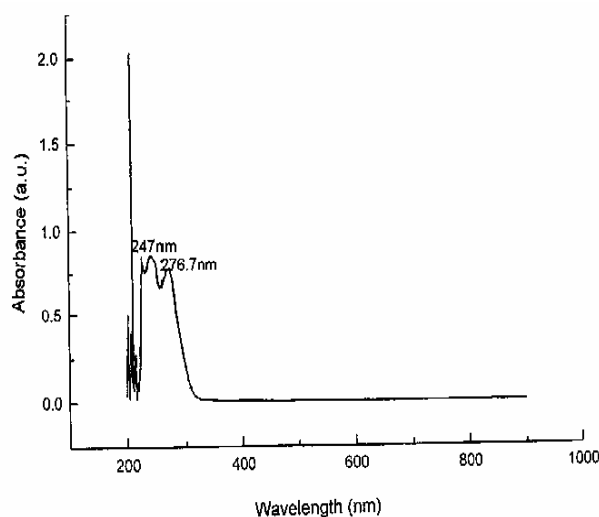


Fig. 2. UV spectrum of Direct Orange - 102 after degradation by *Pseudomonas fluorescens*.

¹HNMR spectrum of Direct Orange - 102 showed two multiplets at 4.72-4.88 ppm and 3.61-3.67 ppm and a singlet at 1.80 ppm (Fig. 3). After degradation the compound showed two singlets, one at 4.8 ppm and another at 3.64 ppm. Hence, it is concluded that new compounds might have been formed (Fig. 4).

Infra Red spectrum of Direct Orange - 102 showed a medium peak at 1100 cm⁻¹, a weak peak at 1450 cm⁻¹, a medium peak at 1575 cm⁻¹, a weak peak at 1750 cm⁻¹, and a broad peak at 3350 cm⁻¹

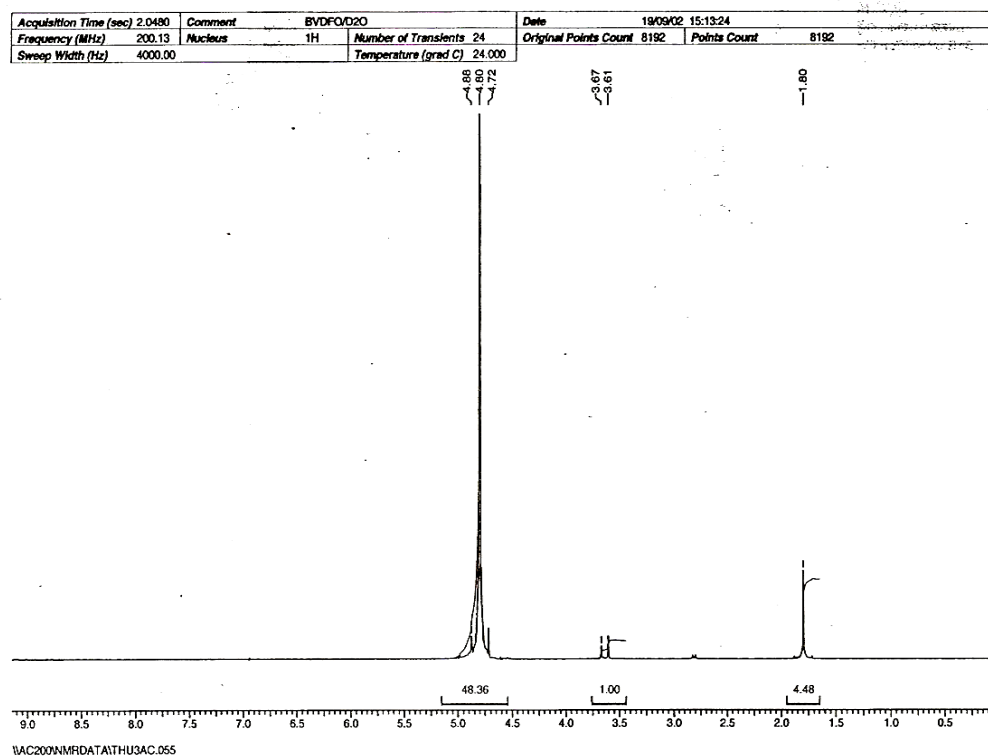


Fig. 3. ¹HNMR spectrum of Direct Orange - 102 before degradation by *Pseudomonas fluorescens*.

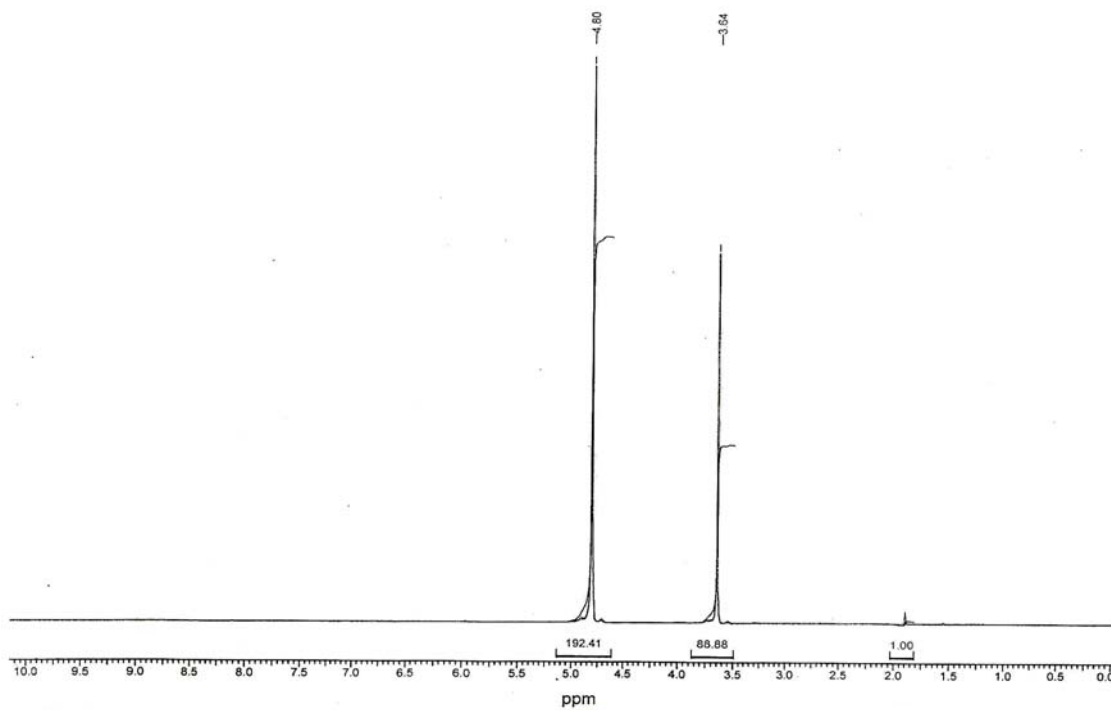


Fig. 4. ¹HNMR spectrum of Direct Orange - 102 after degradation by *Pseudomonas fluorescens*.

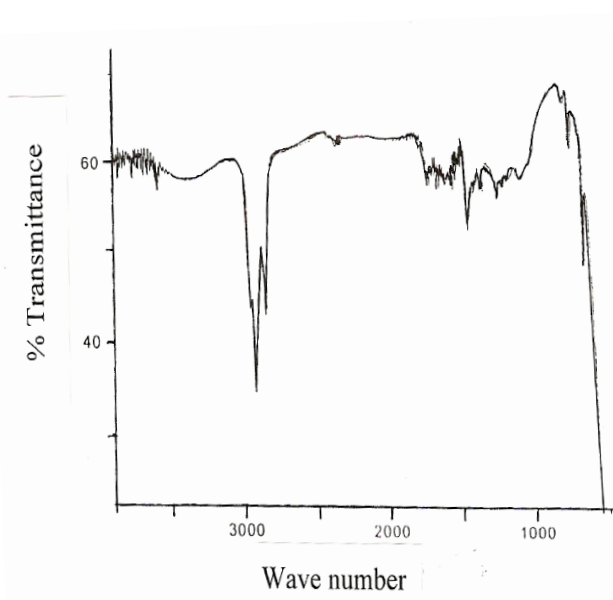


Fig. 5. Infra Red spectrum of Direct Orange - 102 before degradation by *Pseudomonas fluorescens*.

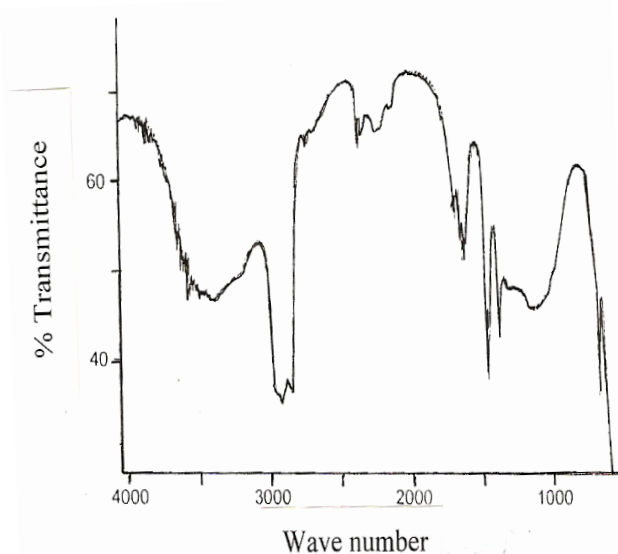


Fig. 6. Infra Red spectrum of Direct Orange - 102 after degradation by *Pseudomonas fluorescens*.

(Fig. 5). After degradation IR spectrum of the test sample showed a broad peak at 1150 cm^{-1} , a medium peak at 1350 cm^{-1} , a strong peak at 1440 cm^{-1} , a medium peak at 1620 cm^{-1} , three weak peaks at 1680 cm^{-1} , 2100 cm^{-1} , 2200 cm^{-1} , and a broad peak at 3400 cm^{-1} . This indicated the presence of C-O, C=O, NHC = ONH, N=N, NH or the OH. The acid carbonyl groups would have disappeared in degraded compounds (Fig. 6).

Discussion

The UV, ^1H NMR and IR spectra after degradation of Direct Orange - 102 by *Pseudomonas fluorescens* showed that Direct Orange - 102 degraded into another compound. The possible pathway of degradation is that Direct Orange - 102 changed into an intermediate compound (Fig. 7). This intermediate compound again changes into 3, 7-diamino-4-hydroxy-naphthalene-2-sulfonic acid sodium salt. This compound has changed again into 7-amino-3,4-dihydroxy-naphthalene-2-sulfonic acid sodium salt or 3-amino-4,7-dihydroxy-naphthalene-2-sulfonic acid sodium salt or 1,3,4,5,6,7,8-heptahydroxy-naphthalene-2-sulfonic acid sodium salt (Fig. 7). A perusal of the literature revealed that the end products obtained as a result of biodegradation of the dyes by the *P. fluorescens* were non-toxic in nature (Buckingham & Macdonald 1996). A similar result has been reported for the Acid Yellow-9 by Pandey & Upadhyay (2006). After degradation of Acid Yellow-9 three intermediate compounds were identified, which were 2,4-dihydroxybenzene sulphonic acid sodium salt, 2-amino-4-dihydroxy-benzene sulphonic acid sodium salt or 4-amino-2-hydroxy-benzene sulphonic acid sodium salt. Bumpus & Brock (1988) reported the biodegradation and mineralization of Crystal Violet by white rot fungus. They observed that the initial degradation starts with the sequential demethylation of Crystal Violet. Three degradation products were identified as N, N, N', N', N''-penta, N, N, N', N''-tetra, and N, N', N''-trimethylpararosalinine.

Different types of organisms such as bacteria, actinomycetes, yeasts and fungi have been reported to decolorize and degrade different types of triphenyl dyes (Azmi *et al.* 1998). A German patent (Akad 1991), states a new process by which the xenobiotic dyes of triphenyl methane series are degraded using *Corynebacterium* sp. and *Mycobacterium* sp. Sulphonated azo dyes such as Orange I and Orange II exhibit two features not found among natural compounds that are present in azo bridge and aromatic sulpho group, the azo bridge disappearance in the cleavage of the dye molecule occurring as first step of bacteria degradation (Kula 1981). Biological treatment is the only way for ultimately controlling the pollution generated by textile and dye-stuff industries. However, more research is needed to develop a viable alternative process for the treatment of dye waste-water using microorganisms.

It may be necessary to find out the enzymatic

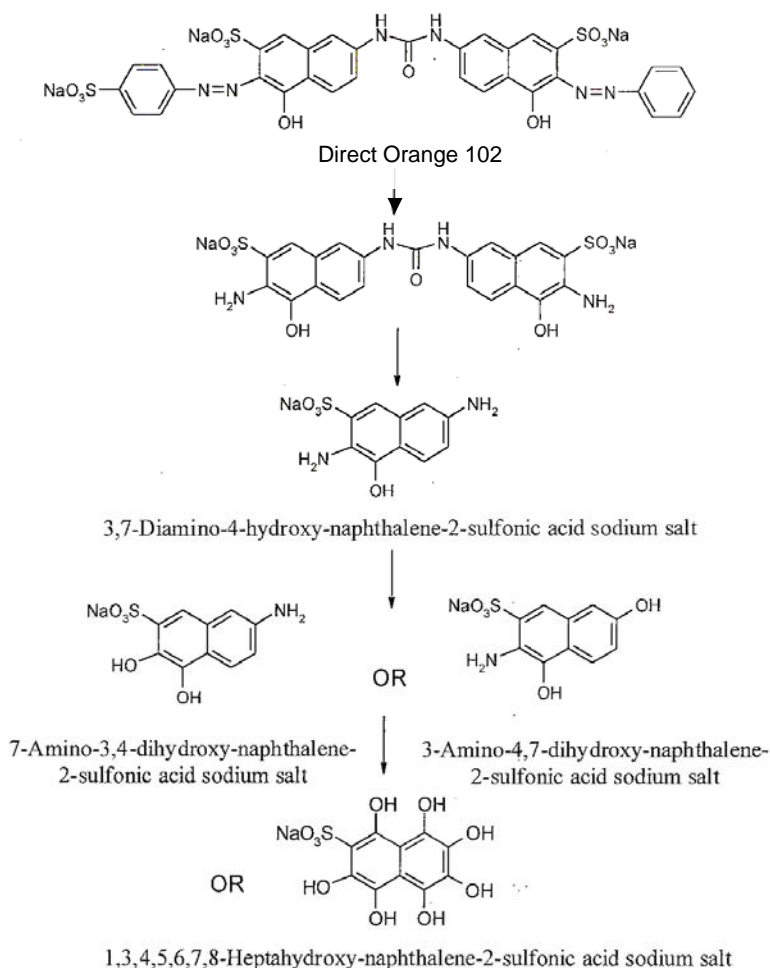


Fig. 7. Possible pathway of degradation of Direct Orange - 102.

system responsible for the decolorization and degradation of dyes. It is necessary to estimate the toxicity with respect to decolorization, because decolorized materials may remain toxic unless the product remains in the cells. Genetic engineering may be useful in bioremediation. One of the objectives of genetic engineering of toxic chemical degrading microorganisms is to develop a so called "Super bug" capable of detoxifying or degrading a large number of pollutants. Designing of potential pathways of degradation and improvements in enzymes taking part in this process could be done by genetic engineering. The genes for degradation of xenobiotics are mostly carried by plasmid. Manipulation of genes and putting them in microorganisms make them very powerful.

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