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Exploring by-products generated by the anaerobic degradation process of synthetic wastewater containing indigo dye

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ABSTRACT

This work aims to study the by-products generated by the anaerobic degradation process of synthetic wastewater containing indigo dye. These by-products were analysed and identified by both high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H NMR). HPLC results showed the dependence of the by-products to the operating conditions. The obtained HPLC chromatogram at the end of the experiments (Run 1) reveals different fractions, with at least eight distinguishable by-products. Increasing the hydraulic retention time from 1 to 5 days (Run 5) which corresponding to the start up of the bioreactor caused a significant change of the obtained HPLC chromatogram, with the decrease of the number of these by-products to only 3 ones. ^{1}H NMR analysis was realised with three representative fractions. The results showed that hypothetical structure of the by-products corresponding to aromatic cycles 1,2-disubstituted and possessing an axial symmetry similar to the phthalate groups.

Keywords: Anaerobic; Biodegradation; Decolourization; Indigo; Nuclear magnetic resonance

1. Introduction

The textile industry plays a crucial role in the world economy but at the same time, it consumes large quantities of water and generates enormous amounts of wastewaters [1]. The principal chemical pollutants present in textile wastewater are dyes containing carcinogenic amines, toxic heavy metals, pentachlorophenol, chlorine bleaching, halogen carriers, free formaldehyde, biocides, fire retardants, dispersing agents, surfactants, electrolytes, acids, and other different organics that have been washed from dyed material. These wastewaters are characterized by a high colour and chemical oxygen demand (COD) content with pH ranging from 2 to 12 [2–4]. Various reports have mentioned the direct and indirect toxic effects of the dyes that can lead to the formation of tumours, cancers and allergies besides growth

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inhibition of bacteria, protozoan, algae, plants and different animals including human beings. Without adequate treatment, these dyes are stable and can remain in the environment for an extended period of time [5–8].

Several methods are used to treat textile effluents including physicochemical methods such as filtration, coagulation, carbon activated and chemical flocculation. These methods are effective but they are expensive and involve the formation of a concentrated sludge that creates a secondary disposal problem [9,10]. Recently, new biological processes have been developed for dye degradation including aerobic, anaerobic processes [11–13]. Several microorganisms such as bacteria, fungi including yeasts and also algae have been isolated and shown to decolourize and biotransform the toxic dyes [14].

Anaerobic digestion techniques are becoming increasingly important and intensively studied since they are cost-effective and environmentally safe. Several mechanisms have been proposed for the decolourization of dyes under anaerobic conditions [15]. The mechanism of the dye reduction is still a subject of debate. Some researchers suggested that it should be a specific reaction by intracellular azoreductase, while others reported it to be an extracellular unspecific reduction process. Under anaerobic conditions, in the presence of a labile carbon source, decolourization of dyes is achieved with the cleavage of the bond, thus rendering the dye colours, with the formation of corresponding aromatic amines. The formed aromatic amines can be degraded in a sequential aerobic environment [16,17]. Various bacteria can participate in the dye decolourization. Among the anaerobes, sulphate-reducing bacteria can degrade the dye, but competition for this substrate may exist with other anaerobic bacteria, notably the fermentative acidogens involved in this anaerobic process. However, the latter microorganisms may not be the only microorganisms responsible for the decolourization [18].

Indigo dye who is a monoazo and vat dye with a brute chemical formula $C_{16}H_{10}N_2O_2$ (Fig. 1), is quite recalcitrant and difficult to degrade by biological treatment processes. In the literature, any reported study on degradation of indigo dye using pure cultures or anaerobic mixed cultures was found [16]. Very few reports are available on the generated by-products of indigo dyes. As has been proposed by many authors, the degradation of indigo produces isatin (indole-2,3 dione) which was further degraded to anthranilic acid (2-aminobenzoic acid) [19] or the formation of 5-Isatin-sulfonic acid [20].

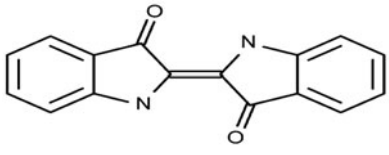


Fig. 1. The structure of the indigo.

This work aims to study the intermediates generated by the anaerobic degradation process of synthetic wastewater containing indigo dye in order to make a better understanding of the degradation pathway of indigo dye. The by-products were analysed and identified by both high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H NMR).

2. Materials and methods

2.1. Bioreactor operating condition

The experiments were carried out in an up-flow anaerobic fixed bed bioreactor with a total liquid volume of 0.7 L. The anaerobic sludge was cultured in a glass cylinder filled with Flocor (Ø 3L3, porosity 95%, surface area 230 m^2 m^{-3}) as a support made in polyethylene for the growth of microorganisms [21]. The bioreactor was maintained at 37°C by running water through its outer mantle. The anaerobic synthetic medium for bioreactor feeding contained (per liter) indigo: 100 mg, starch: 1 g and 10 mL of trace metal solution. The trace metal solution was prepared according to the composition mentioned previously [22], but with an extra addition of $CaCl_2$ (5 g L^{-1}). It contained in g L^{-1}: $MgSO_4 \times 7H_2O$: 5, $FeCl_2 \times 4H_2O$: 6, $COCl_2$: 0.88, H_3BO_3: 0.1, $ZnSO_4 \times 7H_2O$: 0.1, $CuSO_4$: 0.05, $NiSO_4$: 1, $MnCl_2$: 5, $(NH4)_6$ $MO_7O_{24} \times 4H_2O$: 0.64, $CaCl_2 \times 2H_2O$: 5. The pH influent was adjusted to 7.0–7.2 by adding HCl (2 M). The medium was sterilized by autoclaving for 20 min at 121°C. Before and after inoculation, the bioreactor was flushed with N_2 [23]. The bioreactor was inoculated with mixed non-defined cultures obtained from an anaerobic sludge from a (i) digester treating sulphate effluent, (ii) the microflora from the cow rumen, and (iii) a mixed sludge obtained from an anaerobic reactor treating industrial effluents. First the system was operated batch-wise with circulation for one month until biofilm formation was established. The indigo concentration of the feed was 30 mg L^{-1} and 2 mM ethanol was fed as the carbon and energy source for the microorganisms. Continuous feeding with the synthetic medium (soluble COD: 1,700 mg L^{-1}, OD 620 nm: 0.45 (maximum absorbance spectra was obtained at this wavelength after spectral scanning))

was started at low organic loading rate (OLR) of 0.34 g L^{-1} d^{-1} and was increased with time [24]. Bioreactor operating conditions are presented in Table 1.

2.2. UV–vis spectral analysis HPLC and NMR spectroscopy

2.2.1. HPLC-UV analyses

The by-products of the textile wastewater containing indigo dye was performed on a semi-preparative column (S5OD2-25F Spherisorb ODS2, 5 μm, 250 mm × 4.6 mm i.d., Agilent) by high performance liquid chromatography HPLC-UV along with a Agilent™ serial 1100 equipped with a quaternary pump, photodiode array absorbance detector and a BPSU-36 automatic fraction collector. The system was completely piloted by the software Bruker Hystar version 2.3. Samples were filtered on cellulose (Ø 0.2 μm) and injected automatically through an automatic injector with a flow rate of 1 mL min^{-1}. For by-product detection, the mobile phase consisted of acetonitrile (ACN) and water. The elution program began with 20% of ACN and 80% of water with an acquisition time of 40 min and analysis time of 55 min. The percentage of ACN was linearly increased to 95% over 20 min [25].

2.2.2. ^{1}H NMR spectroscopy

All fractions were dried by evaporation under vacuum, in order to determine their respective masses. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer (^{1}H-500.13 MHz) equipped with a 5 mm triple resonance inverse Cryoprobe TXI (^{1}H–^{13}C–^{15} N) with a z gradient. Spectra were recorded with 1.7 mm NMR capillary tube in 40 μL of CD_3OD-99.99% solvent (δ_{1H} 3.31 ppm–δ_{13C} 49.00 ppm) at 300 K. The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data are reported in ppm [25].

Table 1
Bioreactor operating conditions

	Run 5	Run 4	Run 3	Run 2	Run 1
Time period (d)	Day 1–53	Day 54–88	Day 89–113	Day 114–129	Day 130–137
HRT (d)	5	4	3	2	1
OLR (g L^{-1} d^{-1})	0.34	0.42	0.56	0.85	1.7

Notes: HRT: hydraulic retention time; OLR: organic loading rate.

3. Results and discussion

3.1. The effect of hydraulic retention time (HRT) on the by-products (HPLC identification)

HPLC-UV studies were used for detection of the produced intermediates by the anaerobic activated sludge degradation of the textile wastewater containing indigo dye at different runs ((Run 1 (HRT = 1 d corresponding to the end of the experiment); Run 2 (HRT = 2 d); Run 3 (HRT = 3 d); Run 4 (HRT = 4 d); Run 5 (HRT = 5 d corresponding to the start up of the bioreactor)). The obtained HPLC chromatograms are shown in Fig. 2. The analytical study indicated that the parent dye compound was degraded by the anaerobic sludge into different compounds. In fact, HPLC analysis further revealed the formation of different compounds from the indigo. The apparition of these compounds varies according to the operating conditions (HRT) that affected the microbial diversity and afterward of the nature of enzymes allowing their formations. In fact, the increase of the HRT affected both the number and the structure of the by-products. The HPLC chromatogram obtained at the end of the experiment (Run 1) reveals miscellaneous fractions, with at least eight distinguishable by-products with different retention times (fraction (c), (f), (g), (h), (i), (j), (l) and (n)). These 8 intermediary products are explained by an incomplete degradation of the dye due to the short HRT (1 day) which is insufficient for the bacterial enzymes biodegradation. Increasing the HRT from 1 to 2 days (Run 2), the majority of products which are partially biodegraded disappeared, giving the apparition to three new compounds (fraction (b), (d) and (e). The increase of the HRT from 1 to 5 days (Run 5) caused a significant change of the HPLC chromatogram, with a decrease of the number of the by-products to only three ones (fraction (b), (e) and (h)) due to sufficient retention time for the biodegradation. Only one similar fraction (h) persisted corresponding to the residual indigo.

In previous works [24], we proved that during the application of this Run 5 (HRT = 5 days), the bioreactor maintained high performances in terms of colour and COD removal efficiencies of 95 and 90%, respectively. In these conditions, the bioreactor presented higher archaeal and bacterial diversity, and the retention time was sufficient to allow anaerobic microorganisms to decolourize the dye. Zee et al. [26] and Bell et al. [27] have reported that longer HRTs and longer sludge retention times are necessary for the decolourization of dyes. However, older cells with reduced supplemental nutrient supply have been shown to have better degradative capacities. Franciscon et al. [11] showed that the chemical

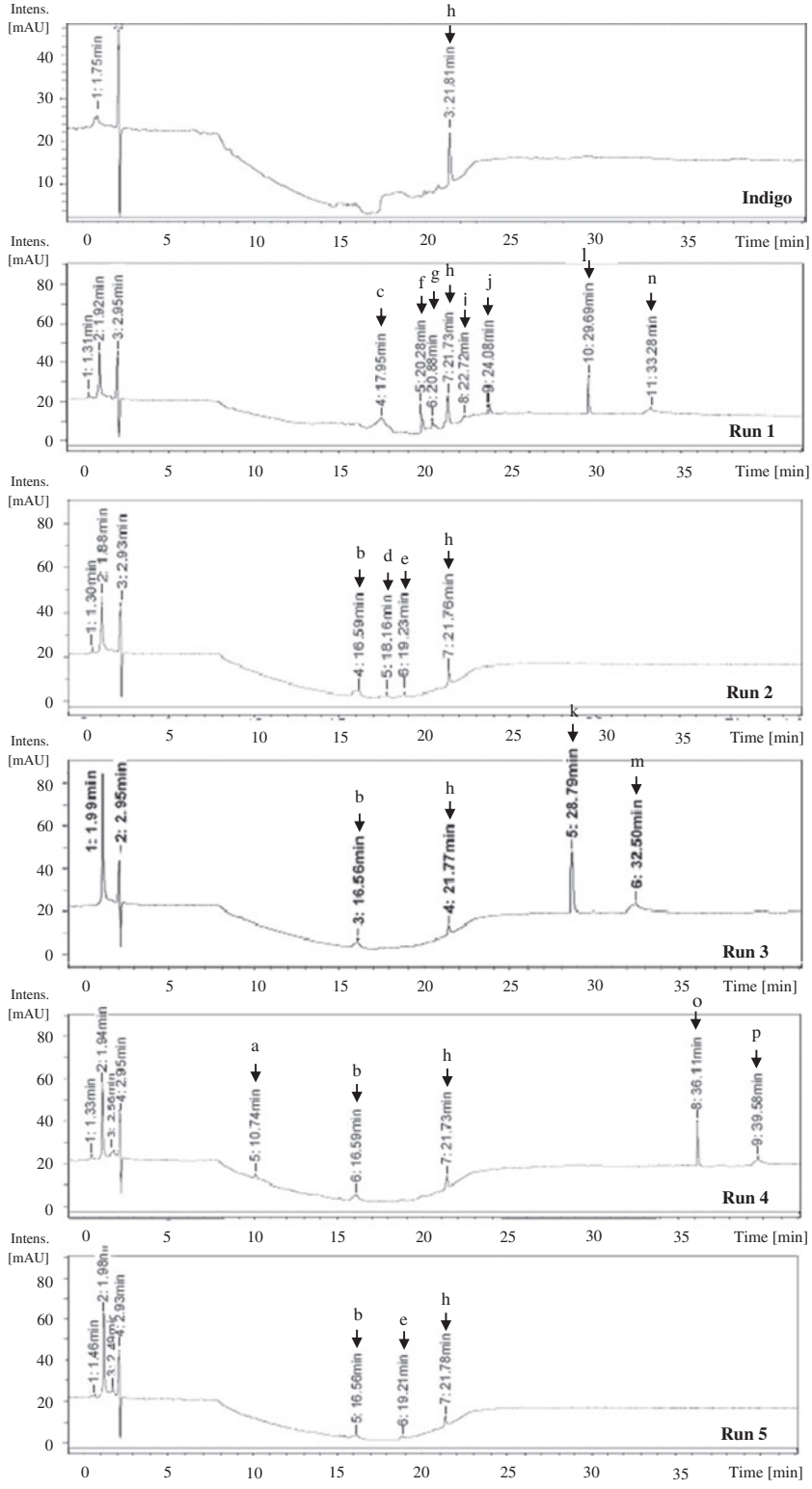


Fig. 2. Anaerobic biodegradation products of the indigo at different runs: Run 1 (HRT = 1 d); Run 2 (HRT = 2 d); Run 3 (HRT = 3 d); Run 4 (HRT = 4 d); and Run 5 (HRT = 5 d).

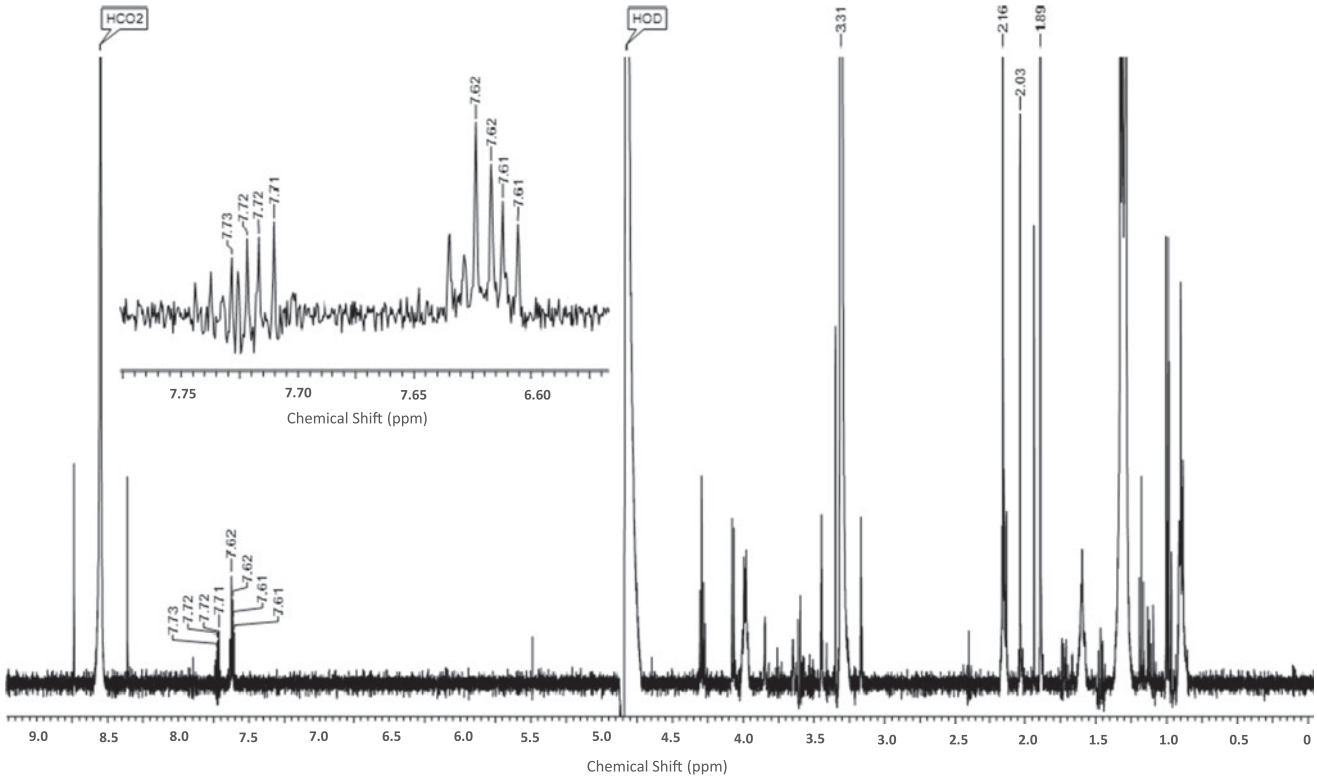


Fig. 3. ^{1}H NMR spectrum of the products generated by the textile wastewater containing indigo dye degraded by the anaerobic activated sludge (fraction (g): RT = 20.88 min (Run 1)).

structures of the dyes profoundly influence both their decolourization rates, and efficiencies. Dyes with simple structures and low molecular weights usually exhibit higher rates of colour removal, whereas colour removal is more difficult with highly substituted, high molecular weight dyes. For this reason, the indigo with simple structure and low molecular weight (monoazo dye) showed a short decolourization time.

These obtained results of colour and COD removal are higher than previously published data. In fact, Cinar et al. [28], on studying the anaerobic colour removal of azo dye in a sequencing batch reactor showed efficient colour and COD removal efficiencies, mostly higher than 80 and 96%, respectively. Debik et al. [13] who studied the colour and COD removal from textile effluents using a static granular bed reactor, proved that with an OLR of 1 kg/m^3 d and a HRT of 48 h, COD and colour removal efficiencies were 74 and 61%, respectively, while the removal efficiencies were 72 and 57%, respectively, with OLR of 1.7 kg/m^3 d and HRT of 24 h. However, anaerobic processes of wastewater treatment system are generally required for the effective biodegradation of dyes. Different anaerobic reactor configurations such as upflow anaerobic sludge blanket, fixed film, membrane bioreactor or fed batch have been used for successful treatment of textile dyestuffs [4].

Azoreductase is the key enzyme responsible for the reductive dye degradation in bacterial species. The advantage of the anaerobic reduction of dyes is that oxygen depletion is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerophilic bacteria to reduce dyes. The reaction takes place at neutral pH values and is extremely unspecific. However, the precise mechanism of anaerobic reduction is still not totally understood. It was recently suggested that microbial anaerobic reduction was linked to the electron transport chain and that dissimilatory reduction was a form of microbial anaerobic respiration. In addition, different models for the nonspecific reduction of dyes by bacteria, which do not require transport of the dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of dyes by anaerobic bacteria, were recently suggested. These results suggested that dye reduction was a strain-specific mechanism that could be performed by an azoreductase enzyme or by a more complex metabolic pathway [11].

Ozkan-Yucel and Gokcay [17] showed effective reduction of the dye after anaerobic treatment. The

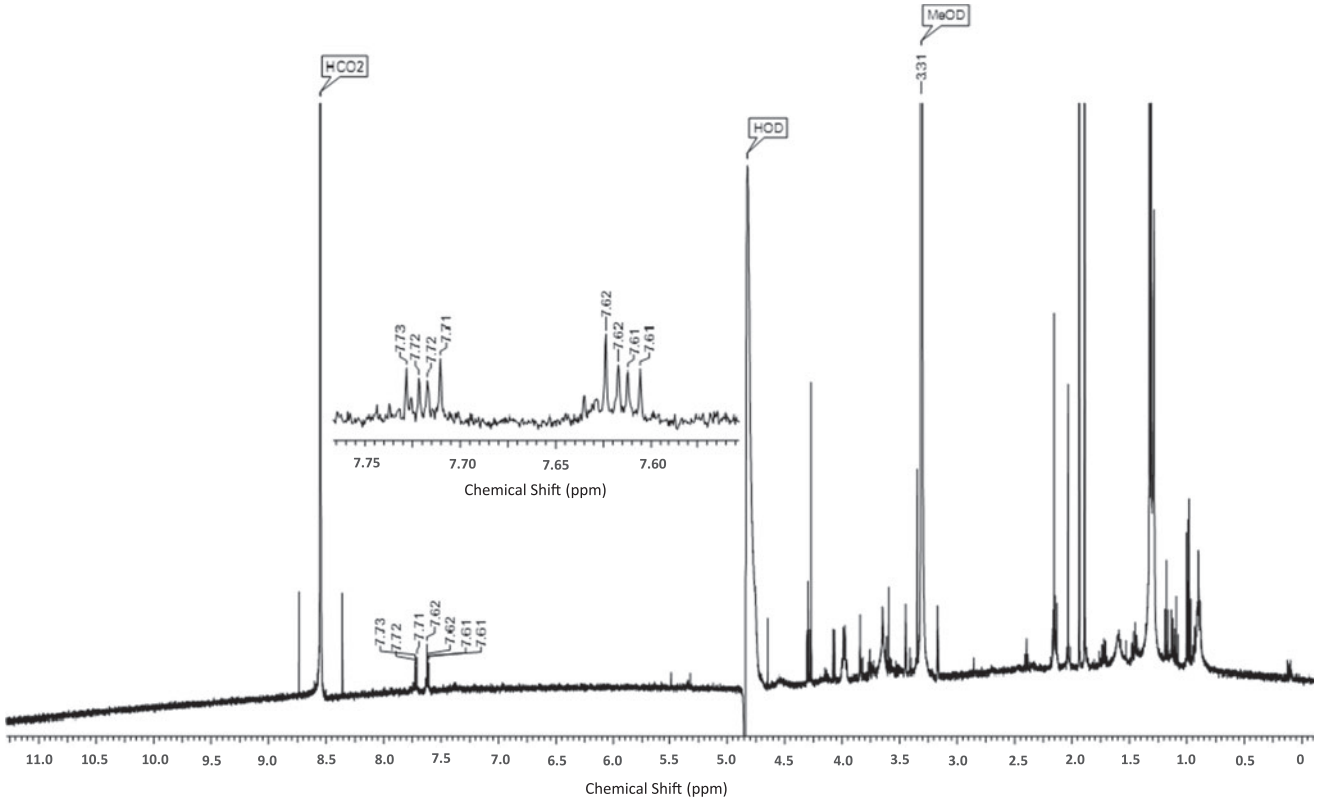


Fig. 4. ^{1}H NMR spectrum of the products generated by the textile wastewater containing indigo dye degraded by the anaerobic activated sludge (fraction (b): RT = 16.59 min (Run 2)).

colourless supernatants of the reactors changed to a light pinkish colour. The colour produced was evidently due to autoxidation of the dye reduction end products. Kudlich et al. [29] also observed a change in the clear colour of the samples to dark blue due to autoxidation of end products that were formed during anaerobic reduction of Naphtol Blue Black B. Zee et al. [30] reported colour development in decolourized samples of several reactive sulfonated azo dyes due to autoxidation upon exposure to air.

3.2. Identification of the main by-products

The ^{1}H NMR analysis was realised with three representative fractions of the main signals observed in the HPLC-UV chromatograms. These fractions were: fraction (g): RT = 20.88 min (Run 1); fraction (b): RT = 16.59 min (Run 2), which was detected in all Runs exceptionally Run 1 and the fraction (e): RT = 19.23 min (Run 2) which was detected in Runs 2 and 5.

For the fraction (g) (Fig. 3) and the fraction (b) (Fig. 4), the ^{1}H NMR spectrum showed the presence of two main characteristic signals of aromatic protons in 7.72 ppm and in 7.62 ppm. The shape of multiples is characteristic of couplings of the first and the second order of protons of an aromatic cycle 1,2-disubstituted which possessed an axial symmetry similar to the phthalates groups (aromatic compounds).

For the fraction (e) (Fig. 5), the ^{1}H NMR spectrum the presence of three main characteristic signals of aromatic protons in 8.32, 7.97 and 7.76 ppm. The fine structure both multiple's in 8.32 and in 7.76 ppm, is characteristic of couplings of the first and second order of protons of an aromatic cycle 1,2-disubstituted of which are similar but not identical. Other signals observed in the ^{1}H NMR spectrum can correspond to the residues of solvent, to the fat or to the still phase of the column HPLC.

The fraction (h) (RT = 21.81 min) observed in all HPLC chromatograms and in all Runs, is similar to the control (indigo). This fraction corresponding to the residual indigo dye in the medium which is not completely mineralized for all these operating conditions (maximum colour removal of 95% observed at the Run 5).

Hypothetic structures corresponding to aromatic cycles 1,2-disubstituted similar to the phthalates

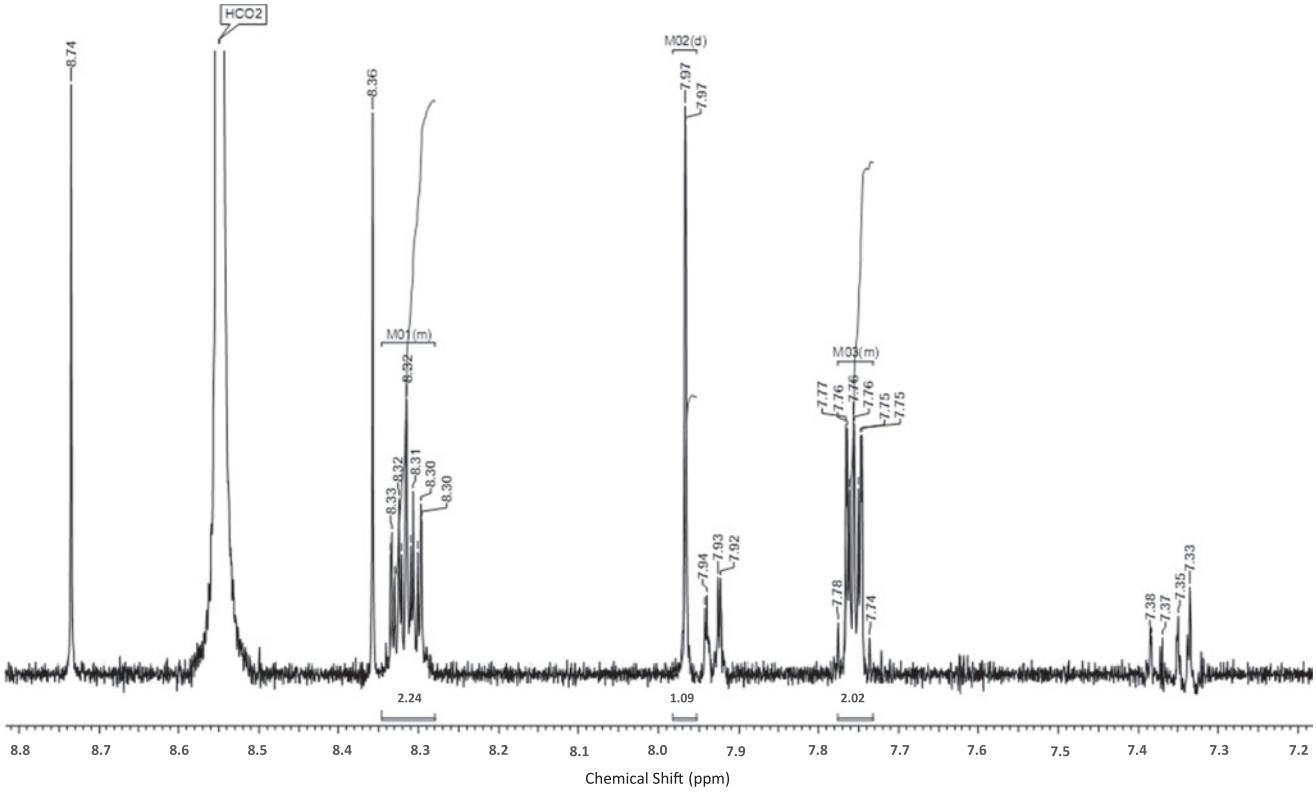
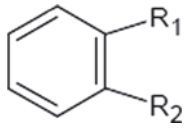


Fig. 5. ^{1}H NMR spectrum of the products generated by the textile wastewater containing indigo dye degraded by the anaerobic activated sludge (fraction (e): RT = 19.23 min (Run 2)).

groups were observed (Fig. 6), with axial symmetry R1 = R2 (for the fraction (g) and the fraction (b)), or similar but with no identical substituent's (R1–R2 (fraction (e)).

Very few reports are available on the intermediates of resulting from the biodegradation of the indigo dye. Podgornik et al. [20], studied the decolourization of indigo carmine by extracellular enzymes of *Phanerochaete chrysosporium* and they showed that the by-products may be 5-Isatinsulfonic acid or slightly modified 5-Isatinsulfonic acid. Balan and Monteiro [19] who studied the decolourization of textile indigo dye by ligninolytic fungi showed that the degradation



R1 ~ R2: fraction(e): RT = 19.23 min (Run 2)

R1 = R2: fraction (g): RT = 20.88 min (Run 1)

Fig. 6. Hypothetic structure of generated products by the textile wastewater containing indigo dye degraded by the anaerobic activated sludge (phthalates groups).

of indigo by laccases produced isatin (indole-2,3 dione) which was further degraded to anthranilic acid (2-aminobenzoic acid).

In this work, the obtained by-products or phthalate esters are a group of chemicals intensively used in industry as additives particularly in plastics to improve the flexibility of materials and also in pharmaceuticals, lubricants, cosmetics or printing inks. The global annual production of phthalates is about 3 million tonnes. These pollutants mainly come from industrial and municipal wastewater treatment plants. Some studies show that certain phthalates (especially those having long ester hydrocarbon chain) are refractory to biological degradation and can even present toxicity for the microorganisms performing the biological treatment. Their presence in the environment is a major environmental hazard, since some phthalates are suspected of being carcinogenic and/or endocrine disruptors. They have been associated to birth defects, organ damage, infertility and cancer [31–33]. The biodegradation of phthalates by activated sludge under both aerobic and anaerobic conditions has been demonstrated in various recent works [34,35]. However, biodegradation requires a long time to render the concentration of phthalate harmless, and microorganisms

could barely biodegrade or remove them completely from aqueous solution [33]. Ozonation has been investigated as an alternative method to decompose these compounds. In fact, Medellin-Castillo et al. [33] who studied the removal of diethyl phthalate from water solution by adsorption, photo-oxidation, ozonation and advanced oxidation process proved that the best system to treat water polluted with diethyl phthalate is the O_3/AC with degradation percentage of 85 and 90% were achieved at 20 min using activated carbons W and S, respectively. De Oliveira et al. [32] using the same coupling O_3/AC, proved that it enhance diethyl phthalate degradation, and it let to a fast and complete pollutant removal. Oh et al. [36] showed that the ozone/UV process was shown to have the highest efficiency for the elimination of diethyl phthalate and its by-products, leading to the complete mineralization of diethyl phthalate.

4. Conclusions

HPLC-UV studies were used for detection of the by-products by the anaerobic activated sludge degradation of the textile wastewater containing indigo dye at different runs. The results showed that the apparition of these by-products varies according to the operating conditions. The HPLC and ^{1}H NMR analyses of these by-products showed the presence of two main characteristic signals of aromatic protons similar to the phthalate groups.

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