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Exploring bioaugmentation strategies for the decolourization of textile wastewater using a two species consortium (*Bacillus cereus and Bacillus pumilus*) and characterization of produced metabolites

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ABSTRACT

Two bacterial strains, *Bacillus cereus* and *Bacillus pumilus*, were isolated from the sludge of an aerobic reactor treating textile wastewater containing indigo dye. These strains were chosen as augmented decolourizers which were mixed in various ratios with the activated sludge and the effect of their concerted metabolism on the decolourization and the biodegradation efficiencies were studied. Results showed that there was an equilibrated ratio between the bioaugmented bacteria and the other microorganisms of the activated sludge. However, the best yields were observed for the combination 33% activated sludge, 33% *Bacillus cereus* and 33% *Bacillus pumilus*, with a colour and carbon oxygen demand (COD) removal of 98% and 99% respectively, obtained after 48 h of incubation. The high performance liquid chromatography (HPLC), Proton Nuclear Magnetic Resonance (^{1}H NMR) analysis of the produced metabolites after the biodegradation of the dye by the bioaugmented consortium showed the presence of protons on an aromatic cycle 1,2-disubstituted and possessing an axial symmetry similar to the phthalate groups.

Keywords: Bioaugmentation; Decolourization; Response surface methodology; HPLC; ^{1}H NMR analysis; Indigo

1. Introduction

Textile industries are some of the leading consumers of water and dyes. Due to large scale production and extensive application, these dyes do not only add pollution problems to water, but they can cause serious health-risk factors [1,2]. Numerous physico-chemical processes have been proposed for the treatment of coloured wastewater for example precipitation, flocculation, membrane filtration and wet oxidation [3–5]. However, these treatment methods are not efficient, they may result in the production of toxic by-products and/or require high levels of energy. Biological decolourizations have been proposed as less expensive and less environmentally intrusive alternative [6]. In the field of wastewater treatment, the effectiveness of adding selected species to a

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complex ecosystem, a procedure called bioaugmentation has been reported as being successful [7,8]. The treatment systems having mixed microbial populations are more effective due to concerted metabolic activities of different strains [9–11]. In fact, the activated sludge contains decolourizers and non decolourizer's species. When these species are mixed with effective decolourizers, the species carrying non-essential functions (i.e., non decolourizer ones) still plays a crucial role to enhance colour removal of decolourizers [9].

Previous work has shown that *Bacillus cereus* and *Bacillus pumilus* are effective decoulourizers, having the ability to reduce keto groups and sulphide-bonds of the indigo dye [12]. In the same context, Pricelius et al. [13] have proposed that the dye decolourizing of the azoreductase enzymes of *Bacillus* species convert dyes by cleaving the molecule leading to free amines.

This work aimed to investigate firstly, the combined effects of two selected species, *B. cereus, and B. pumilus* and their interaction with each other and the microorganisms of the activated sludge to understand their roles in the organic removal. Secondly, the biodegradation products of textile wastewater containing indigo dye were studied by HPLC and Proton Nuclear Magnetic Resonance (^{1}H NMR), and they were identified.

2. Materials and methods

2.1. Bacterial cultures

Both bacterial strains, *Bacillus cereus and Bacillus pumilus*, used in the bioaugmentation experiment, have been previously isolated by Khelifi et al. [12], from the sludge of an aerobic reactor treating textile wastewater containing indigo dye using culture enrichment techniques. Pure cultures were stored on nutrient agar (Difco) slants at 4°C as working stock cultures.

2.2. Bioaugmentation assays

Bacillus cereus and B. pumilus were chosen as augmented decolourizers. Aerobic growth experiments were conducted in batch cultures in various activated sludge ratios. The strains were inoculated as described previously [12], with a total inoculum of 10% (v/v). The culture medium was inoculated with a loopful of the suspension for colony isolation (stock culture of the isolated starin). After 24 h of incubation, the culture broth was transferred into 250 ml flasks containing 100 ml of sterile textile wastewater containing indigo dye (total COD: 1120 mg l^{-1}, colour: 1.4 unit $OD_{620\,nm}$ and pH 7.5), supplemented with a mineral solution, and incubated at 30°C in a rotary shaking bath at 150 rpm for 72 h. The mineral solution contained (in g l^{-1}): Na_2HPO_4: 2, NaH_2PO_4: 1, KNO_3: 2, $MgSO_4$, $7H_2O$: 0.2, NaCl: 5, $CaCl_2$: 0.02, 1 ml trace element solution. The trace element solution contained (in grams per liter): $CaCl_2$, $2H_2O$: 2.0, $FeSO_4$: 1, $NaWO_4$, $2H_2O$: 0.5, $MnSO_4$: 0.5. The pH of the medium was adjusted to 7.0. After inoculation, the flasks were incubated at 30°C under shaking conditions (150 rpm). All assays were performed in triplicate and compared with uninoculated controls.

2.3. Analytical methods

Samples were centrifuged at 6000 g for 15 min. COD and colour measurements were carried out on the clear supernatant. Colour was measured by UV–vis spectrophotometry (Jenway, UV visible spectrophotometer) at 650 nm corresponding to the maximum absorbance spectrum obtained for indigo. The COD was measured following standard methods [1]. All assays were performed in triplicate and compared with uninoculated controls. Measured COD and absorbance values were used for calculation of biodegradation and decolourization efficiencies [1].

2.4. Experimental design

The experimental design provided by the software Minitab (Ver. 14.0, US Federal Government Common wealth of Pennsylvania, USA), was used to optimize the formulation of the microbial consortium. The mixture design was used to study the relationships between the proportion of different variables (*B. pumilus, B. cereus* and the activated sludge) and responses (Colour and COD removal). The mixture design can estimate the relationship between the formulation and the performance through regression analysis in fewer experiment times [14–16]. In this study, *B. pumilus* and *B. cereus* were inoculated to the activated sludge with different proportions ranging from 0% to 100% as shown in Table 1. Decolourization experiments were carried out according to the ratio given by the experimental design [17].

The P-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low P-value indicated a "real" or significant effect. The significance of each variable was determined by applying the Student's *t*-test. The statistical analyses were performed by use of multiple regressions and ANOVA with the softwares Minitab v 14.0 and Essential Regression v 2.2 [2,18].

2.5. HPLC UV–vis spectral analysis and NMR spectroscopy

The biodegradation products of the textile waste water containing indigo dye were detected with high performance liquid chromatography (HPLC-UV Agilent™ serial 1100). The system was completely controlled by the software Bruker Hystar version 2.3. The chromatographic

Table 1
Effect of the mixture between the strains and the activated sludge on the colour and COD removal (%)

Combination	*Bacillus cereus*	*Bacillus pumilus*	Activated sludge	Colour removal (%)	COD removal (%)
1	0.500	0.500	0.000	92 ± 2	91 ± 3
2	0.500	0.000	0.500	89 ± 3	86 ± 2
3	0.166	0.666	0.166	88 ± 4	89 ± 3
4	0.000	0.000	1.000	90 ± 2	91 ± 1
5	0.666	0.166	0.166	87 ± 1	85 ± 2
6	0.000	1.000	0.000	83 ± 2	81 ± 2
7	0.166	0.166	0.666	93 ± 3	94 ± 2
8	1.000	0.000	0.000	82 ± 4	77 ± 3
9	0.333	0.333	0.333	98 ± 2	99 ± 1
10	0.000	0.500	0.500	88 ± 2	83 ± 3

Individual strains were mixed in equal proportion to achieve a final $OD_{600\ nm}$ of 0.8 at the start of the experiment.

column was S5OD2-25F Spherisorb ODS2 (250 mm, 4.6 mm, 5 μm). The column temperature was 25°C. Samples were filtered on cellulose filter (Ø 0.2 μm) before injection. The mobile phase consisted of acetonitrile (ACN) and water. The elution programme began with 20% (v/v) ACN and 80% water with an acquisition time of 40 min. and analysis time of 55 min. The ACN percentage was linearly increased to 95% over 20 min. The flow rate was 1 ml min^{-1}. All Proton ^{1}H NMR spectra were recorded on a BRUKER advance DRX-500 spectrometer equipped with cooled antennas probe (with a triplet resonance TXI 500 MHz, opposite ^{1}H-^{13}C/^{15}N of 5 mm of diameter, gradient Z and of lock ^{2}H). The dried fractions (samples) were solubilised in 40 μl of methanol CD_3OD 99.99% (^{1}H d = 3.31 ppm – ^{13}C d = 49.00 ppm), then they were transferred for the NMR analyses in 300 K without rotation. The ^{1}H spectrum was done with the sequence zgpr (acquisition time = 45 min) [17].

3. Results and discussion

3.1. Bioaugmentation assays

Previous work has shown that *B. cereus* and *B. pumilus* are effective decolourizers, having the ability to reduce keto groups and sulphide-bonds of the indigo dye. If both bacteria have continuous contact, each organism can benefit from the mutual cooperation, and then each one can also do even better by exploiting the cooperation of the other [12].

In this work *B. cereus* and *B. pumilus* were considered as effective augmented decolourizers and they were mixed in various ratios to activated sludge and the effect of their concerted metabolism on decolourization and biodegradation efficiencies were studied (Table 1). In order to confirm the experimental results obtained for colour and COD removal, a mixture contour plot was plotted by MINITAB 14 Software Program. The mixture contour plots between the variables (*B. cereus*, *B. pumilus* and activated sludge) are given in Fig. 1. The lines of the contour plots predicted the values of each response at different proportions of *B. cereus*, *B. pumilus* and activated sludge. When both strains were combined with the activated sludge, the removal yields were enhanced, as compared with each single strain or using only the activated sludge. The best yields were observed for the combination nine with a colour and COD removal of 98% and 99% respectively, obtained after 48 h of incubation (Table 1). As mentioned by Chen [10], although some bacteria of the activated sludge (containing decolourizing and non decolourizing bacteria) are not considered as effective decolourizers, their presence still extensively enhances the decolourization activities of other strains due to extracellular metabolites released as stimulators/enhancers. It is anticipated that all species, irrespective of decolourizers or non decolourizers, in the microbial community may make use of their metabolic activities to reach a goal of pollutant degradation or detoxification for total survival [10]. The same yields were observed for the combination seven with a colour and a COD removal of 93% and 94% respectively, obtained after 48 h of incubation. These yields were somewhat lower than with the combination nine, due to the decrease of the activities of both bacteria. In fact, the addition of *B. cereus* and *B. pumilus* might stimulate the growth of bactivores, which probably induced a strong increase in the grazing pressure exerted on other bacterial species [7]. This suggested that the inoculated bacteria (low ratio of 16.66%) might be eaten by these predators, and that grazing might be responsible for the decline of the bioaugmented bacteria. This result could be explained by a non equilibrated ratio between

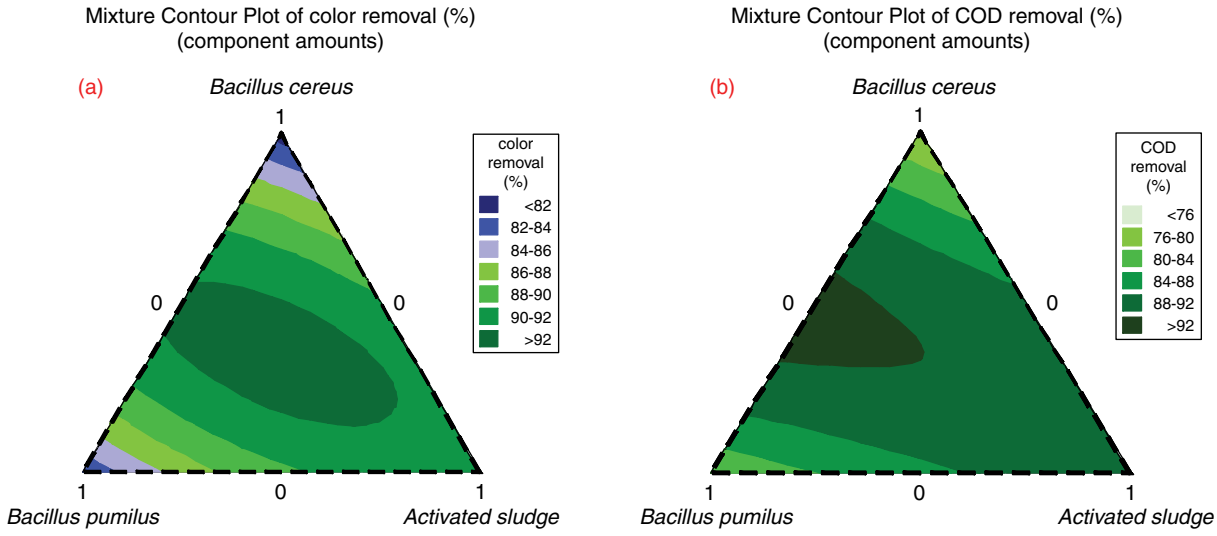

Fig. 1. Mixture contour plots between the variables (*Bacillus cereus, Bacillus pumilus*, and activated sludge) for colour (a) and COD (b) removal (%).

bioaugmented bacteria and the other microorganisms of the activated sludge. However obtained yields were similar to the experiments conducted only with the activated sludge. The importance of an equilibrated distribution of the ecosystem was observed with the massive addition of the bioaugmented bacteria (combination five), which negatively affected the equilibrium of the ecosystem. In fact, the colour and the COD removal efficiencies decreased noticeably to 87% and 85% compared with all the other combinations. However, Bouchez et al. [7], have illustrated that the massive addition of bacteria to induce a biological activity can result in undesirable effects disturbing the ecosystem equilibrium. So, there should be a well-balanced ratio between the bioaugmented bacteria and the other microorganisms of the activated sludge. This balanced distribution of the ecosystem led to improve the colour and COD removal of the treated wastewater.

The obtained *P*-values were $P = 0.65$ and $P = 0.11$, respectively for colour and COD removal, higher than 0.05, meaning consequently that there were significant differences between all 10 combinations (Table 1).

3.2. Model establishment

Regression models of colour and COD removal efficiencies were established (Table 1) and given in the following equations:

$$Y_{\text{colour removal (\%)}} = 80.90A + 82.17B + 90.17C + 42.82A^*B + 18.82A^*C + 13.36B^*C$$
$$R^2 = 76.37\ \%$$

$$Y_{\text{COD removal (\%)}} = 75.35A + 80.25B + 91.16C + 60.78A^*B + 22.60A^*C + 4.60B^*C$$
$$R^2 = 74.88\ \%$$

where *A* is the *Bacillus cereus*, *B* is the *Bacillus pumilus*, *C* is the *activated sludge*.

The adjusted coefficient (R^2) reached 76.37% and 74.88%, indicating that the quadratic models had a good fit with the target ratio formula [19].

3.3. HPLC analysis and NMR (^{1}H NMR) studies

HPLC and ^{1}H NMR studies were used for the textile wastewater containing indigo dye before and after bacterial consortium treatment, for the produced metabolites after biodegradation by the effective combination nine (Table1). The NMR analysis was done with two representative fractions of the main signals observed in the HPLC-UV chromatogram at retention time (RT) of 9.94 and 16.55 min, respectively (Fig. 2). For the first fraction A (RT 9.94 min) the ^{1}H NMR spectrum showed the presence of two main characteristic signals of aromatic protons at 7.72 and 7.62 ppm (Fig. 3). The shape of multiples was characteristic of coupling of the first and second order of protons of an aromatic cycle 1, 2- disubstituted and possessing an axial symmetry similar to the phthalate groups (Fig. 4). For the second fraction B (RT 16.55 min) the ^{1}H NMR spectrum did not show characteristic signals which could correspond to the structure of any compound, even after 45 min of acquisition, maybe due to the too low quantity of sample to observe significant signals in the ^{1}H NMR spectrum.

The fraction C (RT 21.76 min) was similar to the fraction RT 21.81 min of the control (indigo). This fraction did thus correspond to the residual indigo in the medium, which was not completely mineralized (COD removal of 98%). Very few reports are available on the biodegra-

dation products or intermediates of indigo dyes. Podgornik et al. [20] have studied the decolourization of indigo carmine by *Phanerochaete chrysosporium* extracellular enzymes and they have shown that the products of indigo carmine decolourization may be 5-isatinsulfonic acid or slightly modified 5-isatinsulfonic acid; Doralice and Regina [6] have studied the decolourization of textile indigo dye by ligninolytic fungi and shown that the degradation of indigo by laccases produces isatin (indole-2,3-dione) which is further degraded to anthranilic acid (2-aminobenzoic acid).

These produced products (phthalate) are esters which represent a class of chemicals largely used, mainly as plasticisers for polyvinyl chloride in a wide range of domestic and industrial applications. Their volatility and water solubility are very limited. In rodents, the main toxic effects observed pertain to the liver and include hepatic tumours for di-isononyl phthalate and di-(2-ethylhexyl) phthalate. However, it is generally admitted that these effects are not relevant to humans, due to the mechanism of action of these compounds. The reproductive and developmental effects are considered to be the critical endpoints for some phthalates. Di-*n*-butyl phthalate, butylbenzyl phthalate, and di-(2-ethylhexyl) phthalate can produce alterations of the developing and adult male reproductive system, 375 and an embryo/foetal toxicity. Information on their toxic effects in human is rare [21,22].

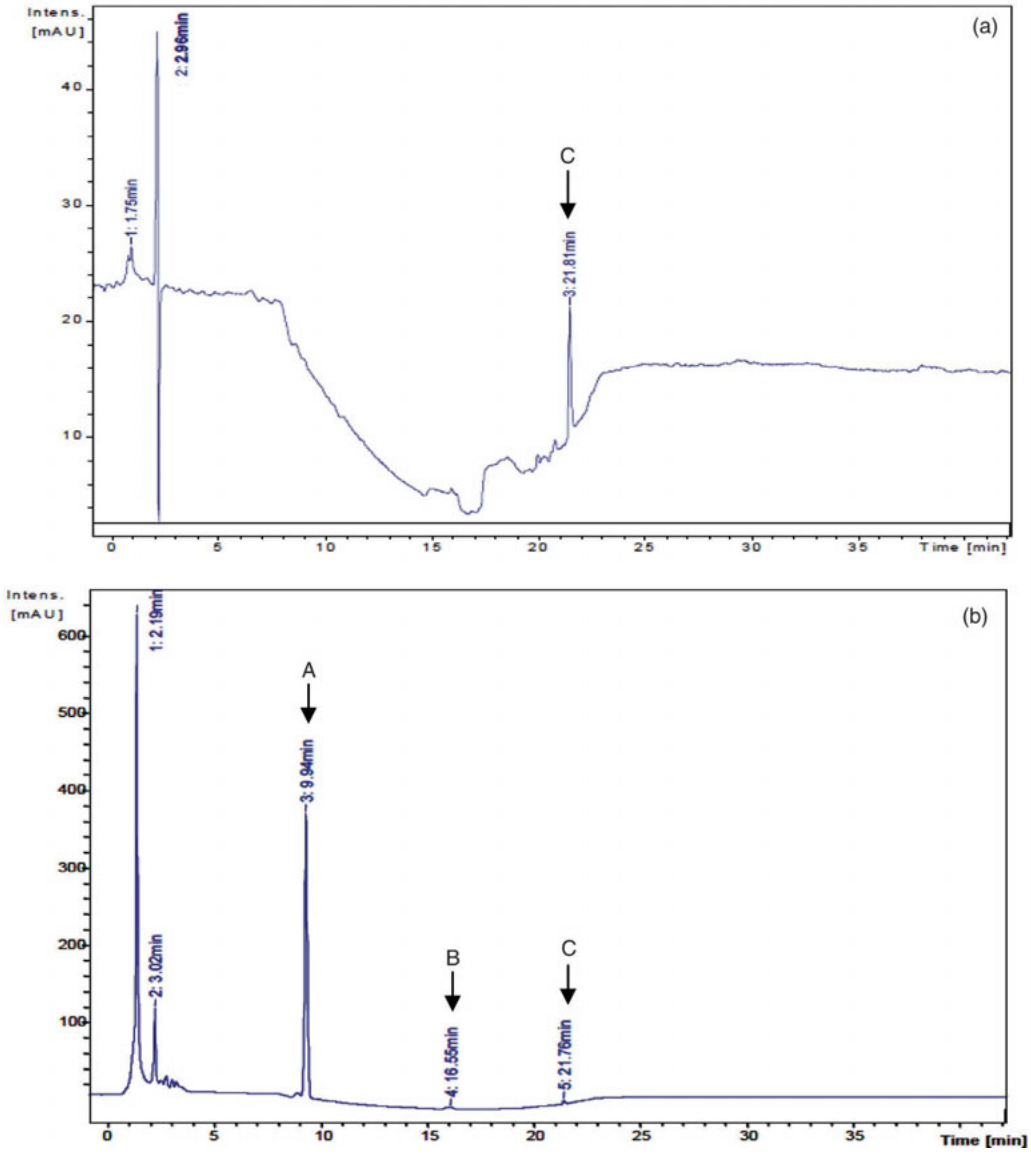

Fig. 2. HPLC spectra of the textile wastewater containing indigo dye before (a) and after degradation (b) by 33% *Bacillus cereus*, 33% *Bacillus pumilus* and 33% activated sludge (48 h of incubation).

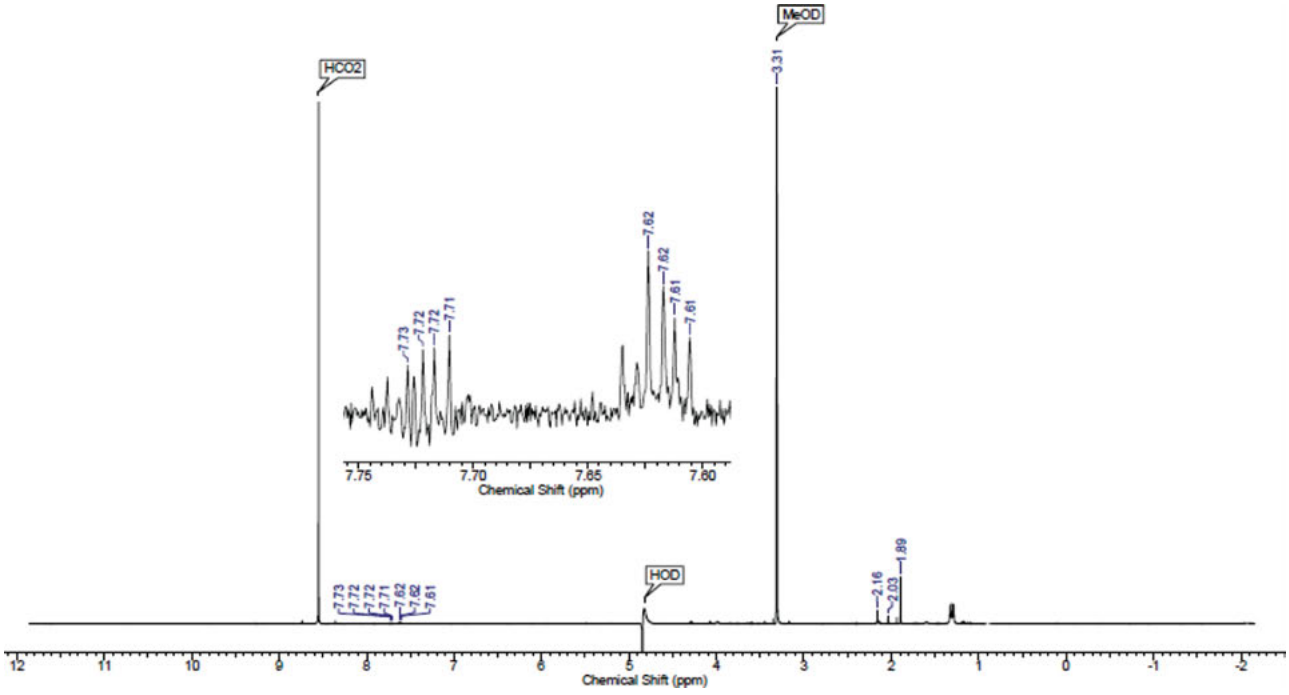

Fig. 3. ^{1}H NMR spectra of the textile wastewater containing indigo dye degradation products by 33% *Bacillus cereus*, 33% *Bacillus pumilus* and 33% activated sludge (48 h of incubation).

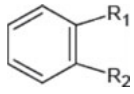

Fig. 4. Hypothetic structure of the biodegradation product present in the fraction.

4. Conclusions

A fractional factorial design was employed to select members to construct a mixed culture community that yields to optimal treatment performance. The present experimentation demonstrated that bioaugmented activated sludge system with both selected strains led to improved colour and COD removal efficiencies. The results showed that there was a well balanced ratio between the bioaugmented bacteria and the other microorganisms of the activated sludge. However, the best yields were observed for the combination nine with a colour and COD removal of 98% and 99% respectively obtained after 48 h of incubation.

The HPLC and ^{1}H NMR analyses of the produced metabolites after biodegradation of the textile wastewater containing indigo dye showed the presence of two main characteristic signals of aromatic protons similar to the phthalate groups.

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