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Effective Decolourization of Textile Azo Dyes by a Novel Bacterial Isolate *Lysinibacillus* SI 01 through Sequential Aerobic - Microaerophilic Process

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A B S T R A C T

Textile effluent polluted soil samples collected from the contaminated sites of Common Effluent Treatment Plant (CETP), Perundurai, Tamil Nadu, India were screened for isolation of potential bacteria capable of decolorizing commonly used textile azo dyes. A novel bacterium, designated as *'Soil Isolate 01' (SI 01)* isolated from the contaminated site was able to efficiently decolourize all the chosen four azo dyes (Reactive Yellow 180, Reactive Red 180, Reactive Red 198 and Red RR) under optimized aerobic - microaerophilic condition in nutrient broth with pH 8 at 37^{o}C and a dye concentration of 100 mg.l^{-1} with decolourization percentage > 85% in all the four dyes at 48 hrs. The biodegradation of the four azo dyes was monitored by UV–Vis analysis. Experiments revealed that the autochthonous bacterium *SI 01* decolorized all the four azo dyes in such a way that its absorption peaks in the visible range disappeared indicating molecular re-arrangement of the dye structure. Scanning Electron Microscopic (SEM) analysis and Live versus inactivated cells studies proved that only live bacterial cells were able to decolorize the dye whereas inactivated cells could not is an evident that decolorization was not due to physical adsorption.

Introduction

Textile industries generate large volumes of contaminated wastewater, which leads to a serious problem for the natural ecosystem (Manai *et al.*, 2016). The toxic effects of textile industrial wastewater are mainly due to the presence of mixture of dye molecules (Verma *et al.*, 2012). It was quantified that more than 80,000 tons/year of dyes are used in textile dyeing process, which needs 70-150 dm^3 of water. It has been reported that the textile industry requires about 40g of dyes for one kilogram of cotton (Mendez-Martineza *et al.*, 2012). Azo dyes are one of the largest classes of synthetic dyes, which are widely used in the wet process of the color industry (Yu *et al.*, 2015). Due to the presence of one or more azo bonds (-N=N-) and aromatic rings in its chemical structure, it is toxic and resistant to degradation (Xu *et al.*, 2007). As a result the release of these dyes without treatment into the environment leads to inimical impact on the aquatic ecosystem. Most of the dyes are reported as

carcinogenic to humans (Tan *et al.,* 2016). Therefore, azo dye containing textile wastewater should be treated before discharge into the environment. Numerous physico-chemical and biological methods have been widely used to treat textile wastewater containing azo dyes (Khouni *et al.,* 2011). However, the physical and chemical treatments have drawbacks due to the high cost involved and release of hazardous secondary pollutants (Saratale *et al.,* 2009a). On the contrary, bioremediation offers a cheaper and more environmentally friendly alternative method for the treatment of textile wastewater containing azo dyes (Jadhav *et al.,* 2010). Several studies have been reported, different microorganisms such as Bacteria (Yu *et al.,* 2015), Yeast (Waghmode *et al.,* 2011), and Fungi (Parshetti *et al.,* 2007) are involved in the subject of color removal; in the mechanism of bioadsorption, biotransformation or degradation. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (–N=N–) with the help of azoreductase under anaerobic conditions resulted into the formation of colorless solutions. The resulting intermediate metabolites (e.g., aromatic amines) are further degraded aerobically or anaerobically (Chang *et al.,* 2000). In the present study we focused our attention on the isolation of potential dye-decolourizing bacteria from contaminated soil of the Common Effluent Treatment Plant (CETP), Perundurai, Tamil Nadu, India and reporting a novel autochthonous bacterium named *Soil Isolate 01(SI 01)* having significant decolourizing ability on several different commonly used textile azo dyes. Physico-chemical conditions have been optimized to achieve maximum decolorization.

Materials and Methods

Dyes and Chemicals

Four commonly used textile dyes, namely Reactive Yellow 180, Reactive Red 180, Reactive Red 198 and Red RR were procured from Jamara textile industry, Erode, Tamil Nadu, India. The dyes were initially studied for the absorption maxima in a UV-Vis spectrophotometer (UV-Vis 1800, Schimadzu, Japan) from 190-800nm. Nutrient broth (Peptone, NaCl, Beef extract and Yeast extract) was obtained from Himedia Labs, Mumbai, India.

Preliminary screening and identification of the novel isolate

The textile effluent polluted soil was aseptically collected from CETP, Perundurai, Erode, Tamil Nadu, India. One gram of soil sample was added in 100 ml of sterile distilled water and serially diluted by the standard protocol. From each dilution 100 µl of sample was plated on nutrient agar medium and kept for incubation at 37˚C for 24 hrs (Elisangela *et al.,* 2009). Seven bacterial cultures (SI 01 to SI 07) were isolated based on the color, morphology and raised as pure culture on a slant containing nutrient agar for further studies. The organisms were used for decolorization studies after preculturing in nutrient broth (g L^{-1}) at 37 ±2˚C for 16-18 hrs (log phase) under shaking condition at pH 7. All the seven isolates were inoculated individually on a dye Reactive Red 198 (chosen as a representative of azo dyes for primary decolorization efficiency test) containing medium and kept for incubation at 37˚C for 72 hrs both in shaking and static conditions. Based on the decolorization efficiency and abundance in preliminary screening (isolation experiments) *SI 01* was chosen for testing decolorization efficiency on other

dyes and was also subjected to identification through biochemical analysis.

Optimization of culture conditions

In order to figure out the optimal physico-chemical parameters required for growth the novel isolate *SI 01* was subjected to growth kinetics study for a period of 24 hours following standard procedure at different culture conditions such as pH (6, 7, 8 and 9), Temperature (30 , 37 and 45 ^{o}C), shaking and static conditions in nutrient broth.

Testing decolorizing ability

The decolorizing ability of the novel isolate *SI 01* was tested individually on the four chosen azo dyes. Inoculum was used at 5 % concentration when the Optical Density reached 1.0, in a 250 ml Erlenmeyer flask containing 100 ml nutrient broth with the dye concentration of 100 mg.l^{-1} in both shaking (120 rpm) and static conditions at 37^{o}C for a period of 72 hrs. Further, a sequential aerobic and microaerophilic process was also carried out to study the decolourization efficiency, where the culture was first subjected to shaking condition (at 120 rpm for first 24 hrs) followed by static condition up to 72 hrs at 37^{o}C in pH 8 (Kalyani *et al.*, 2009). All the experiments were performed in triplicates.

Decolorization assay

Decolorization was detected by UV-Vis spectrophotometer (Shimadzu UV-Vis Spectrophotometer, Japan) at respective λ_{max} using the supernatant from the liquid culture medium after centrifugation at 10,000 rpm for ten minutes in a refrigerated centrifuge (5804R, Eppendorf, Germany). The removal of the color was reported as % decolorization.

[% = A_0-A_t/A_0 x 100]

Where A_0 and A_t were absorbance of the dye solution initially and at cultivation time (t), respectively. Each decolorization value is a mean for three parallel experiments. Abiotic controls (without microorganisms) were also included (Senthil *et al.*, 2016).

Analysis of the decolorized product through UV–Vis spectral analysis

The samples were collected before and after the decolorization processes and filtered through 0.2 µm membrane filters. The filtrate was then scanned in the UV-Vis Spectrophotometer (Schimadzu UV-Vis 1800, Japan) within the range of 190 – 1100nm. Appropriate blank was also subjected to the scanning process. The band width was set to 1 nm during the scanning program. The absorbance was noted at the respective characteristic peak area (λ_{max}) for the interpretation of results (Saratale *et al.*, 2009b).

Adsorption Desorption Assay

Desorption studies were carried out to find out the extent of adsorbed color to the bacterial biomass during the process of color removal. Different desorbing agents like 1 N sodium hydroxide, 1N Hydrochloric acid and double distilled water. Known volumes of thoroughly shaken samples were taken and centrifuged to obtain pellets. The pellets were then treated with above chemicals and extracted twice to remove the adsorbed color, till no more color could be extracted from the biomass. The extent of color adsorbed was estimated by comparing with the actual color of control and the color removed by the bacterial biomass. Controls of pure inocula in buffer, un-inoculated dye-samples/effluent and non-decolorizers were also run parallelly and subjected to the same desorption protocol, to eliminate any color being produced due to the intracellular

contents of the bacteria and the chances of a false reading.

Scanning electron microscopy

The control (unexposed cells) and test samples were fixed with glutaraldehyde, dehydrated with a series of ethanol washes with progressively increasing the ethanol concentrations for 5 min. Finally, the dehydrated samples were air-dried at room temperature and mounted on stainless steel SEM stubs, sputter coated with gold in an ion counter and examined under a scanning electron microscope to study the effect of dyes on the bacterial cell and its mechanism.

Results and Discussion

Structure and absorption maxima of the dyes

The four textile dyes chosen for this study were based on their frequent usage in textile industries and availability of structure in literatures. The absorption spectra of four dyes were studied (350 nm to 750 nm) in a double beam UV-Vis Spectrophotometer. From the optical density at 1 nm bandwidth, the absorption maximum was determined and presented in Table 1.

Preliminary Screening and optimization

Seven different bacterial strains were isolated based on the colony morphology from the contaminated soil samples and were named as *Soil Isolate 01*to *Soil Isolate 07* (*SI 01 to SI 07)* based on abundance. Preliminary screening revealed that SI 01 demonstrated a maximum decolorization of 88.75% on Reactive Red 198 in 72 hours under static condition. Whereas the strain demonstrated maximum decolorization of 87.5% on reactive red 198 in 72 hrs under shaking condition which is insignificantly different. The isolate was then subjected to biochemical based identification and was found to be of genus Lysinibacillus. The biochemical characteristic of the strain is given in Table 2.

Optimization studies

As the *Soil Isolate 01(Lysinibacillus sp.)* was found to be the best decolorizing bacterial strain from preliminary screening on Reactive Red 198, it was subjected for growth kinetics studies in different conditions as mentioned earlier in the materials and methods. The *Soil Isolate 01*demonstrated better growth characteristics at 37^{o}C in nutrient broth at pH 8.0 incubated in the shaking condition *(Data not shown).*

Decolorization under different culture conditions

As the *Soil Isolate 01*demonstrated maximum growth at pH 8, 37^{o}C under shaking condition the same conditions were used for decolourization experiments. There was no significant decolourization in all the four dyes tested until 24 hrs (Table 3, 4, 5 and 6). Similar to most of the bacterial decolourizations *Soil Isolate 01*was not able to decolorize the dye under aerobic (shaking) conditions effectively. Due to poor decolourization the incubation was extended to 48 and 72 hrs in both shaking and static conditions. The static (microaerophilic) condition was found to be optimal for decolorization. Microorganisms able to grow on dye containing medium under shaking condition, but not decolorize dyes under aerobic condition (Anliker 1979). However, cell growth was poor under static condition as compared to shaking condition. Reports indicate that oxygen is deleterious to the activity of enzyme (azoreductase) which is responsible for azo bond reduction, where as oxygen

favors bacterial growth under shaking condition (Ogugbue *et al.,* 2012).

A sequential aerobic and microaerophilic process which involved both shaking and static conditions was also performed to check decolourization efficiency of *Soil Isolate 01in* all the four dyes. From the result it could be inferred that the sequential aerobic and microaerophilic process (shaking and static) has increased the rate of decolorization and has reached its maximum within 48 hrs in all the four dyes when compared to that of independent static and shaking conditions (Table 3,4,5 and 6). Our results were in accordance with the previous reports and indicate that the bacterial cultures under static condition were more efficient in decolorizing dyes used in textile industry. Ghodake *et al.,* 2009 also reported decolorization rate of Direct Brown MR at static 91.3% and 59.3% under shaking condition. Similar kinds of results were reported in the previous studies in case of Psuedomonas SUK1 (Kalyani *et al.,* 2009) and in case of *Psuedomonas aeruginosa* BCH (Jadhav *et al.,* 2011).

UV–Vis analysis and scanning electron microscopy

The biodegradation of the four azo dyes was monitored by UV–Vis analysis. Experiments revealed that the *Soil Isolate 01*decolorized all the four azo dyes in such a way that its absorption peaks (Fig. 1, 2, 3 and 4) in the visible range disappeared while the absorption peak in the UV range did not diminish. Inspecting the cell mats also showed that *Soil Isolate 01*retained their natural color after decolorization of all the dyes. According to the literatures decolorization of dyes by bacteria could be due to adsorption by microbial cells, or to biodegradation. In the case of adsorption, the UV–Vis absorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears (Fig 5). Dye adsorption can also be clearly judged by inspecting the cell mats. Cell mats become deeply colored because of the adsorbed dyes, whereas those retaining their original colors occur when biodegradation takes place. To observe the effect of decolorization of the textile dyes on the bacterial biomass and its mechanism, Scanning Electron Microscopy was carried out. The surfaces of the cells exposed to dyes were as smooth as seen in the unexposed control cells (Fig 6 a, b). This would mean that biodegradation could be a major contributor to color removal rather mere adsorption. Studying live versus inactivated cells proved that only live bacterial cells were able to decolorize the dye whereas inactivated cells were unable to do so. This proved that decolorization was not due to physical adsorption by inactivated cells. The novel strain (SI 01) isolated from the textile effluent polluted soil rapidly decolourized all the chosen four azo dyes under sequential aerobic and microaerophilic conditions, with differences in decolourization times depending on the dye structure, as was confirmed by the UV–vis analysis. The study reveals that decolourization was strongly dependent on the biomass size in the medium, indicating the need for shaking conditions (aerobic) during decolourization process. High decolourization efficiency was attained in shorter duration under sequential static condition when compared to that of shaking and static conditions separately. Although this bacterium has shown greater dye degradation ability as compared to other bacteria, there is no much available literature on dye decolourization with *Lysinibacillus sps.*

Table.1 Adsorption maxima of the chosen four textile azo dyes.¬

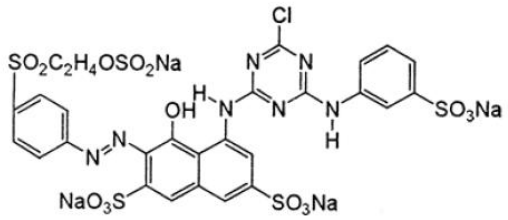
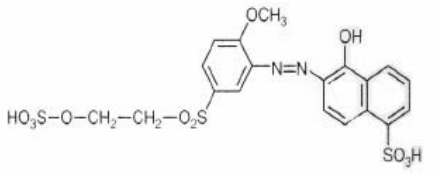
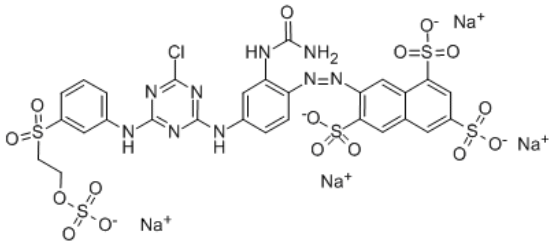
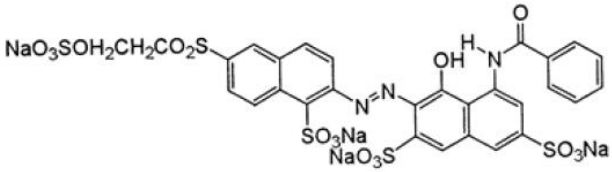
S.No.	Name of the dye	λ max	Dye Structure
1.	Reactive Yellow 180	450 nm	
2.	Red RR	520 nm	
3.	Reactive Red 180	520 nm	
4.	Reactive Red 198	520 nm	

Table.2 Colony morphology and biochemical characteristics of the novel Soil Isolate SI 01.

Sl. No.	Tests Performed		Results
1.	Colony morphology on Nutrient Agar		Opaque, unpigmented and smooth colonies
2.	Gram stain		Positive
3.	Haemolysis		Non-haemolytic
4.	Motility		Motile
5.	Sugar Utilization	Glucose	Positive
6.		Lactose	Negative
7.		Sucrose	Positive
8.	H_2S Production		Negative
9.	Urease Test		Negative
10.	IMViC Test		Negative
11.	Nitrate Reduction		Positive
12.	Phenylalanine		Positive
13.	Casein hydrolysis		Positive
14.	Citrate utilization		Positive
15.	Tween 20 hydrolysis		Positive
16.	Oxidase		Positive
17.	Catalase		Positive
18.	Starch Hydrolysis		Positive
19.	Gelatine Hydrolysis		Positive
20.	Huge-Leifsion		Oxidative

Table.3 Decolourization efficiency of Soil Isolate 01on Reactive Yellow 180 dye under shaking, static and sequential aerobic and microaerophilic process conditions.

Duration in Hrs	% of the Decolorization under Shaking condition	% of the Decolorization under static condition	% of the Decolorization under Sequential aerobic and microaerophilic process (24 hrs shaking followed by static condition upto 72 hrs)
24hrs	6.34±1.6%	35.17±5.3%	5.26±4.0%
48hrs	37.02±4.2%	50.62±2.1%	86.84±1.6%
72hrs	60.43±1.8%	85.58±1.9%	86.84±2.6%

Table.4 Decolourization efficiency of Soil Isolate 01 on Reactive Red RR dye under shaking, static and sequential aerobic and microaerophilic process conditions

Duration in Hrs	% of the Decolorization under Shaking condition	% of the Decolorization under static condition	% of the Decolorization under Sequential aerobic and microaerophilic process (24 hrs shaking followed by static condition upto 72 hrs)
24hrs	10.45 ± 2.4 %	25.36 ± 4.2 %	13.75±2.2%
48hrs	34.81±2.5%	45.28±1.8%	85±3.1%
72hrs	67.04±1.5%	85.22±1.6%	86.25±3.5%

Table.5 Decolourization efficiency of Soil Isolate 01 on Reactive Red 180 dye under shaking, static and sequential aerobic and microaerophilic process conditions

Duration in Hrs	% of the Decolorization under Shaking condition	% of the Decolorization under static condition	% of the Decolorization under Sequential aerobic and microaerophilic process (24 hrs shaking followed by static condition upto 72 hrs)
24hrs	6.98 ± 2.6%	12.36 ± 1.4 %	7.14 ± 0.9%
48hrs	29.32 ± 1.5%	35.69 ± 1.5%	80.61 ± 1.6%
72hrs	50 ± 1.7 %	88.88 ± 2.8 %	90.81 ± 1.9%

Table.6 Decolourization efficiency of Soil Isolate 01 on Reactive Red 198 dye under shaking, static and sequential aerobic and microaerophilic process conditions

Duration in Hrs	% of the Decolorization under Shaking condition	% of the Decolorization under static condition	% of the Decolorization under Sequential aerobic and microaerophilic process (24 hrs shaking followed by static condition upto 72 hrs)
24hrs	42.5 ± 1.9%	33.75 ± 3.2%	25 ± 2.7%
48hrs	87.5 ± 2.4 %	85 ± 4.1%	85 ± 4.8 %
72hrs	87.5 ± 3.4%	88.75 ± 3.7 %	85 ± 3.1 %

Fig.1 Spectral analysis of Reactive Yellow 180: a - native dye, b – after biological treatment

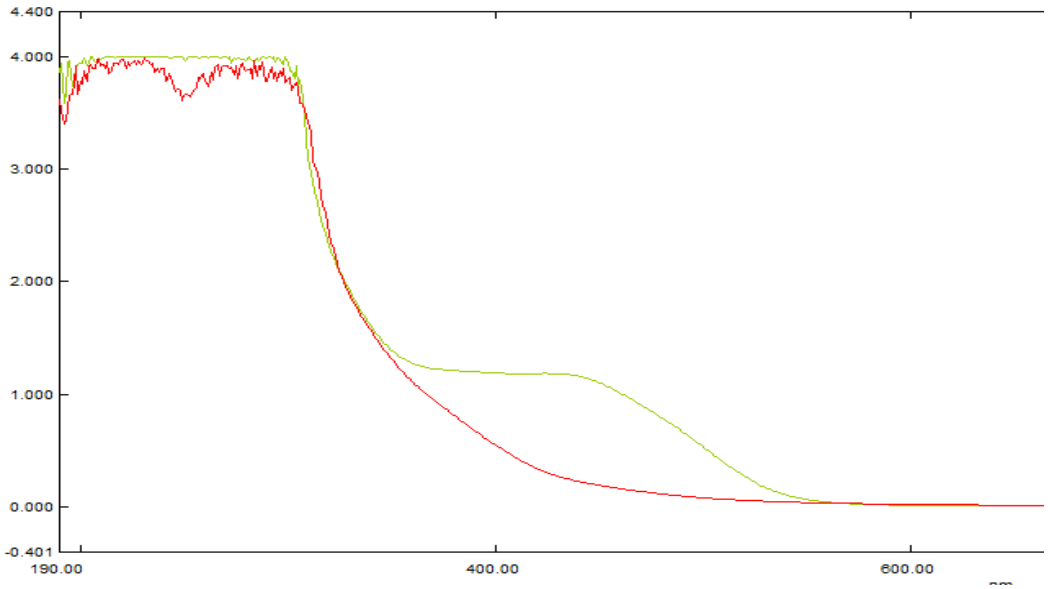


Fig.2 Spectral analysis of Red RR: a - native dye, b – after biological treatment.

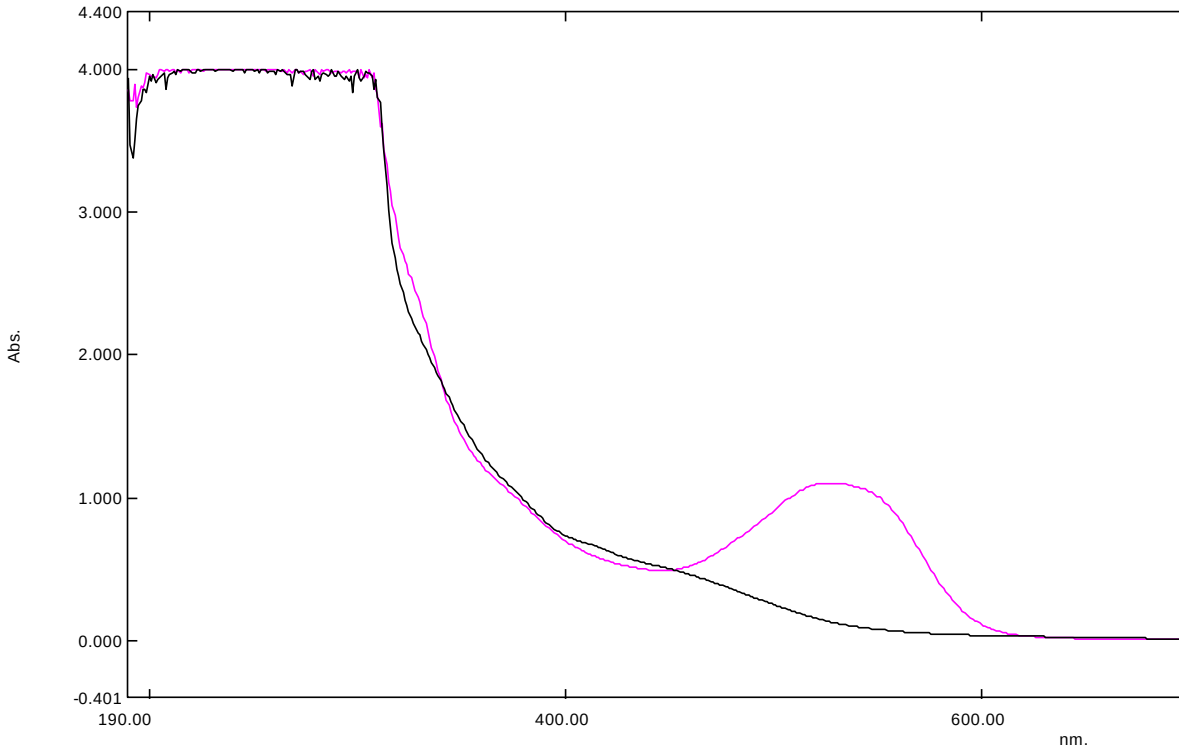


Fig.3 Spectral analysis of Reactive Red 180: a - native dye, b – after biological treatment.

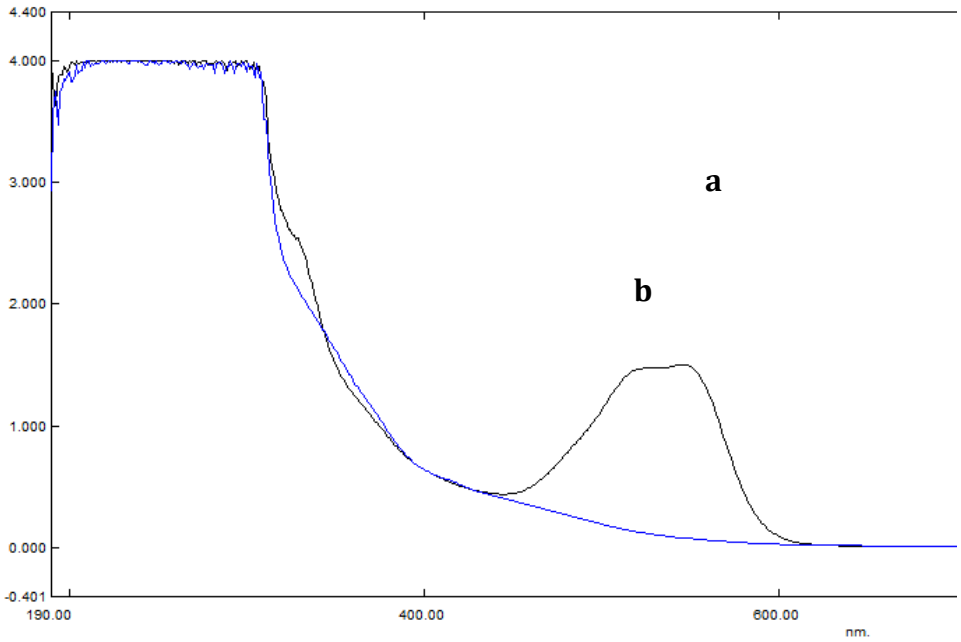


Fig.4 Spectral analysis of Reactive Red 198: a - native dye, b – after biological treatment.

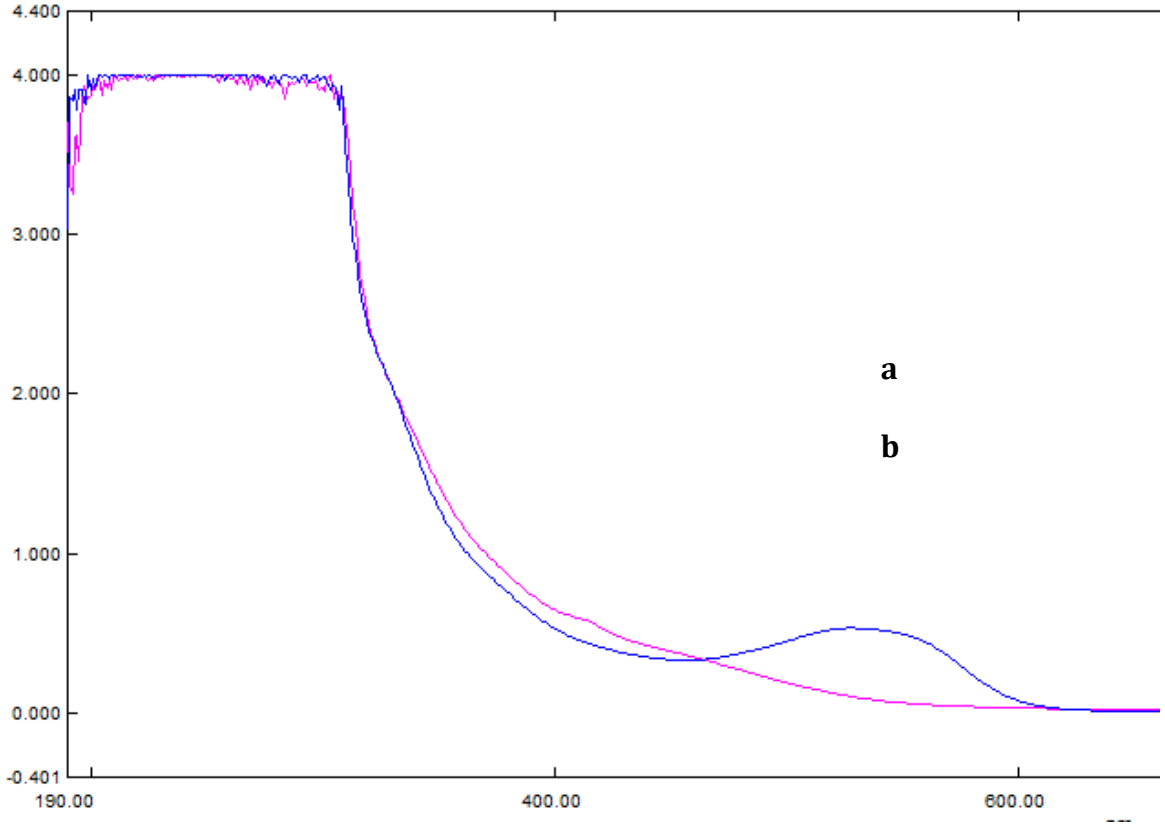


Fig.5 Spectral analysis of the Adsorption – Desorption Assay of the Isolate SI 01

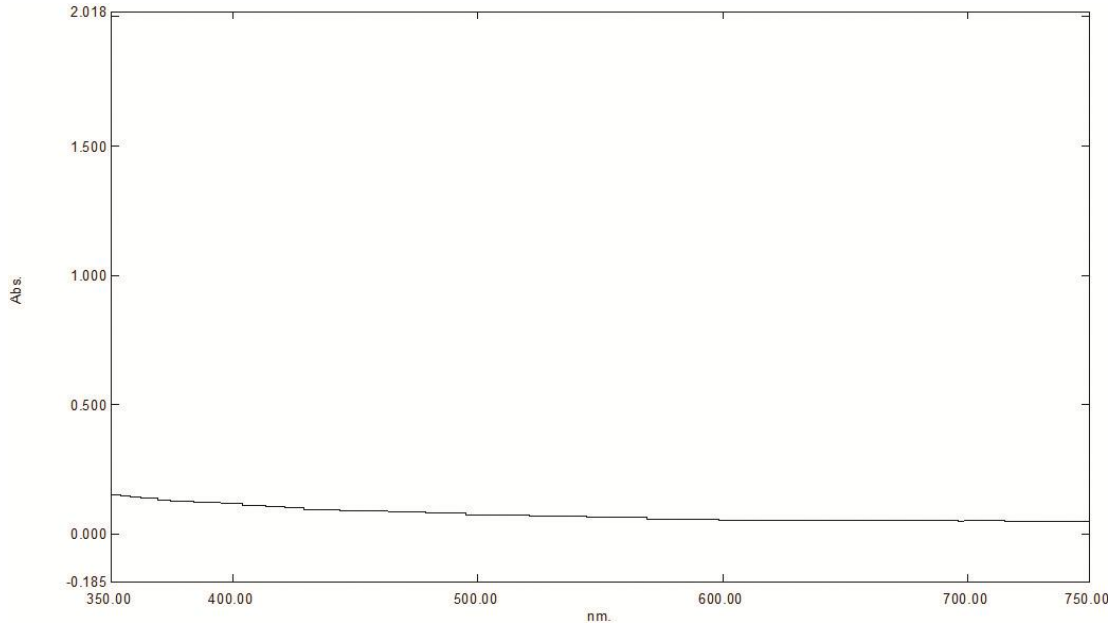
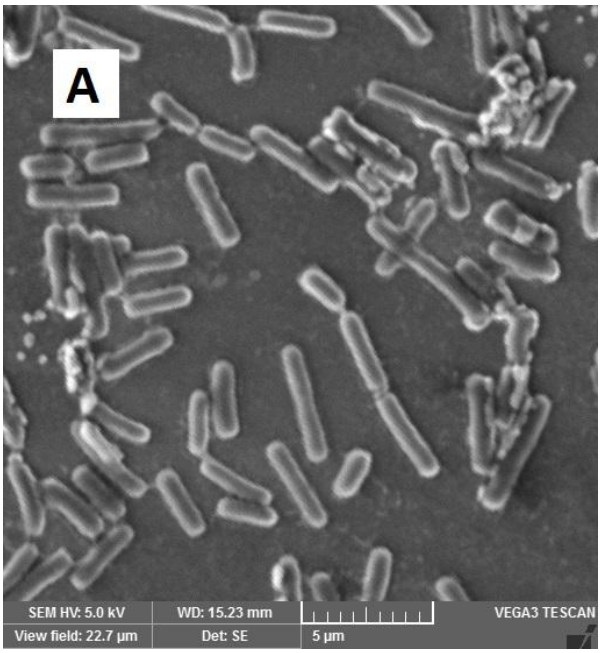
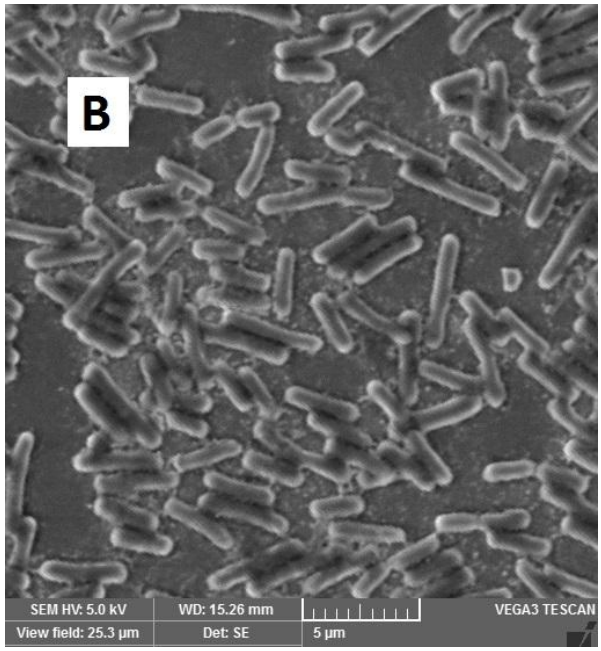


Fig.6 Scanning Electron Micrograph of the Soil Isolate 01; A - before decolorization (unexposed cells); B- after decolorization (exposed cells)





Azoreductase is the key enzyme responsible for the reductive azo dye degradation in bacterial species. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH, thus impeding electron transfer from NADH to the azo bond. The advantage of the anaerobic reduction of azo dyes is that oxygen depletion is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerophilic bacteria to reduce azo dyes. It is evident from the present study as the decolourization was not significant when maintained in shaking condition. However, the precise mechanism of anaerobic azoreduction is still not totally understood. It was recently suggested that microbial anaerobic azoreduction was linked to the electron transport chain, and that dissimilatory azoreduction was a form of microbial anaerobic respiration. In addition, different models for the nonspecific reduction of azo dyes by bacteria, which do not require transport of the azo dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested. These results suggested that azo dye reduction was a strain-specific mechanism that could be performed by an azoreductase enzyme or by a more complex metabolic pathway. Thus, due to the lack of information about the metabolism of *Lysinibacillus* the usual true time dependant kinetic determinations of the azoreductase activity using the azo dye as substrate were not performed, and the azo reduction mechanism of this novel *Soil Isolate 01*(*Lysinibacillus)* will be the subject of a future specific study. This methodology using a single microorganism in a sequential aerobic – microaerophilic process was shown to be very effective in azo dye decolourization. In a single reactor with a single bacterium, only changing the agitation conditions, it is possible not only to

decolorize the dyes, but also to achieve a good degree of mineralization and low toxicity, with low running and maintenance costs.

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