

Decolorization of Dyehouse Effluent and Biodegradation of Congo Red by *Bacillus thuringiensis* RUN1

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A dye-decolorizing bacterium was isolated from a soil sample and identified as *Bacillus thuringiensis* using 16S rRNA sequencing. The bacterium was able to decolorize three different textile dyes, namely, Reactive blue 13, Reactive red 58, and Reactive yellow 42, and a real dyehouse effluent up to 80–95% within 6 h. Some non-textile industrially important dyes were also decolorized to different extents. Fourier transform infrared spectroscopy and gas chromatography-mass spectrometer analysis of the ethyl acetate extract of Congo red dye and its metabolites showed that the bacterium could degrade it by the asymmetric cleavage of the azo bonds to yield sodium (4-amino-3-diazenyl-naphthalene-1-sulfonate) and phenylbenzene. Sodium (4-amino-3-diazenyl-naphthalene-1-sulfonate) was further oxidized by the ortho-cleavage pathway to yield 2-(1-amino-2-diazenyl-2-formylvinyl) benzoic acid. There was induction of the activities of laccase and azoreductase during the decolorization of Congo red, which suggests their probable role in the biodegradation. *B. thuringiensis* was found to be versatile and could be used for industrial effluent biodegradation.

Key words: Decolorization, dyehouse effluent, Congo red, UV-visible analysis, GC-MS analysis

The release of harmful wastewaters in and around the heavily industrial areas is a serious environmental and health concern. Most of the industries, the textile industries in particular, produce huge amount of wastewaters with spent dyes that are extremely difficult to be removed.

These dyes result in coloration of wastewaters, thereby reducing the dissolved oxygen and leading to oxygen sag in the receiving water bodies [1]. In addition, the formation of tumors, cancers, and various allergies have been associated with the presence of some of these dyes in wastewaters [19]. Therefore, developing low cost and effective methods of degradation of these dyes is vital.

Most of the methods used in textile effluent treatment are channeled towards color removal. These methods are either ineffective or expensive and moreover generate more toxic products that are recalcitrant to biodegradation processes [2, 11]. Microbial decolorization and detoxication, however, pose as a cost-effective and eco-friendly alternative [22]. Yet, anaerobic decolorization of colored effluent often produces aromatic amines, which are more toxic, mutagenic, and/or carcinogenic than the parent dyes [5]. Although, the aromatic amines could be degraded aerobically, aerobic decolorization of colored effluent is a subject of discussion [21], thus leading to make 2-phase aerobic/anaerobic systems popular; there are however concerns about the time requirements of such processes. The need to develop textile effluent biotreatment processes that would not only decolorize but also mineralize the toxic metabolites generated during wastewater treatments could not be overstressed.

Studies on mineralization are only possible with specific dyes with well-known structures, as the effluents are complex in nature [16]. Congo red is the sodium salt of benzidinediazo-*bis*-1-naphthylamine-4-sulfonic acid. It is a secondary diazo dye that is immensely used for the coloration of paper products, in medicine (as a biological stain), as an indicator since it turns from red-brown in basic medium to blue in acidic, and to color textiles and wood pulp. Congo red is recalcitrant to most of the treatments and is thus found in effluents of many industries, particularly

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the paper factories. The dye also acts as a potent carcinogen and mutagen because of the presence of aromatic amine groups [8].

Bacillus thuringiensis, a facultative anaerobic, motile, Gram-positive, spore-forming bacterium, has been used extensively in the environmental control of insect dwelling in lakes and rivers. The organism has also been used in agricultural pest control systems, and its gene has been incorporated into different types of crops in what is known as *Bt* crops [4]. The organism and its anti-pest proteins have not been reported to have any known effect on wildlife such as mammals, birds, fish, or aquatic vertebrates. Very few reports on the ability of this organism to decolorize or degrade dyes have been published.

In the present investigation, we have evaluated the decolorization of dyehouse effluent, textile dyes, and other non-textile industrially important dyes by a newly isolated strain of *B. thuringiensis*. Enzymes involved in the degradation were assayed and its ability to mineralize Congo red was also investigated.

MATERIALS AND METHODS

Dyes and Chemicals

Methyl red and Congo red were obtained from Merck (Mumbai, India). Veratryl alcohol was obtained from Himedia Pvt. Ltd., India. Catechol, *o*-tolidine, *n*-propanol, and other chemicals were purchased from Sisco Research Laboratory, India. Dyehouse effluent was collected from a local textile industry at Abeokuta, Ogun State, whereas the technical-grade reactive dyes used were collected at Atlantic Textile Mills, Lagos, Nigeria.

Isolation and Screening of Organism for Dye Decolorization

Azo dye-decolorizing bacteria were isolated from soil collected around the refuse site of Redemption City, Ogun State, Nigeria. The isolation was done using enrichment culture: soil sample was suspended in sterile distilled water, and 5 ml suspensions were added to 100 ml conical flasks, each containing 50 ml of nutrient broth, pH 7.2. The nutrient broth was supplemented with a mixture of three azo dyes: Reactive blue 13 (RB13), Reactive red 58 (RR58), and Reactive yellow 42 (RY42), and incubated at 30°C. The nutrient broth contained (g l⁻¹) beef extract 1 g, yeast extract 2 g, peptone 5 g, and NaCl 5 g. The decolorized dye-broth solution was then cultured on nutrient agar plates. Organisms that survived were subcultured into fresh plates for identification. The bacterium used in this study was among the prominent decolorizers of the textile dyes mixture. It was constantly maintained on nutrient agar slants at 4°C.

Identification of Dye-Decolorizing Bacteria

Identification of the dye-degrading microorganism was initially done using colony morphology, microscopy identification, and biochemical characterization. Further identification was carried out using full-length 16S rRNA gene sequencing (Bangalore Genei, India) and 16S-23S ITS (Laragen Inc., USA). The 16S rRNA gene sequence was initially analyzed at the NCBI server (<http://www.ncbi.nlm.nih.gov>) using the BLASTn tool and corresponding sequences were downloaded.

A phylogenetic tree was constructed by the neighbor joining method using the MEGA4 package [20].

Decolorization Experiments

Approximately 0.05 g dry of biomass of the organism was added into 100 ml of 100 mg/l dye-broth solutions at static anoxic condition. Nutrient broth was added to the dyehouse effluent and sterilized prior to inoculation. A 3 ml sample was withdrawn at every 3 h interval for 24 h; the samples were centrifuged at 2,236 ×g for 15 min to remove biomass and the biomass content was determined according to the study carried out by Olukanni *et al.* [16]. The decolorization of the dyehouse effluent (DE), Congo red (CR), Reactive blue 13 (RB), methyl red (MR), malachite green (MG), and crystal violet (CV) were estimated at their predetermined λ_{max}: 548, 480, 568, 420, 617, and 585 nm respectively.

Percentage decolorization was recorded as $(A_0 - A_t)/A_0 \times 100$, where A₀ and A_t are initial and final absorbance units, respectively [14]. Decolorization of the respective dyes was also monitored using the absorption spectrum (700–300 nm) with a Hitachi UV-visible spectrophotometer (Hitachi U-2800). The spectra were compared with those of abiotic controls of dye solutions.

Biodegradation Analysis

Biodegradation was determined by comparing the Fourier transformed infrared spectroscopy (FTIR) peak profiles of the metabolite of Congo red and those of its abiotic control. Attempt was also made to identify the dye metabolites using their gas chromatography-mass spectroscopy (GC-MS) spectra. The decolorized Congo red solution, withdrawn after 24 h and centrifuged at 8,944 ×g for 10 min, was extracted using ethyl acetate. The extract was dried in a rotary evaporator and re-dissolved in high-performance liquid chromatography grade methanol for GC-MS analyses. FTIR analysis of biodegraded Congo red was carried out using a Shimadzu 800 spectrophotometer and compared with that of the control dye. The FTIR analysis was done in the IR region of 400–4,000 cm⁻¹ with 16 scan speed. The samples were mixed with spectroscopically pure KBr for pellets formation and the pellets were used for the analyses.

The identification of metabolites formed after degradation was done using a QP2010 GC-MS system (Shimadzu, Japan). Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80°C for 2 min, after which it increased linearly at 10°C/min to 280°C, which was held for 7 min. The ionization voltage was 70 eV. The temperature of the injection port was 280°C and the GC-MS interface was maintained at 290°C. The helium carrier gas flow rate was 1.0 ml/min. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta; Shimadzu) of the GC-MS.

Enzyme Assays

Activities of laccase and veratryl alcohol oxidase were determined using the procedure reported earlier by Olukanni *et al.* [16]. Azoreductase enzyme activity was assayed using the method of Maier *et al.* [11]; the assay mixture (2 ml) contained 50 mmol/l phosphate buffer (pH 7.4), 1 mmol/l NADH, 0.25 mmol/l substrate, and 0.2 ml of crude enzyme. The reaction mixture was pre-incubated for 4 min, followed by the addition of NADH, and monitored for the decrease in color intensity at 430 nm. Lignin peroxidase activity

was determined as the H₂O₂-dependent oxidation of veratryl alcohol to veratrylaldehyde; the increase in absorbance at 310 nm due to the oxidation of veratryl alcohol to veratrylaldehyde was monitored [6]. The reaction mixture of 2 ml contained, 0.2 ml veratryl alcohol (10 mM), 1.6 ml citrate buffer (pH 3.0), and 0.2 ml enzyme. The reaction was initiated with the addition of 100 µl of 0.5 mM H₂O₂ and monitored over 3–5 min. Tyrosinase activity was determined in a reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) by measuring liberated catechol quinone at 495 nm [24]. Enzyme activity was recorded as absorbance units min⁻¹ mg protein⁻¹.

RESULTS

Phylogenetic Position of Isolate

To analyze the phylogenetic position, the full-length 16S rRNA gene sequence of strain RUN1 (1,307 bp, KC153529)

was determined. Fig. 1A shows the phylogenetic relationship between the strain and other related microorganisms found in the GenBank database. The homology assay result indicated that the strain was either *B. cereus* or *B. thuringiensis*. Further analysis using the strain's 16S-23S intergenic spacer region (233 bp, KC153530), however, confirmed that the strain was in the phylogenetic branch of *Bacillus thuringiensis* (Fig. 1B).

Decolorization of Dyehouse Effluent and Various Dyes

The result of the decolorization experiment showed that approx. 0.05 g dry cell mass (DCM) of the bacterium decolorized 100 ml of the dyehouse effluent (84.74 ± 2.21%), methyl red (91.15 ± 0.53%), and Reactive blue 13 (97.30 ± 2.03%), within 6 h. The organism also decolorized Congo red (72.84 ± 3.25%) and malachite green (82.02 ± 0.35) in 12 h and 24 h, respectively. Crystal violet was

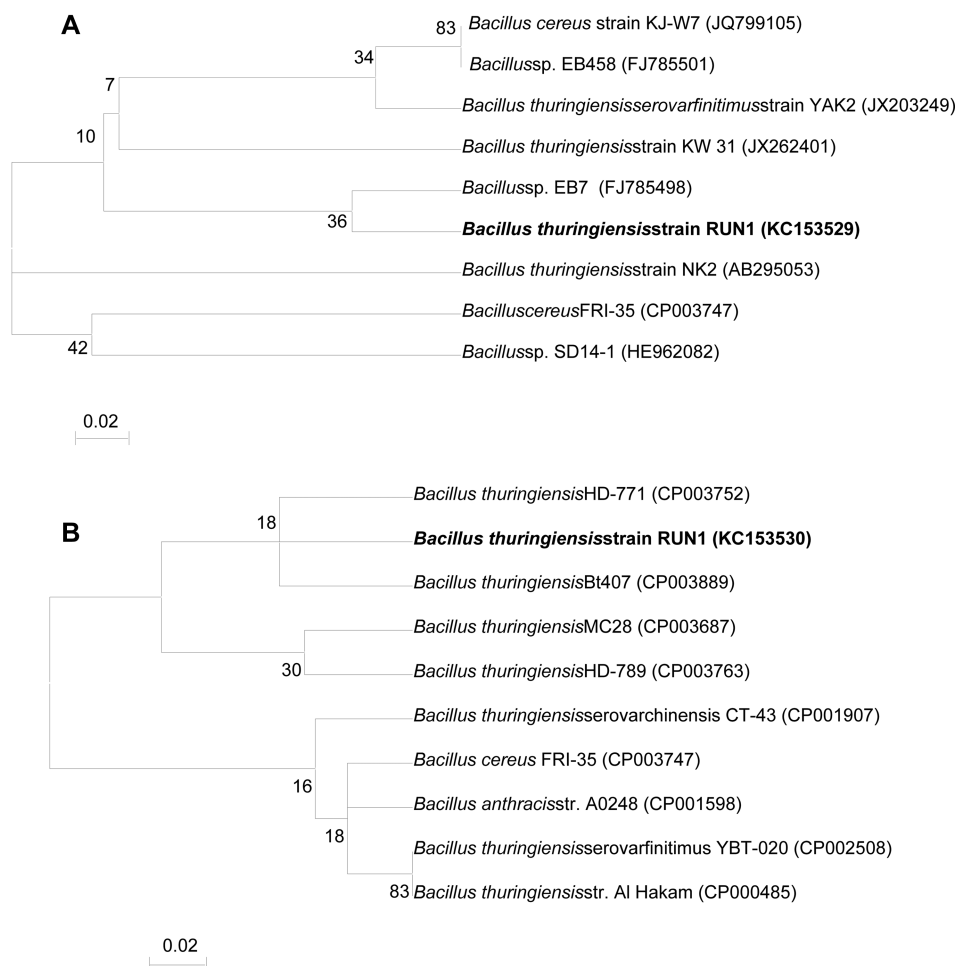


Fig. 1. Phylogenetic analysis of (A) 16S rRNA gene sequence and (B) 16S-23S intergenic spacer sequence of bacterial isolate *Bacillus thuringiensis* strain RUN1.

The distance tree was constructed using the neighbor-joining method by using MEGA4. The sequences have been retrieved from the NCBI, showing the phylogenetic relationships of *Bacillus thuringiensis* RUN1 and other related microorganisms found in the GenBank database. Numbers at nodes show the level of bootstrap support based on data for 1,000 replications. Bar, 0.01 substitutions per nucleotide position; numbers in parenthesis represent GenBank accession numbers.

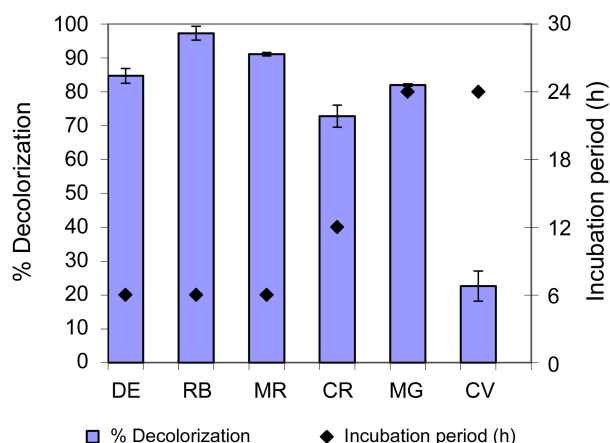


Fig. 2. Decolorization of dyehouse effluent and other known recalcitrant dyes by *Bacillus thuringiensis* RUN1. DE, Dyehouse effluent; RB, Reactive blue 13; MR, methyl red; CR, Congo red; MG, malachite green; CV, crystal violet.

however recalcitrant to decolorization with only 22.62 ± 4.46 , even at 24 h (Fig. 2). UV-Vis scan (400–800 nm) of Congo red, when compared with its metabolites formed after degradation, showed removal of the peaks responsible for the λ_{\max} of the dye (Fig. 3).

Analysis of Enzyme Activities

The cell-free extract of the *B. thuringiensis* grown in nutrient broth showed the activities of enzymes known for biodegradation, both in the absence and presence of dye as inducer. Azoreductase, laccase, lignin peroxidase, veratryl alcohol oxidase, and tyrosinase activities were recorded during the time course of Congo red degradation (Table 1). The activities of azoreductase, laccase, and lignin peroxidase were noticed in both extracellular and intracellular cell-

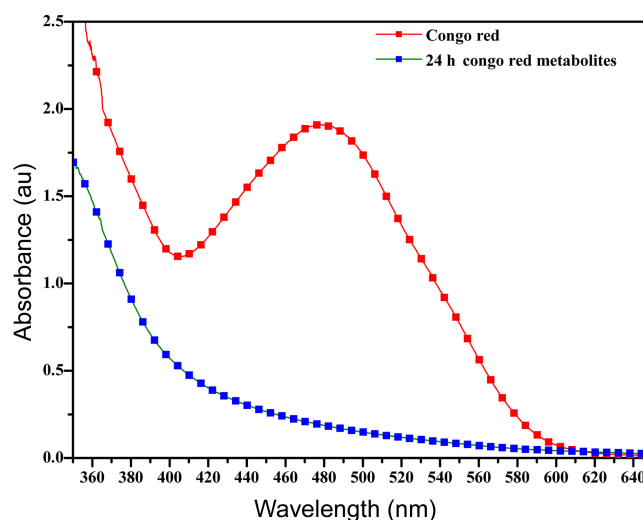


Fig. 3. UV-visible spectra of Congo red solution and that of its 24 h metabolites, showing the removal of peak due to maximum absorbance.

free extract of the *B. thuringiensis* culture. The activities of azoreductase and laccase were however higher when Congo red was added to the culture as the inducer. Tyrosinase activity was only found in the extracellular extract when Congo red was added as the inducer.

Biodegradation Analysis

The ability of the bacterium to degrade the dyes was investigated using FTIR and GC-MS analyses of Congo red. The FTIR spectrum of the dye when compared with that of the extracted metabolites (24 h) is shown in Fig. 4. Peaks in the control dye spectrum represent the stretching vibrations of S=O at $1,047 \text{ cm}^{-1}$, symmetric stretching at $2,948 \text{ cm}^{-1}$, and asymmetric stretching at $2,868 \text{ cm}^{-1}$ for

Table 1. Specific activities of important biodegradation enzymes in both intracellular and extracellular extracts of *Bacillus thuringiensis*.

		Extracellular ^a	Intracellular ^a	Substrate
Azoreductase	(Control ^b)	0.13 ± 0.07	0.54 ± 0.04	Methyl red (+ NADH)
	(Induced ^c)	0.03 ± 0.02	1.35 ± 0.07^d	
Laccase	(Control)	0.37 ± 0.04	ND	<i>O</i> -Tolidine
	(Induced)	0.70 ± 0.03	14.23 ± 0.25^d	
Veratryl alcohol oxidase	(Control)	1.71 ± 0.08	ND	Veratryl alcohol
	(Induced)	0.90 ± 0.05	ND	
Lignin peroxidase	(Control)	0.91 ± 0.06	4.59 ± 0.31	Veratryl alcohol (+ H ₂ O ₂)
	(Induced)	1.27 ± 0.03	4.51 ± 0.70	
Tyrosinase	(Control)	ND	ND	Catechol
	(Induced)	0.27 ± 0.07^d	ND	

^aEnzyme activities, Units/min/mg of protein.

^bControl, 24 h culture without the addition of dye.

^cInduced, After 24 h culture with dye (Congo red).

^dEnzymes were probably induced.

ND, Activity not in detectable range.

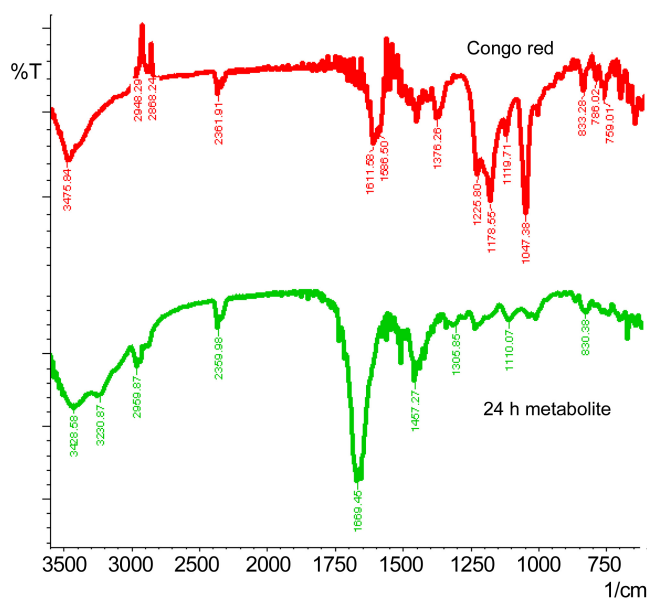


Fig. 4. FTIR spectra of Congo red and its 24 h metabolites showing the distortion of aromatic peaks and formation of the carbonyl peak.

C-N. The $3,475\text{ cm}^{-1}$ peak represented the presence of free N-H group from the parent dye structure and the stretching between C-H was reported at $2,948\text{ cm}^{-1}$. The -N=N- stretching of the azo group was reported between $1,578$ and $1,611\text{ cm}^{-1}$, whereas the peaks at $769\text{--}833\text{ cm}^{-1}$ were those of aromatic C-H bending. The FTIR spectrum of 24 h extracted metabolites showed significant changes in the positions of peaks when compared with the spectrum of control dye (Fig. 4). The comparison showed the disappearance of the peaks at the fingerprint region, the removal of the peak at $1,047\text{ cm}^{-1}$, the widening of the $3,428\text{ cm}^{-1}$ peak, and the deformation of the $769\text{--}829\text{ cm}^{-1}$ peaks. New peaks were also formed at $1,457$ and $1,669\text{ cm}^{-1}$. The result of the GC-MS analysis revealed nine major peaks, whose molecular weight were lower than that of the dye (Table 2).

DISCUSSION

B. thuringiensis has been widely used in the environment as a biopesticide. This study considered the potential of this bacterium in the biotreatment of industrial effluent. The result of the decolorization of dyehouse effluent showed the disappearance of the peaks in the visible region of the UV-visible analyses. This has been reported as an indicator of decolorization [10, 15], thus suggesting that the organism could be effective in the treatment of textile effluent. The ability of *B. thuringiensis* to decolorize recalcitrant triphenylmethane dyes such as malachite green

Table 2. The GC-MS parameters of Congo red's 24 h metabolites.

Peak #	Retention time	Area %	m/z	Base peak
I	12.359	7.65	224	73.10
II	15.869	4.93	217	73.10
III	20.041	7.91	205	128.15
IV	21.378	15.35	196	154.15
V	21.560	2.68	177	156.15
VI	21.702	5.23	192	70.10
VII	22.594	14.68	202	154.15
VIII	22.784	32.66	200	154.15
IX	22.889	8.91	201	154.15

and diazo dye (Congo red) showed that the strain could be useful in the management of other industrial effluents. Malachite green is used in the fish industry [18], whereas Congo red is a common dye in the pulp and paper industries [3].

The presence of azoreductase and laccase activities suggested both specific and nonspecific reduction of the azo bond prior to mineralization. Laccase is known to attack aromatic rings *via* two steps of electron transfer in the presence of molecular oxygen [13], whereas veratryl alcohol oxidase has been implicated in the prevention of polymerization of reactive rings produced by laccase [12], thereby making laccase products susceptible to ring cleavage. Veratryl alcohol oxidase has also been reported to decolorize dyes *in vitro* [7]. The induction of tyrosinase as an exclusively extracellular enzyme suggested that there is no need for the dye or its metabolites to cross the cell membrane prior to ring cleavage. Recent reports have shown that certain bacteria produced the lignin-degrading enzyme, which has ability to degrade polymeric dyes from the textile industry and paper whitening through oxidative cleavage [9, 10, 17]. Generally, lignolytic enzymes are known for their immense roles in the biodegradation of pollutants [23].

The disappearance of the peaks at the fingerprint region in the FTIR spectrum of 24 h extracted metabolites is clear evidence of biodegradation. The removal of the peak at $1,047\text{ cm}^{-1}$ suggested desulfonation of the parent compound, and the widening of the $3,428\text{ cm}^{-1}$ peak indicated formation of compounds with a hydroxyl (OH) group. A new peak at $1,457\text{ cm}^{-1}$ represented C-H deformation of acyclic CH_2 . The C-H deformation between 769 and 829 cm^{-1} pointed towards removal of the aromatic ring, and the sharp peak at $1,669\text{ cm}^{-1}$ suggested the formation of compounds with C=O stretching. GC-MS analysis of the dye metabolite showed the presence of derivatives of naphthalene and tolidine with clear evidence of ring cleavage. Based on the enzyme assays results and the identified metabolites, a pathway was proposed for the biodegradation of Congo red by *B. thuringiensis* (Fig. 5). Our approach of the experiment

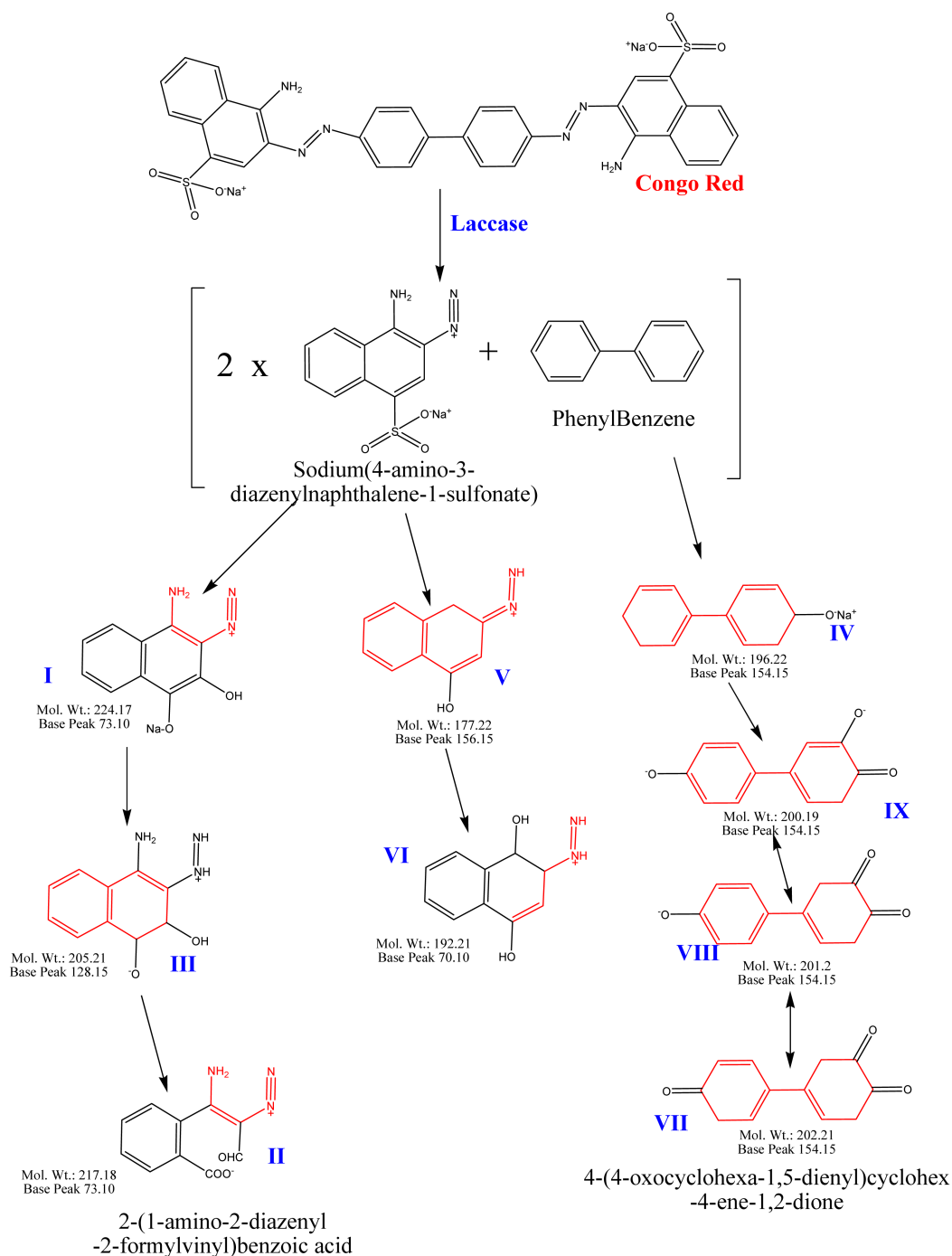


Fig. 5. Proposed pathway for the biodegradation of Congo red by *Bacillus thuringiensis* RUN1.

Structures responsible for the base peaks are in red. Blue numberings are peak numbers as eluted by the GC-MS. (For interpretation of the references to color in this figure, the reader is referred to the web version of the article.)

showed various steps involved in the degradation: either the laccase or the lignin peroxidase catalyzed the initial asymmetric cleavage of Congo red to produce two molecules of sodium (4-amino-3-diazenylnaphthalene-1-sulfonate) and a molecule of phenylbenzene. Other enzymatic activities resulted in intermediate products, some of which were

identified as 3-diazonium naphthalen-1-ol with the retention time of 21.56 min and a mass peak of 177; sodium phenylphenoxide with the retention time of 21.38 min and a mass peak of 196; and 4-(4-oxocyclohexa-1,5-dienyl)cyclohexa-4-ene-1,2,-dione with the retention time 22.59 min and a mass peak of 202. The metabolite with m/z value of 217.18

and base peak of 73.10, identified as 2-(1-amino-2-diazenyl-2-formylvinyl) benzoic acid, supports the ortho cleavage of one of the metabolites of Congo red, thus suggesting the ability of the organism to mineralize the dye.

In conclusion, it can be said that a strain of *Bacillus thuringiensis* that can decolorize and degrade industrially important dyes was successfully isolated. The strain harbors important biodegradation enzymes such as laccase, veratryl alcohol oxidase, and azoreductase. A pathway for the biodegradation of Congo red was proposed based on metabolites identified using GC-MS and enzyme assays. The research also suggested that *B. thuringiensis* might be doing more than pest control in the environment.

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