Biodegradation of Malachite Green by Extracellular Laccase Producing *Bacillus thuringiensis* RUN1

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Abstract: A bacteria strain *Bacillus thuringiensis* RUN1 identified by 16s RNA gene phylogenetic analysis was used to decolorize malachite green, a triphenylmethane dye in a simulated wastewater. The ability of the organism to produce extracellular laccase and degrade the dye were also investigated. Results showed that the organism decolorized (84.67 ± 1.19 %) malachite green at 40 mgl^{-1} within 6 h; and the decolorization was associated to laccase production by the organism experimentally. Laccase activity increased as the decolorization process progressed, with the highest activity value of 0.1043 ± 0.02 U/min/ mg protein recorded after 24 h of incubation using ABTS as substrate. In addition, FTIR analysis showed that the strain actually degraded the dye. It was therefore concluded that this strain of *Bacillus thuringiensis* will be relevant in the biotreatment of industrial effluent containing malachite green and in the production of laccase, an industrially important enzyme.

Keywords: Laccase, malachite green, decolorization, biodegradation, *Bacillus thuringiensis*.

INTRODUCTION

In textile dyeing and printing processes 10-15% of the dyes remains as unreacted dyestuffs and are lost directly in the effluents of textile units, rendering such wastewater highly colored [1-3]. It is estimated that 280,000 tonnes of textile dyes are discharged annually in such industrial effluents worldwide [4]. The presence of dye in water even at a very low concentration (10-50mgl^{-1}) is highly visible and affects the aesthetic quality, water transparency and gas solubility of water bodies [2, 5]. Due to reduced light penetration caused by the presence of dyes in receiving water, photosynthetic activity in such polluted aquatic environment may be significantly affected [6].

Among many classes of synthetic dyes used in the industries, triphenyl methane group of dyes such as malachite green and crystal violet constitute a major and versatile group that play a predominant role in almost every type of application [5]. Malachite green (MG) is an N-methylated diaminotriphenylmethane dye that has been used extensively for dyeing silk, wool, jute, leather, ceramic and cotton [7]. It is highly soluble in water and has also been used in aquaculture industry as a fungicide, parasiticide and disinfectant [8]. MG has however been found to be highly toxic to mammalian cells; promote hepatic tumor formation in rodents and cause reproductive abnormalities in rabbits and fish [9]. It was nominated by the Food and Drug Administration as a priority chemical for carcinogenicity testing by the National Toxicology Programme of the USA [7]. The need for effective removal of such dyes from wastewater is therefore expedient.

Amongst the methods used in biological treatment of wastewater containing dyes, the microbial decolorization and degradation of dyes has been of considerable interest [10]. Bacterial treatment offers a cheaper and environment friendlier alternative for color removal in textile effluents [11]. The potential of microbes to degrade synthetic dyes have been linked to the production of enzymes during degradation [5, 10]. It is therefore imperative to study the metabolic and enzymatic systems of dye-degrading bacteria in order to gain insight into their degradation metabolisms.

Lignolytic enzymes majorly produced by the white rot fungi have been widely studied for their potential application in bioremediation [12]. The three major classes of these enzymes include laccases, lignin peroxidases and manganese dependent peroxidases. Laccase has been shown to be of critical importance to the dye-degrading potentials of fungi [13, 14] and a recent study has demonstrated the versatility of laccase as industrial biocatalyst [15]. Laccases carry out one-electron oxidation and reduce molecular oxygen to water. The biotechnological importance of

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this enzyme lies in its ability to oxidize both phenolic and non-phenolic lignin-related compounds [1] as well as highly recalcitrant environmental pollutants [16]. This type of degradation is expected to be very useful for the treatment of wastewater and dye degradation [17], especially for the textile and the paper and pulp industry.

The enzymatic treatment of wastewater requires the production of large amounts of enzymes, in this case laccases, at low cost. The current commercial price of laccases is high, constituting a drawback for its use. A low-cost process for the production of laccases is necessary for a sustainable enzymatic wastewater treatment. In this study, the ability of *Bacillus thuringiensis* to produce dye-degrading extracellular laccase during the decolorization and biodegradation of malachite green was investigated.

MATERIALS AND METHODS

Chemicals and Media

Malachite green (MG) was obtained from SD-Fine Chemicals Limited (Biosar, India). 2,2-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was obtained from Sigma Aldrich Chemicals Company (St. Louis, MO, USA). Guaiacol was obtained from SRL Chemicals, India. Syringic acid, peptone, nutrient broth, beef extract, yeast extract and agar powder were obtained from Hi-Media laboratory, India. All chemicals were of highest purity and analytical grade.

Strain Isolation and Characterization

Dye-decolorizing bacteria were isolated from soil samples obtained from a garbage disposal site around Redemption City, Mowe, Ogun State, Nigeria (6°34'10"N, 3°21'59"E). The organisms were first screened for the ability to decolorize textile dyes using 60 mgl^{-1} dyes in nutrient broth after which they were maintained on nutrient agar slants at 4^oC. The pure culture was grown in 250 ml Erlenmeyer flask containing 100 ml nutrient broth (g/L): beef extract 1 g, yeast extract 2 g, peptone 5 g and NaCl 5 g, at 37^oC for 24 h. The 24 h culture was kept at 4^oC and used as the seed inoculums.

The identification of the dye decolorizing microorganisms was initially done using colony morphology, microscopy identification and biochemical characterization. Further identification was done for the best decolorizing strain using 16S rRNA gene sequence analyzed at Laragen Inc, California, USA. The 16S rRNA gene sequence obtained was compared with those deposited at the GenBank database at NCBI server (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) to identify and download corresponding sequences. Phylogenetic tree was then constructed by Neighbour-Joining (NJ) method using the Molecular Evolutionary Genetics Analysis (MEGA4) package by Tamura *et al.* [18].

Decolorization Assay

The decolorization of malachite green by the isolated bacterial strain was studied in 250 ml Elenmeyer flasks containing 100 ml sterile nutrient broth (13 mgl^{-1}) containing MG. 2 ml of bacterial suspensions ($O.D_{600}$ = 2.011) were inoculated aseptically into each culture flask and incubated at 30^oC. All decolorization experiments were performed in triplicates with an abiotic control set up in each case. In order to evaluate the removal of MG from the culture medium, an aliquot of 2 ml was withdrawn at one hour time intervals between 0 h and 6 h. Biomass was removed by centrifugation at 4000 rpm for 20 mins. Decolorization was followed by monitoring the absorbance of the cell-free supernatant at the dye's predetermined maximum absorbance wavelength (λ_{max}) of 618 nm using UV/VIS spectrophotometer (UV-1650PC Shimadzu, Japan).

Percentage decolorization was then calculated according to Olukanni *et al.* [11] as:

$$P = [(A_o - A_t)/A_o]x\ 100\% \text{ ----------} \quad \text{(Eq.1)}$$

Where P = Percentage decolorization of MG, A_o = Initial absorbance of MG and A_t = Absorbance of inoculated effluent (or final absorbance) after time t.

To determine the effect of dye concentration on decolorization activity of *Bacillus thurigiensis*, decolorization assays with varying initial dye concentrations between 10 and 100 mgl^{-1} were carried out.

Biodegradation Analysis

Fourier Transform Infrared Spectroscopy (FTIR) analysis of the metabolites was performed to confirm the biodegradation of MG. The decolorized medium was centrifuged at 4000 rpm for 20 minutes and the cell free supernatant obtained was used to extract metabolites with equal volume of ethyl acetate. The extracts were allowed to evaporate and dried in a desiccator. FTIR analysis of extracted metabolites was

done on Fourier Transform Infrared Spectrophotometer (FTIR-8400S Shimadzu, Japan) instrument and compared with control dye in the mid IR region of 400-4000 cm^{-1} with 16 scan. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95 prior to analyses.

Enzyme Assay

The activity of laccase was assayed spectrophotometrically in cell free extract as described by Wolfenden and Willson [19], by monitoring the oxidation of (ABTS) at 420 nm in a reaction mixture containing 0.1 mM ABTS in 0.1 M acetate buffer (pH 4.0). All enzyme assays were carried out at room temperature (25°C). Reference blanks contained all components except the enzyme. The ability of the crude enzyme to utilize other common laccase substrates such as guaiacol and syringic acid was also investigated; these were done by monitoring the substrate disappearance at 465 nm for guaiacol and at 272 nm for syringic acid [16]. Extracellular protein was determined by Lowry method, using bovine serum albumin as standard. Specific activities of the enzyme were described as change in absorbance unit per minute per mg protein. The results were expressed as means of triplicate $\pm$ standard deviation.

RESULTS AND DISCUSSION

Characterization of the MG-Degrading *Bacillus thuringiensis* Strain

The phylogenetic tree obtained when the 16s RNA gene sequence of the organism was analysed using MEGA4 is shown in Figure **1**. The sequence was subsequently deposited at the GenBank as Bacillus *thuringiensis strain* RUN1 with accession number HQ873480.

Decolorization of MG by *Bacillus thuringiensis*

The strain demonstrated 84.67 ± 1.19 % decolorization of the malachite green at 40 mgl^{-1} concentration, within 6 h (Figure **2**). The result is quite commendable when compared with other works involving the degradation of the dye by individual organisms or microbial consortia (Table **1**). Similarly, the UV-Visible spectral scan showed a reduction in the absorbance peaks of MG at its λ_{max}, 618 nm. Absorbance values decreased from 1.553 at 0 h to 0.766 at 3 h and further to 0.264 at 6 h (Figure **3**) thus depicting progressive color removal by *Bacillus thuringiensis.* It was also observed that decolorization efficiency increased with increasing concentration of MG up to 40 mgl^{-1} at 6 h (Figure **2**) and over 90%

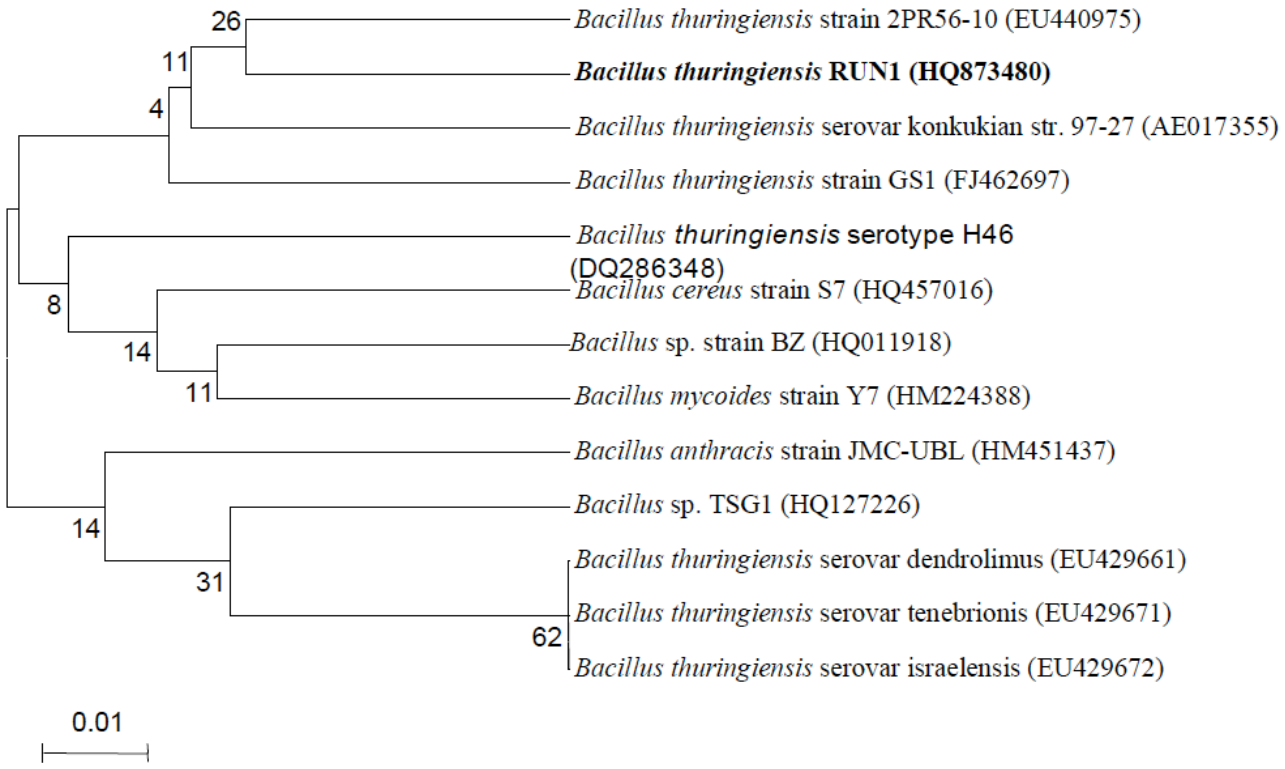

Figure 1: Phylogenetic analysis of 16S rRNA gene sequence of bacterial isolate *Bacillus thuringiensis* strain RUN1.

decolorization was observed at 24 hours at 50 mgl^{-1} MG concentration (data not shown). The initial increase in decolorization as the concentration increased may be due to induction of enzymes involved in decolorization. It has been shown that dyes that act as inducers of enzyme production in a culture medium are in turn decolorized by the enzymes, and the highest inducer is decolorized highest [20]. Significant induction in DCIP reductase and MG reductase activities were observed during decolorization of MG by *K. rosea* [10]. When the concentration was increased to 80 mgl^{-1} however, decolorization efficiency dropped to 49.32 $\pm$ 0.46%, indicating that MG may be toxic to the organism at the higher concentration levels. It has been shown that 1 mM concentration of MG completely inhibits the growth of bacterium, which indicates toxicity of MG at higher dye concentration [3], while Cha *et al*. [9] and Youssef *et al.* [21] have also reported similar observation for inhibition of fungal growth at higher concentration of MG.

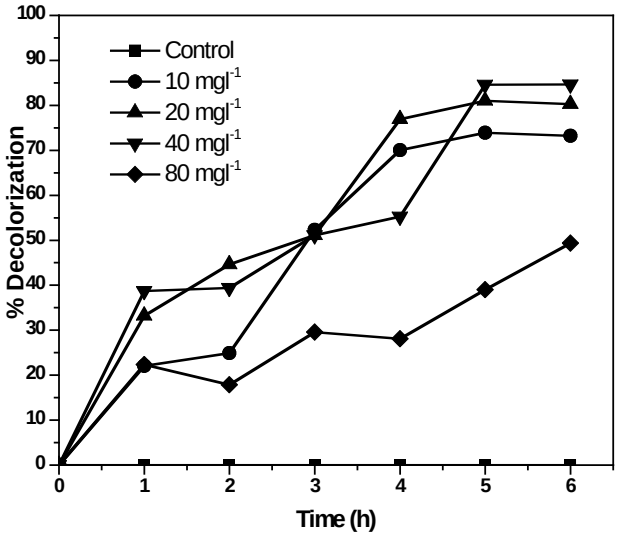

Figure 2: Time course plot showing the decolorization of various concentrations of MG. Organism was grown in 100 ml of different concentrations of MG: Control (■), 10 mgl^{-1}(●), 20 mgl^{-1}(▲), 40 mgl^{-1}(▼) and 80 mgl^{-1}(♦).

Biodegradation of MG by *Bacillus thuringiensis*

The absorbance peak of MG at its predetermined λ_{max} (618 nm) was observed to diminish progressively, while the λ_{max} value shifted to lower wavelength in the decolorised medium (Figure **3**). This result is an indication of the degradation of the dye and suggested that demethylated products may have been formed. A shift in the λ_{max} value of a dye is often attributed to its degradation [17, 10, 21]. It is also known that demethylated products of MG show absorption maxima at wavelengths lower than that of MG [10].

The FTIR spectra of control MG and its 24 h extracted metabolites (Figure **4**) showed marked variations in the fingerprint region (1500 to 500 cm^{-1}). Prominent peaks observed in the spectrum of control MG in the fingerprint region (1500 to 500 cm^{-1}) for the mono-substituted and para-disubstituted benzene rings had diminished in the spectrum of the 24 h extracted metabolites. Parshetti *et al*. [10] reported the variations in the FTIR spectra of MG and that of its 5 h as an indication of biodegradation. Thus it was confirmed that this strain of *B. thuringiensis* decolorised MG broth culture by degrading the dye. This suggests the potential ability of the strain for the removal of malachite green from dyed wastewater, moreso that the organism is non-pathogenic and a well known biopesticide [22].

Extracellular Enzyme Production

The specific activities of the extracellular crude laccase increased from 0.0104 U/mg protein/min at 3 h to 0.0392 U/ mg protein/min at 5 h during decolorization (data not shown). This is consistent with the results in Figure **2**, which shows increasing decolorization efficiency with incubation time, thus suggesting that laccase produced by this strain of *B. thuringiensis* is induced by the dye and may be involved in the decolorization process. The involvement

Table 1: Comparison of Microbial Decolorization of Malachite Green in Recent Works

Organism	Concentration	Decolorization	Time (h)	Author
Kocuria rosea MTCC 1532	50 mgl^{-1}	~100%	5 hours	Parshetti *et al.* [10]
Fomes fomentarius	100 mgl^{-1}	<30%	20days	Jayasinghe *et al.* [12]
Acremonium kiliense	5 mgl^{-1}	95.4%	72 hours	Youssef *et al.* [21]
Mixed culture (Pseudomonas sp. and E.Coli)	100 mgl^{-1}	98%	24 hours	Tom-Sinoy *et al*. [25]
Bacillus thuringiensis	40 mgl^{-1}	84.6%	6 hours	This work
Bacillus thuringiensis	50 mgl^{-1}	> 90%	24 hours	This work

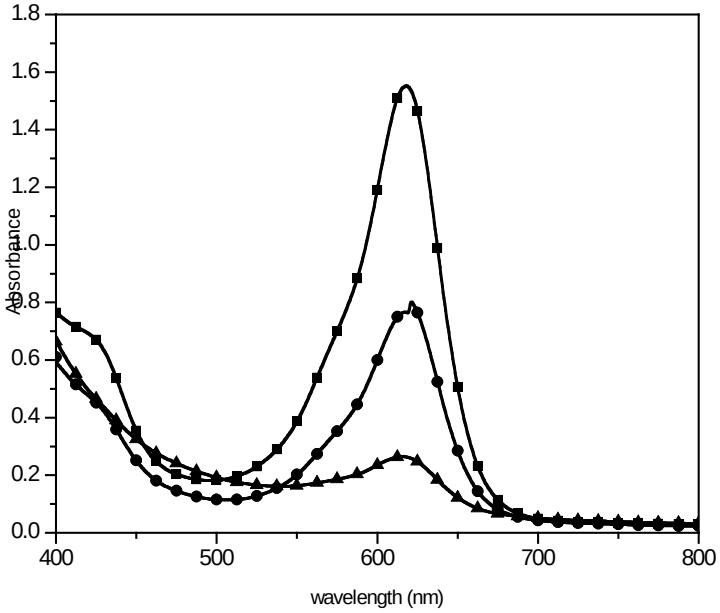

Figure 3: UV-Visible spectral scan of MG at 0 h (■, control) and after degradation by *Bacillus thuringiensis* (●, 3 h; ▲, 6 h).

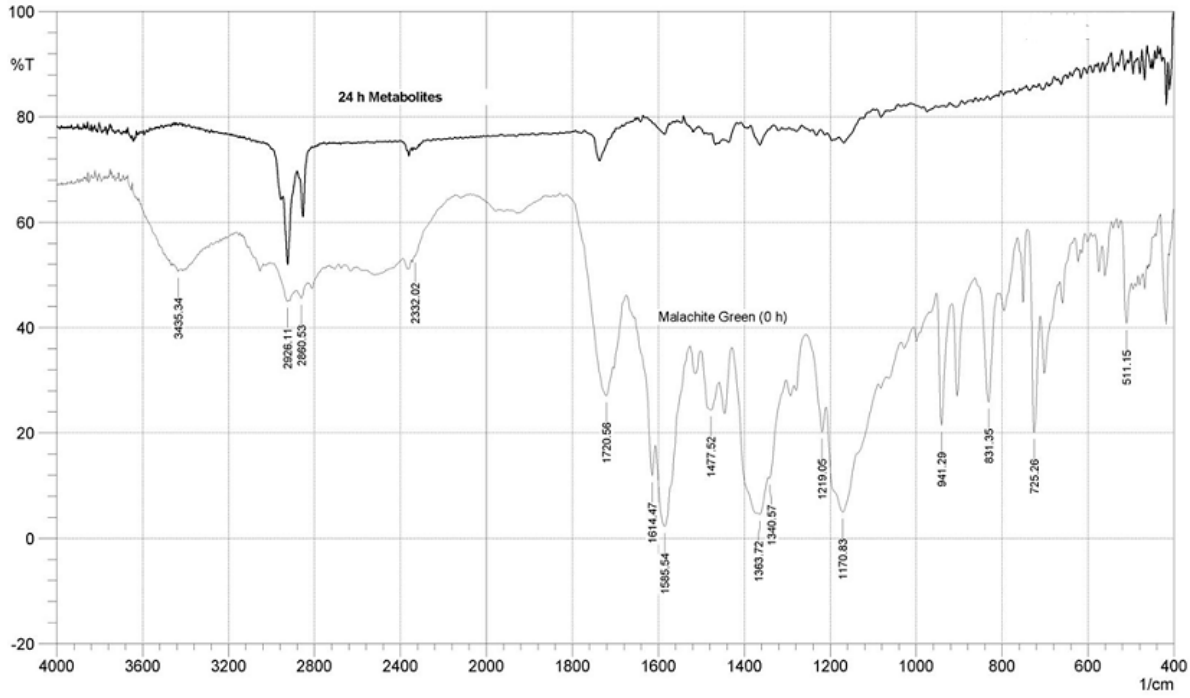

Figure 4: FTIR spectra of malachite green dye 0 h (control) and the dye's metabolites extracted after 24 h of degradation by *B. thuringiensis.*

of laccase and manganese-dependent peroxidase in the decolorization of crystal violet, another triphenylmethane dye, by *Pleurotus ostreatus* has also been reported by Kunjadia *et al*. [23]. The crude extract of the enzyme was able to oxidize common laccase substrates with a preference for syringic acid (0.3409 $\pm$ 0.14 U/mg protein /min) while it demonstrated 0.0348 $\pm$ 0.02 U/mg protein/min and 0.0402 $\pm$ 0.02 U/mg protein/min activities for ABTS and guaiacol respectively (Figure **4**). Zouari-Mechichi *et al*. [24] however, showed that laccase from a white-rot fungus, *Trametes trogii* preferred ABTS to syringic acid. It has been reported that different laccases showed different preference to different substrates [16]. The ability of the extracellular crude enzyme to utilize common laccase substrates suggested the production of laccase during the degradation of MG degradation by *B. thuringiensis*.

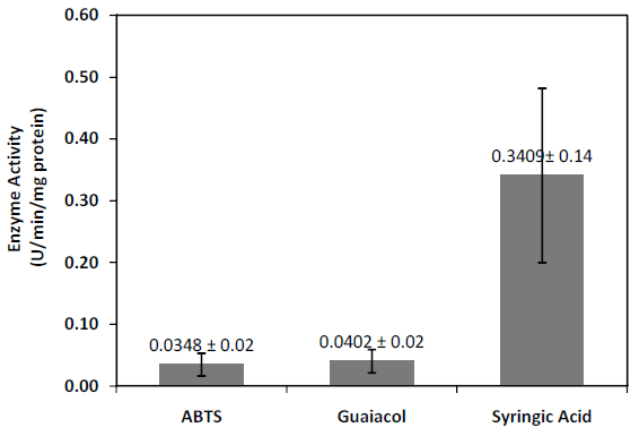

Figure 5: Extracellular laccase activities during MG decolorization using three common laccase substrates; values in mean ± standard deviation of triplicate readings.

CONCLUSIONS

The strain *Bacillus thuringiensis* RUN 1 decolorized malachite green by degradation and produced extracellular laccase in the process. The biodecolorization appeared to be coupled to laccase production. It was therefore concluded that this strain of *Bacillus thuringiensis* has a good potential for use in the treatment of industrial effluent containing malachite green and in the production of laccase, a highly versatile and industrially important enzyme.

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