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Biodegradation of Textile Dye by Ligninolytic Bacteria Isolated from Western Ghats

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ABSTRACT

Improper disposal of synthetic dyes in waste water causes severe environmental problems. The dye methylene blue is used in various industries and it causes several mutagenic and toxicological effects in living organisms on its exposure. Bioremediation is an emerging technique for effluent treatment. In our study, we aimed to isolate Methylene blue degrading ligninolytic bacteria originated from Western Ghats soil. Totally twenty-one bacterial strains were isolated and among them, nine strains were found to be ligninolytic and able to decolorizing methylene blue. Potential strain with maximum methylene blue decolorizing efficiency was selected and identified by 16S rRNA gene sequencing studies. Influence of environmental parameters such as pH, initial dye concentrations and the mechanism of dye removal were studied with the selected strain. The bacterial strain B1 have the ability to decolorizes up to 71% in nutrient media containing methylene blue and was selected as potent strain and identified as *Bacillus cereus* with 100% similarity. Maximum decolorizing efficiency was found to be in a nutrient medium containing up to 50mg/l of MB dye at pH 5. The isolated ligninolytic bacterium *Bacillus cereus* WGB1 has good potential for the degradation of methylene blue and can be applied at textile industry for the treatment of textile dye wastewater for environmental safety.

Keywords: Ligninolytic bacteria, biodegradation, FTIR, *Bacillus cereus* WGB1, Methylene blue

INTRODUCTION

Improper disposal of synthetic dyes in waste water result in acute toxic effects on aquatic flora and fauna which fate to severe problems to natural ecosystem. [1] Treatment of textile dyeing wastewater is a major concern since it contains the major pollutants. Textile dyeing effluent contaminated sites needs immediate reclamation. Existing treatment methods have limitations and fail to degrade textile dyes. [2] Methylene blue (MB) is a dye and used as a pharmaceutical drug. It contains heterocyclic aromatic chemical compound. Textile, paper, and pharmaceutical industries discharges methylene blue through effluents and it enters water bodies and adversely affects eco-system and aquatic life. Presence of methylene blue in drinking water causes eye and skin irritation, hemolytic anemia, nausea, vomiting, and abdominal pain. Therefore, its removal from the polluted water is of significant importance. [3]

Nowadays the most attracted technologies for treating textile wastewater is the bioremediation-based technologies since it has been proved that the microorganisms able to decolorize and metabolize the dyes. Enzymes such as azo reductases, laccases, peroxidases and many other enzymes are considered as a molecular weapon for bioremediation of azo dyes due their great decolorizing potential. [4] Ligninolytic bacteria produces extracellular oxidative enzymes which includes peroxidases. Peroxidases have diverse applications and they have been involved in the removal of phenolic pollutants, synthetic dye decolourization. [5] The enzymes reported to be involved in bacterial lignin

degradation are manganese peroxidases, lignin peroxidases, laccases, glutathione S-transferases, monooxygenases, phenol oxidases and ring cleaving dioxygenases. [6] The bacterial ligninolytic potential is still largely unexplored and bacterial enzymes may be superior to their fungal counterparts with regard to specificity, thermostability and mediator dependency. [7]

A large extent of research has been done with microbes which are suitable for industrial use but still vast amount of microflora remains unexplored. Western Ghats is one of the world's ten "Hottest biodiversity hotspots" and a good source of microbes which are hidden in soil. They comprise a large portion of life's genetic diversity and their potential in industrial dye effluent treatment is still not well explored. Hence this study demonstrated the dye decolorizing potential of the ligninolytic bacterial strains isolated from Western Ghats.

MATERIALS AND METHODS

Sample collection

Soil samples were collected at the foothills of Marudhamalai, a southward extension of the Western Ghats (76°- 45‟ and 76°-55‟ E and 11°-0‟ and 11°-5‟N). Three soil samples were collected from the surface to a depth of about 5 to 10 cm using sterile spatula and sterile zip lock covers and there were kept at 4°C for further studies.

Isolation of ligninolytic bacteria

Bacteria from soil samples were isolated by standard serial dilution technique and isolates were screened for the production of lignin peroxidase and manganese peroxidase by azure B agar plate assay. Azure B is a recalcitrant thiazine lignin-model synthetic dye [8] widely used for screening the production of lignin peroxidase and manganese peroxidase. The isolated bacteria were streaked onto Luria Bertani agar plate added with Azure B (25 mg/L). The plates were incubated at 37˚C for 7 to 10 days. The agar plates were monitored for bacterial growth and appearance of halo zones around bacterial colonies which indicates the production of lignin peroxidase and manganese peroxidase.

MB Decolorization assay

The bacterial isolates were screened MB decolorization [9] and the dye degradation assay [10] was performed with the bacterial isolates. 0.1 ml of bacterial culture was inoculated in 250 ml Erlenmeyer flask containing 50 ml nutrient broth media added with methylene blue at a concentration of 10 mg/l and incubated at 37°C for 24hrs. Dye containing uninoculated medium served as control. 2 ml of the culture media was withdrawn at different time intervals and centrifuged at 5000 rpm/min for 20 min. Decolorization potential was evaluated by measuring the absorbance of culture supernatants at the maximum absorption wavelength of MB (665 nm) and percentage of decolorization was determined by the following formula. [11]

$$\% \text{ Decolorization} = (A_0 - A_t / A_0 \times 100)$$

Where, A_0 denotes the initial absorbance of sample and A_t denotes the absorbance at different time intervals.

Molecular taxonomic identification of potent strain by 16s rRNA sequencing studies

The genomic DNA was isolated from the selected strain and PCR amplification of 16S rRNA gene was carried out with universal primers 33F (5' TCCTACGGGAGGCAGCAGT 3') and 533R (5' TTACCGCGGCTGCTGGCAC 3'). The amplicon was electrophoresed in a 1% Agarose gel, visualized under UV and concentration was checked in a Nanodrop ND 8000. The amplicon was purified using Pure Link purification column (Invitrogen) and sequenced with forward and reverse primers in ABI 3730XL cycle sequencer. The forward and reverse sequences were assembled and contig was generated after trimming the low quality bases using Fastqc 0.11.4 tool and analysis was carried out using tool BLAST of NCBI. Based on maximum identity score first few sequences were selected and aligned using multiple

sequence alignment software ClustalW. The similarity matrix and phylogenetic tree were generated with MEGA 7 software by neighbor-joining method. [12]

Optimum conditions for dye decolorization

The concentration of methylene blue, effect of pH and incubation period on decolorization were evaluated at various concentrations of MB (50mg, 100mg, 150mg, 200mg and 250mg/l), [13] at different incubation time (0, 1, 2, 3, 4, 5 days) and at different pH (3, 5, 7, 9 and 11).

Assessment of Dye adsorbing potential

The dye adsorbing potential of the bacterial isolate was evaluated with Heat-killed bacterial cells. The Heat-killed cells were prepared by growing the isolate MB supplemented nutrient broth, centrifuged at 6000 rpm for 5 min and then washed three times with potassium phosphate buffer (pH 6.2). The resulting pellet is resuspended in small volume of the buffer and cells are heat-killed at 70°C for 10 min and then added to 50 ml NB media added with MB (10mg/l) and kept for incubation at 37°C. The assay was consequently carried out with live cells for 24 hours. The percentage of dye decolorization was calculated at every 2 h interval.

Spectral study for Assessment of Dye degrading potential

The UV – visible spectra of control and the sample treated with bacterial isolates were recorded in the range between 500-700 nm in Multiskan GO Elisa reader in order to observe the changes in the peak values of the degraded products [10]. The structural changes in the methylene blue structure before and after decolourization was studied by FT-IR Spectroscopy. The samples were analysed in the mid infrared region 400–4000 cm^{-1} with 16 scan speed and the % of transmittance was recorded. [14]

RESULTS AND DISCUSSION

Isolation of Ligninolytic Bacteria

Textile dye degradation by ligninolytic bacterial strains were carried out in the present study. Totally twenty one bacterial strains were obtained from the soil samples. Among the 21 isolates, 11 strains showed visible decolorization zone on Azure B agar plate. These strains were able to produce lignin peroxidase and manganese peroxidase (Fig.1). Similar results were observed in the previous studies. [8] Lignin peroxidase activities which are the high redox potential agents required in the dye decolorization mechanisms. [15] Azure B dye decolorization was also reported in the bacterial strain *Bravibacillus agri* which showed significant ligninolytic action in the pulp and paper mill effluent. [16]

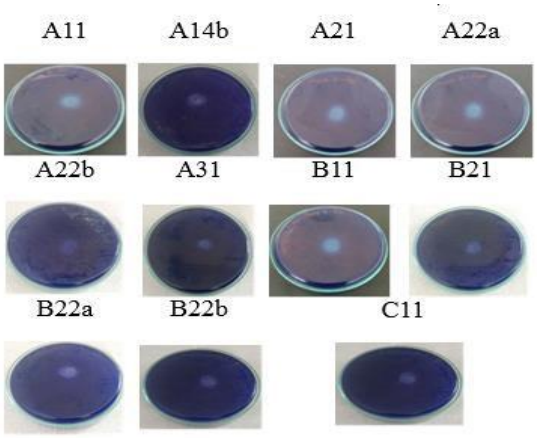
MB decolorization assay

Further ligninolytic bacterial strains were assessed for the MB decolorization. Nine isolates were able to form clear zones (Fig.2) on solid medium containing MB. The results showed that all the isolates have significant decolorization potential compared to the uninoculated control sample because of the adsorption of cationic dye i.e., methylene blue to the negatively charged outer surface of the bacterial cells. The highest percentage of methylene blue decolorization was observed at 24h of incubation (70.9%) by the bacterial strain B1 (Fig.3). The bacterial biomass was blue in colour when it is separated from the cell contents indicated that both biodegradation and biosorption processes are involved in the decolorization of MB. [17] The bacterial strain *Stenotrophomonas maltophilia* decolorized 53% of methylene blue in nutrient broth medium. [14] A ligninolytic bacterial strain *Brevibacillus agri* produced clear zone on methylene blue agar plate. [16]

Molecular identification of potential isolate B1

The potential bacterial isolate B1 was taken for molecular level taxonomic identification. The partial 16s rRNA gene sequence of strain B1 was amplified and sequenced. And the sequence was submitted to GenBank (Accession number: MH560276) and used for BLAST analysis. The 16s rRNA gene sequence exhibited maximum identity of 100% with *Bacillus cereus*. To characterize B1 more precisely, the most closely related sequences were

used to reconstruct a phylogenetic tree by neighbor-joining method (fig 4). The phylogenetic analysis of 16s rRNA gene sequence of isolate B1 showed 99% similarity to *Bacillus cereus* KF484676.1. The isolated bacterial strain B1 was identified as *Bacillus cereus* and it was named as *Bacillus cereus* WGB1.



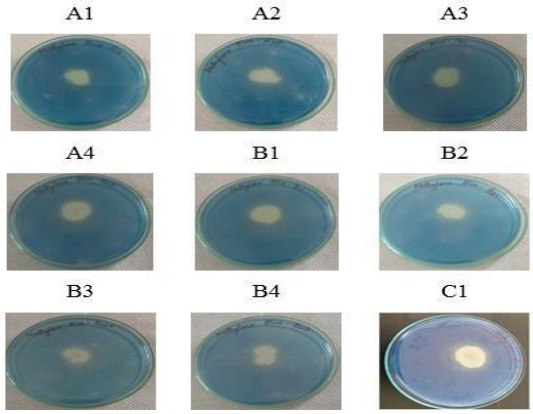


Fig 1. Visible decolorization zone on Azure B agar plate by the bacterial isolates
Fig 2. Methylene blue decolorizing zone on solid medium containing MB by the bacterial isolates

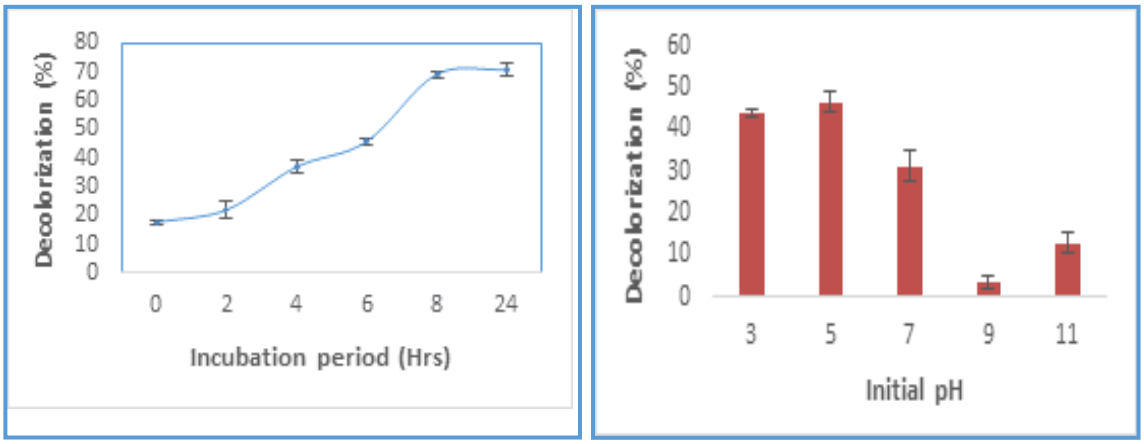


Fig 3. MB decolorization assay. The bacterial strain *Bacillus cereus* WGB1 showed highest percentage of methylene blue decolorization at 24h of incubation (70.9%). The experiments were performed in triplicates and the data given as mean±SD
Fig 6. Influence of pH on Methylene blue decolorization The strain *Bacillus cereus* WGB1 showed its maximum activity in the acidic pH and it was further decreased in alkaline medium. The experiments were performed in triplicates and the data given as mean±SD

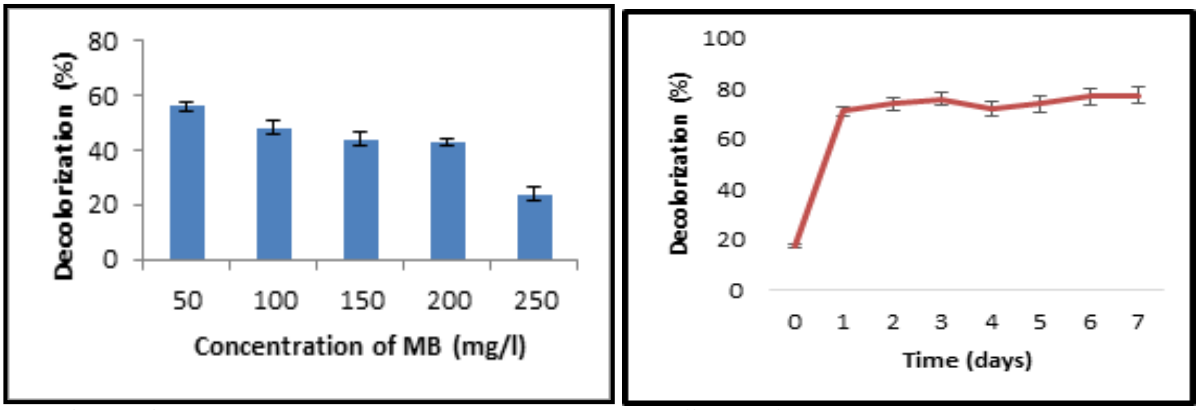


Fig 7. Influence of MB concentration on dye decolorization. The decolorizing efficiency of the strain *Bacillus cereus* WGB1 was 55.9% of MB at 50 mg/l concentration which was gradually decreased when MB concentration was increased. The experiments were performed in triplicates and the data given as mean±SD
Fig. 5 Influence of incubation period on Methylene blue decolorization. The removal of MB by *Bacillus cereus* WGB1 was (70.9%) at 1st day and a gradual increase was observed upto 7th day (77.3%). The experiments were performed in triplicates and the data given as mean±SD

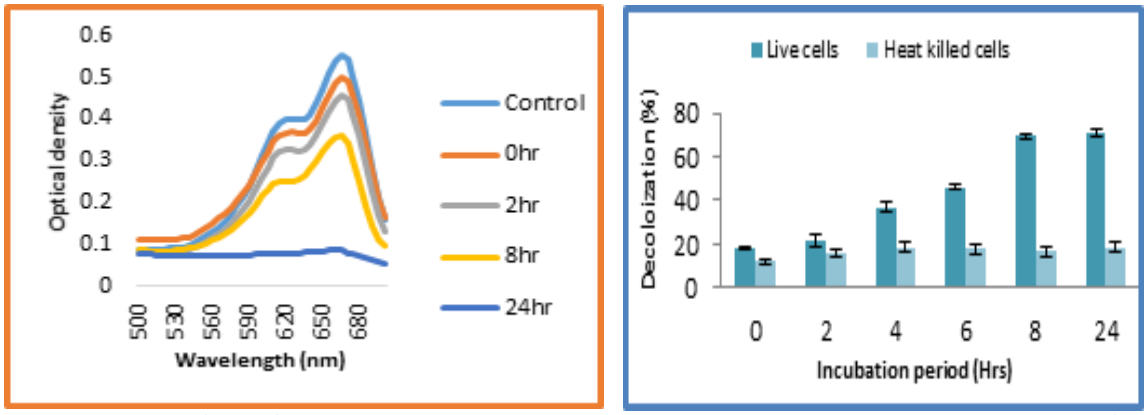


Fig 9. UV spectrum of MB. After treating with *Bacillus cereus* WGB1, the absorbance peak at 665nm decreased gradually. After 24hr of incubation, the absorbance peak shifted from 665nm to 655nm.

Fig 8. Comparison of MB removal by live & heat killed cells of *Bacillus cereus* WGB1. When MB was treated with the heat killed cells, the removal rate was (18.7%) after 2h of incubation. In the medium with live cells, removal rate was increased with the increase in incubation period. The experiments were performed in triplicates and the data given as mean±SD

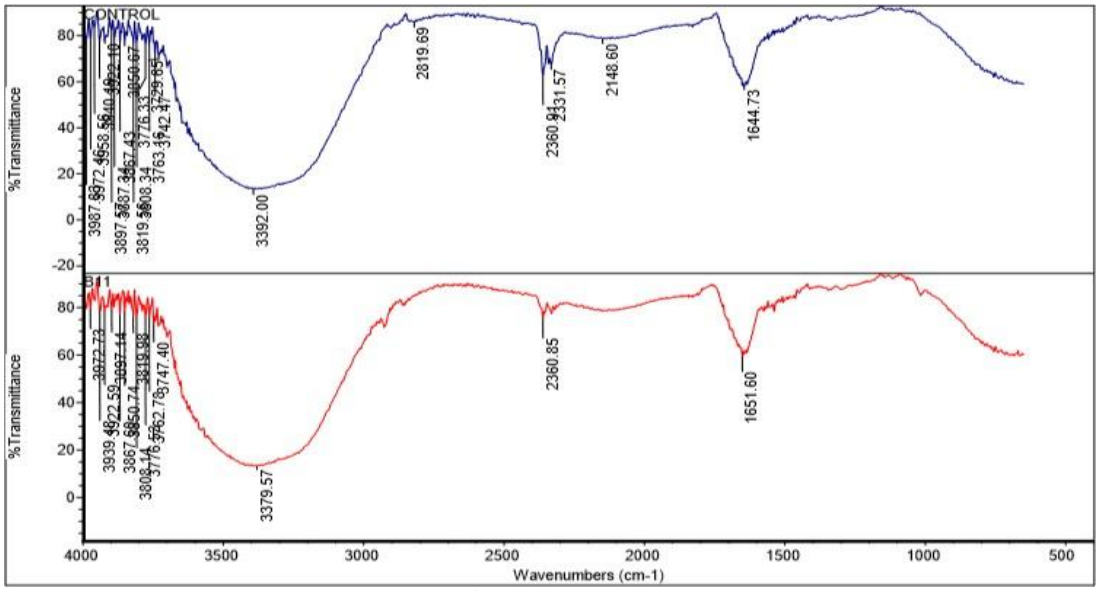


Fig.10 FTIR spectra of control (a) and decolorized sample (b)

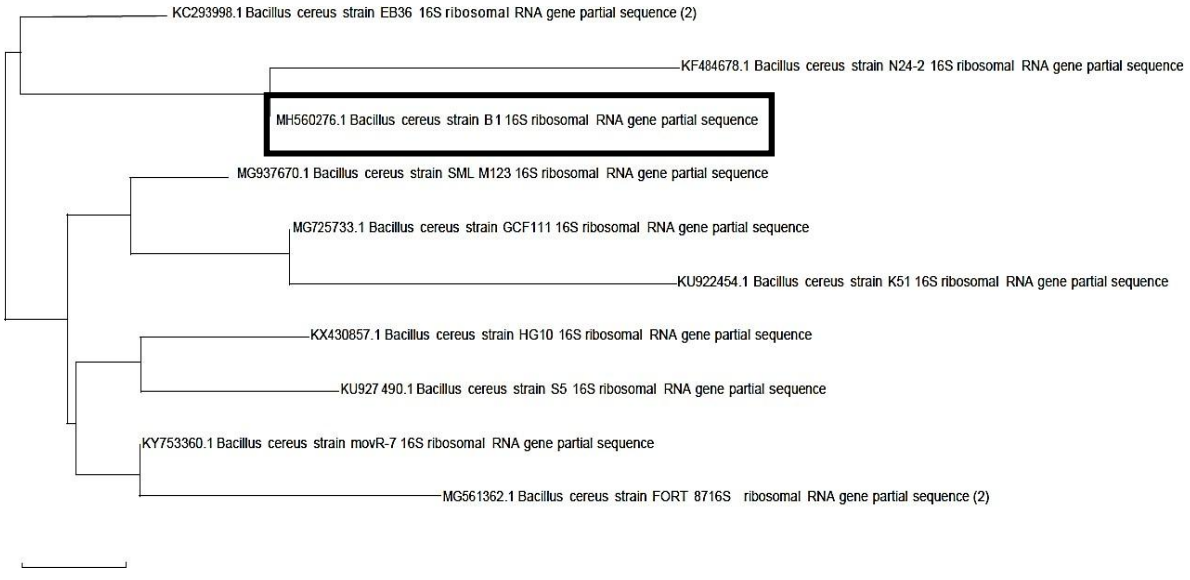


Fig 4. Phylogeneti

Optimum conditions for dye decolorization

The pH has a profound effect on the efficiency of dye decolorization. In this study the decolorization of MB by the strain *Bacillus cereus* WGB1 was found to be occur almost in all the pH range of 3-11 (Fig.6). The strain *Bacillus cereus* WGB1 showed its maximum activity in the acidic pH and it was further decreased in alkaline medium. At pH 3, the strain showed 43.9% of decolorization and reached to 46.4% of decolorization in pH range of 5. At neutral pH, it was decreased to 31.1% and no significant activity was observed in alkaline pH range (3.4% at pH 9 and 12.7 at pH 11). The result is accordance with the observation of *Stenotrophomonas maltophilia* strain showed its maximum decolorization (59.6%) at pH 5 which decreased rapidly at highly acidic or alkaline pH. [11] The decolorizing efficiency of the strain *Bacillus cereus* WGB1 was 55.9% of MB at 50 mg/l concentration which was gradually decreased when MB concentration was increased (Fig.7). It could be attributed to antibacterial properties of MB in high concentrations and lack of microbial biomass in proportion to the increased concentration of MB. [18] The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye at higher concentrations and the ability of the enzyme to recognize the substrate efficiently at very low concentrations. [4] In the present study, the removal of MB by *Bacillus cereus* WGB1 was (Fig.5) found to increase rapidly at 1st day (70.9%) and a gradual increase was observed upto 7th day (77.3%). This result clearly shows the MB decolorizing potential of the strain. In the initial stages, rapid rate of decolorization by *Pseudomonas sp.* was observed and the dye decolorization was decreased gradually with the increase in the concentration of the dye Reactive Red 5. [19]

Assessment of Dye adsorbing potential

Biodegradation and bio sorption are two major mechanisms proposed to be involved in the dye removal process. Biological removal of methylene blue takes place in two stages. Initially, the dyestuff quickly gets adsorbed to cell surface through physical interactions or chemical reactions and then degraded by enzyme catalytic reactions in the next step. To understand the MB decolorization mechanism by *Bacillus cereus* WAGB1, MB was treated with live and heat killed cells of the strain. Figure 7 illustrates the MB decolorization pattern of live and heat killed cells of *Bacillus cereus* WAGB1.When MB was treated with the heat killed cells, the removal rate was rapidly increased during the few min and reached maximum MB removal percentage (18.7%) after 2h of incubation. After that it persisted nearly constant up to 24h. This suggesting that the bio sorption also played significant role in the removal of methylene blue. Besides, it was found that the biomass of the *Bacillus cereus* WAGB1. In the medium which was inoculated with live cells, the percentage of decolorization was increased with the increase in incubation period. Whereas, the medium which is inoculated with heat killed cells showed less than 20% decolorization (Fig 8). From these results, it is inferred that the possible mechanism responsible for decolorization of MB could be biodegradation. The increased MB decolorization by live cells is may be due to the cellular enzymes secreted by the live cells could participate in the biotransformation of MB.

Biodegradation of dye

The UV–visible and FTIR spectral analysis was done to confirm the bacterial degradation of MB dye with untreated control and samples inoculated with *Bacillus cereus* WGB1 (Fig.9). Biodegradation mechanism has technical advantages in comparison to the biosorption mechanism due to the complete conversion of the dye to non-toxic mineral compounds. By treating the methylene blue with *Bacillus cereus* WGB1, the absorbance peak at 665nm decreased gradually. After 24hr of incubation, the absorbance peak shifted from 665nm to 655nm. The reduction in absorbance was likely due to the

degradation of MB chromophore, with the peak shift showing the occurrence of de-methylation reaction by the cell. This result may be correlated with the action of enzyme lignin peroxidase from *Phanerochaete chrysosporium*. N-demethylation methylene blue by Lignin peroxidase (LiP) are involved in the degradation of crystal violet and methylene blue (MB). In this study, it was depicted that decolorization of MB is not only a visible decolorization, it could be substantially due to enzymatic biodegradation. The maximum decolorization efficiency showed at pH range 3-5 (Fig.6) and the peak shift from 665nm to 655nm confirms that the methylene blue decolorization is an enzymatic biodegradation mechanism by the enzyme lignin peroxidase.

Functional group characterization of the dye before and after decolorization was further investigated through FT-IR analysis. FTIR spectrum of control was compared with FTIR spectrum of the decolorized sample (Fig.10). A significant changes in the position of peaks were observed. FTIR spectrum of control sample shows

C=C alkene stretch at 1644.73cm^{-1} which is shifted to 1651.60cm^{-1} in the treated sample. The –C≡C– alkyne stretch at 2148.60cm^{-1} and C≡N nitrile stretches at 2331.57 cm^{-1} were disappeared in decolorized sample. The C≡N nitrile stretches at 2360.91 cm^{-1} was shifted to 2360.85cm^{-1}. The-C-H aldehydic stretches at 2819.69cm^{-1} disappeared in the treated sample. The stretching vibrations of N–H bond (secondary amides) at 3392 cm^{-1} shifted to 3379.57cm^{-1} in the decolorized sample. The stretching vibrations of O–H at 3742.47 and 3763.16cm^{-1} were shifted to 3747.40 and 3762.78cm^{-1} respectively and O–H stretching vibrations at 3729.65, 3958.56 and 3987.83cm^{-1} were disappeared in the decolorized sample.

Changes in the FTIR spectrum confirm the existence of chemical reaction between the bacterial cellular contents and MB. Previous studies also reported the degradation action of bacterial isolates on the structure of the dyes based on the changes in the FTIR spectrum.

CONCLUSION

In this study, the isolated strain *Bacillus cereus* WGB1 has demonstrated for its methylene blue dye degradation. The mechanism of action of the dye removal was identified as biodegradation which is advantageous due to the complete conversion of the dye to non-toxic compounds. During MB dye degradation, *Bacillus cereus* WGB1 produced extracellular lignin peroxidase which revealed the potential of ligninolytic enzymes in the dye degradation process. The isolated bacterium *Bacillus cereus* WGB1 has good potential for methylene blue dye degradation and can be applied at industry level for the treatment of textile wastewater for environmental safety.

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Conflict of interest: Authors have no conflicts of interest.

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