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Biodegradation of malachite degradation by laccsae from Bacillus licheniformis NS2324

Navleen Kaur Chopra
Department of Biotechnology, I.K. Gujral Punjab Technical University, Kapurthala-144603, Punjab, India

Sonica Sondhi*
Department of Biotechnology, Chandigarh Group of Colleges, Landran-140307, Mohali, Punjab, India
*Corresponding author

***Abstract*---**Malachite green, a triphenylmethane dye, is used in aquaculture to limit the growth of protozoans and fungi in fish. It is also employed in the food, medicinal, and textile sectors. MG has high toxicity covering micro organisms and higher eukaryotes. Its toxic effects include organ damage, developmental abnormalities and mutagenic/carcinogenic potentials. Hence it is of utmost importance to degrade MG. Therefore, in this study with the help of catalytic properties of laccase, biodegradation of dye-malachite green was studied with respect to different factors like temperature, pH, enzyme dose and treatment time. The maximum of 99.31±0.97% MG decoloration was obtained under the conditions of 10 U ml^{-1} enzyme, pH 8.0 incubation time 1h and a temperature of 50˚C. MG was degraded without any mediator of laccase in the current study.

***Keywords*---**laccase, Bacillus licheniformis laccase, malachite green, decolorization.

Introduction

Malachite green (MG) is an organic compound that is employed as a colour and, more disputedly, as an antimicrobial in aquaculture(Sudova et al., 2007). Silk, leather, and textile are among the industries where malachite green has historically been used as a dye(Cha et al., 2001). It should be noted that, despite its name, this dye is not derived from the mineral malachite, and the term is derived only from the colour resemblance between the two minerals. Malachite green is classed as a triphenylmethane dye in the dyestuffs sector, and it is also used in the pigment manufacturing industry(Arunprasath et al., 2019). Malachite green is chemically a chloride salt $[C_6H_5C(C_6H_4N(CH_3)_2)_2]Cl$,(Alhendawi, 2011),

however the name malachite green is used more informally and often pertains to the coloured cation rather than the chloride salt in formal terms. The anions have no influence on the colour of the solution. The bright green colour of the cation is due to a high absorption band at 624 nm(An et al., 2010), which is responsible for the colour. Malachite green is a dye that has been used for centuries. MG and similar triphenylmethanedyes are manufactured in large quantities for this purpose on an annual basis, amounting to millions of kilos.

A triphenylmethane dye, malachite green (MG), has high anti-fungal characteristics(Culp & Beland, 1996). It is used to treat Saprolegnia (fungus)(S. Kumar et al., 2020) on fish or as a preventive therapy to protect fish eggs from infection when the fungus is present on the fish. It is a highly common therapy for *Ichthyophthirius multifiliis* in freshwater aquariums, and it is quite effective(Sudova et al., 2007). Because of its genotoxic and carcinogenic effects on humans, there has been an increase in public concern over its use in recent years(Culp & Beland, 1996). Despite the fact that the use of malachite green hazardous to living beings, its cheap cost and effectiveness have led to its widespread usage in the aquaculture business and as an dye. There are many physicochemical approaches which may be utilized to remove colours from wastewater effluent. These approaches, however, have the inherent disadvantages of being monetarily impractical, inefficient at dye removal and causing sludge development, all of which result in secondary pollution when used in their entirety(Kuhad et al., 2004). Bioremediation, which involves the use of microbial enzymes for the breakdown of xenobiotics, seems to be an environmentally friendly solution to the issue of environmental contamination.

Laccase is a commonly utilized enzyme because of its ability to destroy a broad range of dyes in a single reaction(Janusz et al., 2020). There has only been very less studies on the use of bacterial laccase in the degradation of malachite green. Bacterial laccases have more benefits as compared to fungal laccases: Comparatively speaking, bacterial systems are considerably easier to manage than fungal systems; also, because bacteria reproduce at much higher rates than fungus, early enzyme production becomes an additional element, which is undoubtedly a desirable quality; Fungi usually prefer an acidic environment where as bacteria prefer anything from acidophilic or alkalophilic.

Also most of enzymatic degradation studies done on Malachite Green are done using mediator, which in turn cause secondary pollution. In this investigation, the NS2324 laccase was isolated from *Bacillus licheniformis* NS2324 (MTCC 13026) and was used to study the break down of malachite green without the use of mediator. Furthermore optimal conditions for the degradation of malachite green like optimum enzyme dose, temperature, time and pH was also studied in the present study.

Materials and methods

Chemicals

Guaiacol was purchased from Sigma (USA). Malachite green was purchased from Hi-media. The other chemicals that were employed were of analytical quality.

Isolation of laccase producing bacterial species

M162 basal media was used to isolate laccase-producing bacteria (supplemented with 2 mM guaiacol as substrate). Soil samples from places where dyeing industry effluent was released were supplemented on the same media for 24 hours and then plated at optimum dilutions. For 48 hours, the plates were incubated at 37°C. The colonies that had a reddish brown tint were chosen. Selected colony was further characterized biochemically and by 16S rDNA technology.

Laccase Production

Laccase was synthesized in M162 medium supplemented with 0.2 percent yeast extract, 0.2 percent tryptone ans 100 mM $CuSO_4$. Bacillus licheniformis NS2324 was inoculated into the medium at a concentration of 0.1 percent using a 24-hour-old culture. Flasks were held at 37°C with shaking at 150 rpm for a total of 48 h. A 15-minute centrifugation at 7826 x g was performed after the incubation period. The extracellular enzyme was obtained from the supernatant.

Laccase Assay

The enzyme assay was carried out for 5 minutes at 55°C using a substrate of 2 mM guaiacol in 0.1 M Tris-HCl buffer (pH 8.0).The guaiacol oxidation was measured at 465 nm (= 12000 M-1 cm-1) for changes in absorbance owing to oxidation. One unit of enzyme activity was defined as the quantity of enzyme that oxidised micromoles of substrate oxidised by one ml of enzyme in one minute under normal assay conditions.

Degradation of Malachite green dye by NS2324 laccase

The enzymatic degaradation of malachite green dye was performed without any mediator. The dye's absorbance was measured at 624 nm before and after treatment. Dye degradation was measured as the decrease in absorption maxima. The following formula was used to get the percent degradation.

$$\% \text{ Degradation} = \frac{A1-A2}{A1} * 100 \quad \ldots(1)$$

Where,
A1 = Absorbance without enzyme treatment
A2 = Absorbance after treatment with NS2324 laccase

Optimization of treatment condition for malachite green degradation

OVAT approach was used to optimize the effect of several treatment parameters on malachite green degradation, such as enzyme dosage, temperature, reaction time, and pH. In following reactions, the conditions that were optimized in the prior reaction were employed.

Impact of enzyme dosage

In a total reaction volume of 10 ml containing 50ppm Malachite green dye, the enzyme dosage was varied through 10-50 $IUml^{-1}$. For each reaction, the appropriate substrate and enzyme control were created. Treatment was carried out for 4h and change in absorbance was calculated as per equation 1.

Impact of time

The enzyme and dye reaction mixture was incubated for various periods of time (1-4 h). In a total reaction volume of 10 ml, the optimized enzyme dose of NS2324 laccase was added to 50ppm malachite green dye. The same conditions were used for the appropriate substrate and enzyme control. Treatment was carried out and change in absorbance was calculated as per equation 1.

Impact of temperature

The malachite green dye and enzyme reaction mixture was incubated for optimized time and enzyme at temperatures ranging from 40 to 70 °C to investigate the effect of temperature on its degradation. In a total reaction volume of 10 ml, optimized enzyme dose of NS2324 laccase was added to 50ppm malachite green. The same conditions were used for the appropriate substrate and enzyme control. Treatment was carried out and change in absorbance was calculated as per equation 1.

Impact of pH

The dye was prepared in various buffers with pH ranging from 6 to 9 to investigate the effect of pH on dye degradation. In a total reaction volume of 10 ml, optimized enzyme dose of NS2324 laccase was added to 50ppm malachite green dye. The study was done carried out for optimized time duration at optimized temperature. The same conditions were used for the appropriate substrate and enzyme control. Treatment was carried out and change in absorbance was calculated as per equation 1.

Statistical Analysis

All of the tests were done in triplicates, and the mean and standard deviation have been plotted on a graph to demonstrate their results. Using Sigma Stat version 2.03, the data was evaluated using analysis of variance (ANOVA), and only statistically significant values (p values less than 0.05) were taken into consideration.

Results

Isolation and screening of laccase producing bacteria

Laccase producing bacteria were isolated from industrial effluent of textile industry on M162 medium. It was observed that 8 colonies out of 20 were showing reddish brown appearance on guaiacol containing medium owing to

secretion of laccase and was selected for further work. After screening for extracellular enzyme production, it was observed that colony no. 14 was producing extracellular laccase. Therefore, isolate no. 14 was selected for further work.

Biochemical and 16S rDNA analysis of isolate 14

Biochemical characterization of isolate showed that it was able to ferment glucose, sucrose, lactose and mannitol. MR/VP, indole, oxidase and citrate tests were negative. Catalase test was positive. Bacterium was able to reduce nitrate as well. On 16S rDNA anaylsis, isolate was found to be belonging to *B. licheniformis* and hence it was named as *B. licehniformis* NS2324. The 16S rDNA sequence was submitted to Genbank under accession number of *MT186173***.** The culture was deposited to MTCC, IMTECH, Chandigarh with MTCC no. 13026.

Optimization of treatment condition for malachite green degradation
Impact of enzyme dose

The maximum degradation of 88.66±1.34 was observed with the enzyme dose of 10U/ml after which a plateau was observed. Hence the optimum enzyme concentration for degradation of malachite green was concluded to 10 U/ml(Figure 1).

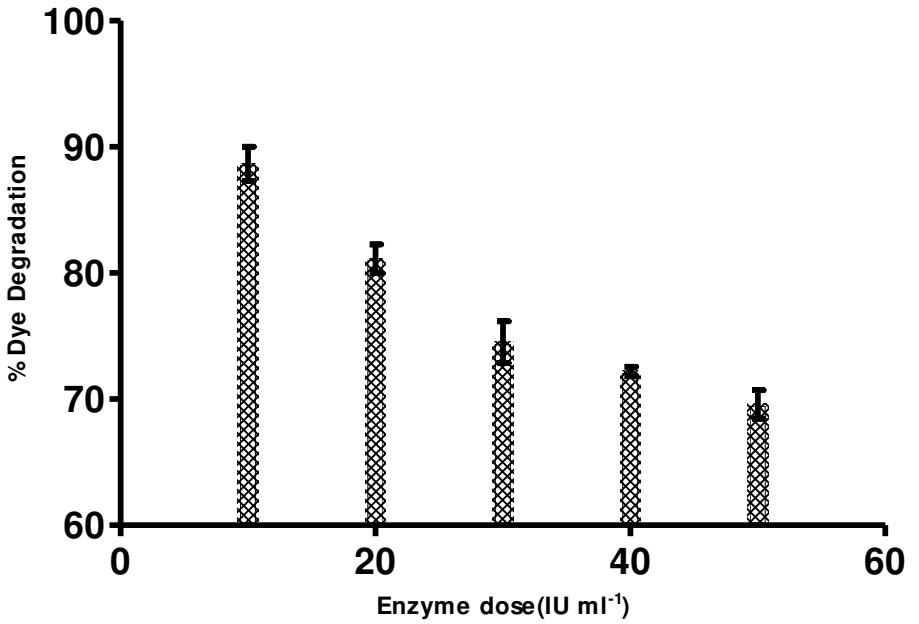

Figure-1:-Impact of enzyme dose on Degradation of Malachite green dye

Impact of Time

The degradation of malachite green dye was observed to be maximum atfter 1h. Further increase in time have no effect on dye decolorization. The maximum decolorization 89.84±2.90 was observed by the 1 h (Figure-2).

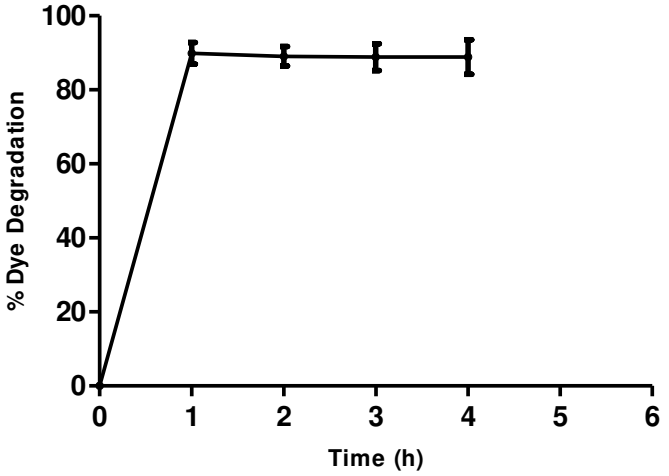

Figure-2: Impact of time on Degradation of Malachite green dye

Impact of Temperature

The maximum degradation of malachite green dye was observed at 50^{0}C which observed to 92.95±1.65% with further increase in temperature the rate of decolorisaton decreased and a downward curve was observed (Figure-3).

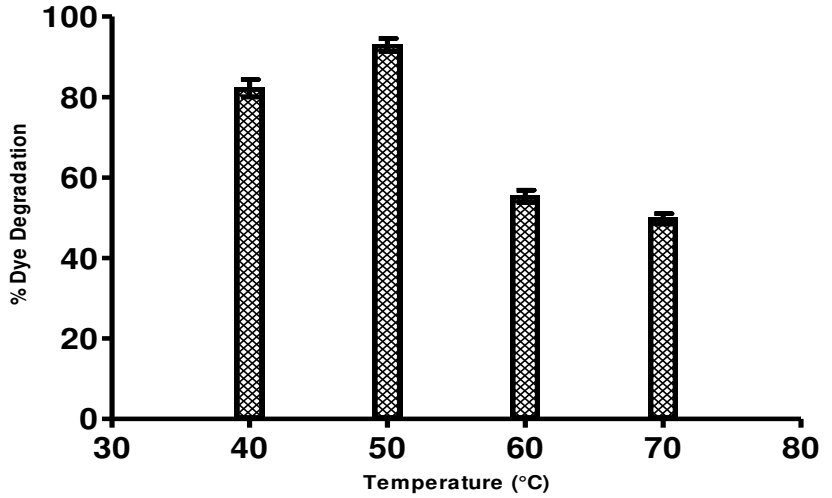

Figure-3: Impact of temperature on Degradation of Malachite green dye

Impact of pH

There was increase in rate of degradation of malachite green with the increase of pH however the maximum degradation 99.31±0.97% was observed at pH 8 (Figure-4) with further increase in pH there was a little decrease in the degradation rate (Figure-5).

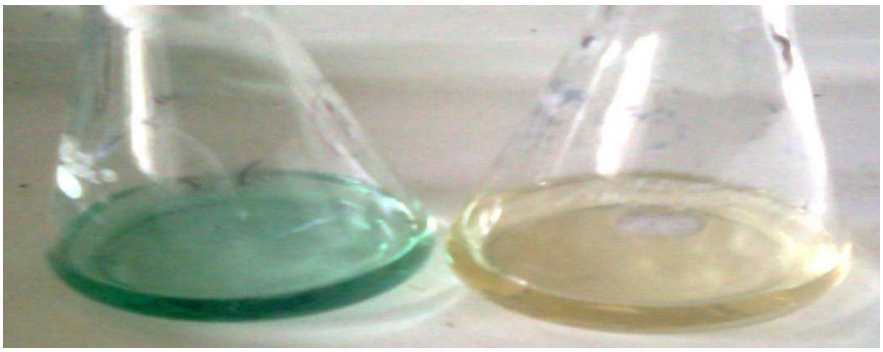

Figure-4: Degradation of Malachite green dye at pH 8

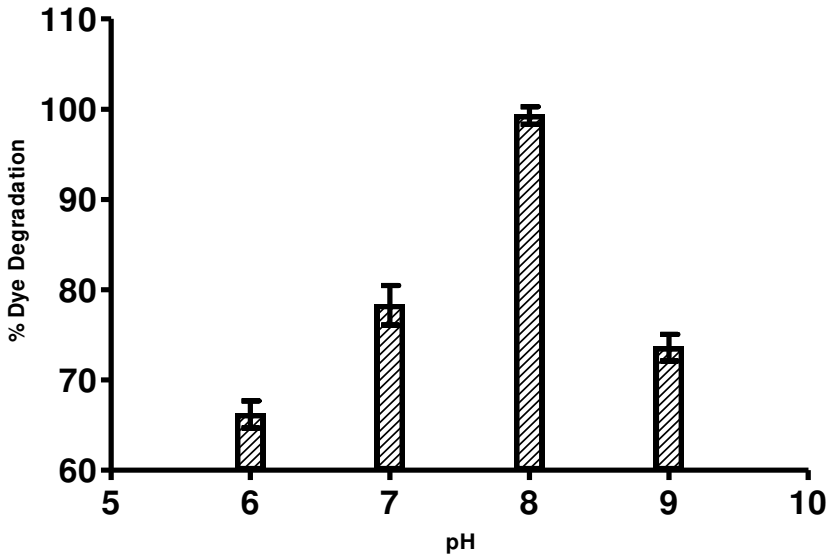

Figure-5:Impact of pH on Degradation of Malachite green dye.

Discussion

Malachite green is basically a Triphenylmethane (TPM) dye chemically. Many industries employ TPM dyes, such as those in the leather, textiles, and paper sectors. Their ability to produce vibrant colours is well-known. These dyes are difficult to degrade using enzymes because of their slow decomposition rate(Forootanfar et al., 2012) . Based on its ability to suppress seed germination, Malachite Green has been described as being hazardous to plants (Kalyani et al.,

2008; V. V. Kumar et al., 2012; Yang et al., 2015) by researchers. TPM dyes have been shown to be degradable by laccases, with N-demethylation by laccase being the most important factor in their breakdown (Casas et al., 2009). Malachite Green's decomposition has been divided into two distinct processes. Demethylation of MG's chromophore structure might lead to breakdown or polymerization of the molecule. The carbinol form is readily destroyed in the second step, where hydroxylation occurs (Fischer et al., 2011).

In the present study, malachite green was degraded with bacterial laccase without any mediator molecule. Degradation of malachite green, dye widely found in the aquatic environment, was also assessed using spore laccase from *Bacillus vallismortis* fmb-103, isolated from textile industry disposal sites by Zhang et al (2012) 76.84%, of malachite was reported to be degraded after 24 hours wih the help of mediators. Balan et al (2012) also reported about 96% degradation of 95.80 ppm of Malachite Green in 3.02 hours using 2.16 U/ml of laccase isolated from *Pleurotus florida* NCIM 1243 in the presence of 0.85 mM ofredox mediator (HBT).

In another study, laccase from white-rot fungus *Cerrena* sp. showed strong decolorizing ability towards MG. They obtained maximum MG degradation of 91.6% (Yang et al., 2015). Shanmugam et al (2017) studied biodegradation of malachite green (MG) by a laccase from *Trichoderma asperellum*. The maximum degradation of MG was achieved under optimal parameters with the enzyme concentration of 1.50 U/mL, the dye concentration of 122.66 mg/L, pH of 6.75, and incubation period of 98.58 minutes. In a separate investigation, a new laccase from Geothermobacter hydrogeniphilus (Ghlac) was cloned and produced in Escherichia coli. Ghlac version Mut2 with improved thermostability was developed for industrial usage. In three hours at 70 °C, Mut2 was able to decolorize roughly 100 mg/L of malachite green dye (Mao et al., 2021).

Recently,by Thoa et al. (2022) the laccase produced from Fusarium oxysporum HUIB02 fungus strain was used for the direct breakdown of MG by the enzyme. After 20 hours of treatment, the crude laccase was able to eliminate 80% of the MG. Cu^{2+} ions facilitated MG breakdown, while Fe^{2+} ions and anions like Cl and I slowed the process down. A temperature of 40 °C was ideal. MG breakdown was enhanced by up to 99 percent with the addition of mediators such as syringaldehyde, 1-hydroxybenzotriazole, and vanillin (Thoa et al., 2022).Laccase from *B. licheniformis,* in the current study, is degrading MG to 99.31±0.97% without the use of any mediator, Copper, and inducers of laccase. Thus the present study revealed useful results for degradation of MG and can be used for at industrial level.

Conclusion

The findings of present study propose an effecient environment friendly and economical method to degrade Triphenylmethane dye Malachite Green without mediator. Further studies using the above research can be done for the application enzymatic degradation by laccase NS2324 at industrial so that the pollution caused by industrial effluents can be treated effectively without causing any secondary pollution.

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Author Contribution

Ms Navleen Kaur Chopra is currently pursuing PhD from I.K. Gujral Punjab Technical University, Kapurthala-144603, Punjab, India. Her main area of research is degradation of leather and textile dyes enzymatically from laccase enzyme. She contributed in experimentation, data analysis and manuscript preparation

Dr. Sonica Sondhi is currently working as an Associate Professor in Department of Biotechnology, Chandigarh Group of Colleges, Landran, Mohali, Punjab, India. She has done Ph.D. from Panjab University, Chandigarh. Her main area of research is enzymology and applications of enzymes in industry and environment. She is the corresponding author of the manuscript and contributed in analysis of results, manuscript preparation and all correspondence related to submission.

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