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Biodegradation and Detoxification of Malachite Green Dye Using Novel Enzymes from *Bacillus cereus* Strain KM201428: Kinetic and Metabolite Analysis

Wycliffe Chisutia Wanyonyi*[a], John Mmari Onyari[b], Paul Mwanza Shiundu[c], Francis Jackim Mulaa[d]

[a]*Department of Physical Sciences, School of Science & Technology, University of Kabianga, P. O. Box 2030- 20200 Kericho, Kenya.*
[b]*Department of Chemistry, College of Biological and Physical Sciences, University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya.*
[c]*Department of Chemical Sciences and Technology, Technical University of Kenya, Haile Selassie Avenue, P.O. Box 52428 - 00200, Nairobi, Kenya*
[d]*Department of Biochemistry, College of Health Sciences, University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya.*

Abstract

Enzyme based degradation of organic pollutants is a promising detoxifying approach due to the promiscuous nature of the enzyme, efficiency, cost effective and ecofriendly. In the present study, we have carried out detailed decoloration and degradation studies on a model triphenyl methane group of dyes (Malachite Green dye (MG)) using a newly isolated enzyme from *Bacillus cereus* KM201428 under the static condition. Biodegradation of dyes was monitored by UV-VIS spectrophotometer and the resultant metabolites analyzed by Liquid Chromatography–Hybrid Quadrupole Time of Flight Mass Spectrometry (LC–QToF-MS) and Gas Chromatography/Mass Spectrometry (GC - MS). Metabolite analysis results revealed that enzymatic degradation of MG dye resulted in complete mineralization and benzene ring-removal; the latter known for organic dye toxicity. Kinetic study results revealed that first-order kinetic model was best applicable for describing MG dye decoloration. Michaelise-Menten kinetics, Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the dye decoloration. Lineweaver–Burk plot provided the best theoretical correlation of the experimental data with maximum rate (V_{max}) of 17.70 mg $l^{-1}h^{-1}$ and Michaelis constant (K_m) of 124 mgl^{-1}. Results provide evidence that crude enzyme from *Bacillus cereus* strain KM201428 offers an effective, renewable, ecofriendly and affordable biotechnology for treatment of industrial effluents polluted with organic dye.

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Keywords: Biodegradation, Malachite Green dye, *Bacillus cereus*, Kinetic, Metabolite

* Corresponding author. Tel.: +254 721810325
E-mail address: wwanyonyi@kabianga.ac.ke

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1. Introduction

Synthetic dyes are widely used in textile, leather tanning, cosmetic, printing, drug and food processing industries to color products. Currently, dyes are continuously being upgraded and replaced by superior compounds that have enhanced fastness, stability, brightness and resistance to natural degradation such as sunlight. Although extensive utilization of dyestuffs makes them valuable to human life, they also have a negative impact on the environment and human health because of their residuals in effluent [1]. Discharge of colored wastewater from these industries into natural streams has caused many significant problems, such as increased toxicity, chemical oxygen demand of the effluent, and reduced light penetration, which has adverse effects on photosynthetic phenomena [2]. Dyes usually have a synthetic origin and complex aromatic molecular structures which make them more stable and more difficult to biodegrade [3]. Furthermore, dyes are not only recalcitrant and refractory pollutants that constitute a significant burden on the environment, but are also toxic, mutagenic and carcinogenic.

Malachite oxalate green dye (Fig.1) is a dark green, crystalline solid and a controversial compound, due to its effects on the immune and reproductive systems as well as its potential genotoxicity and carcinogenicity [4,5]. Despite its high toxicity, MG is currently used extensively worldwide for dyeing due to its relatively low cost, readily available and efficiency. Human exposure to MG could result either from the consumption of treated fish, working in the dye and aquaculture industries. Several physicochemical methods have been used for the removal of dyes from wastewater effluent [6–8]. However, implementation of these methods has the inherent drawbacks of being economically unfeasible, inefficiency in dye removal and sludge formation which result in secondary pollution. Moreover, dye containing wastewaters from textile industry are discharged at high pH, temperature and high levels of salinity thereby causing problems for conventional physicochemical treatment methods. Development of new technologies which are economical, renewable and highly effective to cope with the treatment of dye-bearing effluents in textile wastewater cannot be over emphasized. Biodegradation of dye is rapidly gaining prominence as an environmentally friendly and less expensive alternative.

Fig 1. Structure of Malachite Green Oxalate (MG) dye.

Extremophile microorganisms like alkaliphiles bacteria exhibit the ability to grow at the extremely harsh environmental conditions such as high pH, temperature, levels of salinity and pressure which critically influence their growth. It is worth noting that this is almost similar environment condition under which industrial wastewaters containing organic dyes are released into the receiving water bodies. Application of enzyme from these extremophile microorganisms from East African Rift Soda Lake in bioremediation of organic dye has been severely lacking. The present study aims at investigating the efficacy of crude alkaline protease from extremophile bacteria *Bacillus cereus* strain KM201428 from Lake Bogoria (a Soda Lake in Kenya) in biodegradation of MG dye. Correlation of the kinetic properties with substrate (dye) concentration and other rate-dependent environmental parameters were investigated. The resultant metabolites were further analyzed by GC-MS and LC-QToF-MS. This study affords the opportunity to develop a new green biotechnology and provide fundamental information of utilizing enzyme from extremophile microorganism in detoxification of organic dyes contained in industrial wastewater.

2. Materials and Methods

2.1 Chemicals

A cationic basic dye, Malachite green oxalate (MW: 929.03g) was obtained from Loba-Austria and used without further purification. Stock solutions were prepared in double distilled water and pH adjusted by adding either 0.1 M HCl or NaOH. All the culture media, organic and inorganic compounds, and reagents used in GC - MS and LC–QToF-MS analysis were obtained from Sigma Aldrich. Crude alkaline protease was produced by *Bacillus cereus* strain KM201428 using 1% casein under submerged fermentation as described by Wanyonyi [9]

2.2 Dye decolorization under different conditions

Batch experiments were conducted in 100ml conical flask on a Thermolyne Orbital shaker at 150 rpm running at different time intervals at 25˚C. All experiments were conducted in triplicate and mean value reported. UV–Vis Spectrophotometer was set to the maximum wavelength value (617 nm) for all the absorbance measurements. Effect of contact time was investigated using 10ml of crude protease mixed with 40 ml of 9.30mg/L MG dye solution at pH 8.5 in 100 ml conical flasks. The biodegradation of the MG dye was monitored by UV–Vis Spectrophotometer (U-2810 Hitachi High-Technologies Co., Tokyo, Japan). After different time interval, aliquots from the reaction mixture were analysed for MG dye residual. Effect of initial dye concentration was investigated by varying the initial MG concentrations ranging from 0.93mg/L to 9.30mg/L. 10ml of crude protease was mixed with 40 ml dye solution. Absorbance readings were taken at intervals of 10 minutes till equilibrium was attained. Effect of temperature was investigated at temperature range of 25 - 70˚C and pH 8.5. 40 ml of MG dye of initial concentration of 9.30mg/L was mixed with 10 ml of crude protease both pre equilibrated at the working temperature for 30 minutes before mixing in 100ml conical flask. Absorbance reading was taken at an interval of 10 minutes till attainment of equilibrium. The decolorization efficiency of the enzyme was calculated using the following equation:

$$\text{Decolorization (\%)} = \frac{A_0 - A}{A_0} \times 100 \tag{1}$$

Where A_0 is the initial absorbance and A is the absorbance of medium after dye decolorization at λ_{max} (nm)

2.3 Determination of Maximum Dye Consumption Rate (V_{max}), Decolorization Rate Constant (K_m) and Reaction Kinetics Order Models (k_0, k_1 and k_2)

Determination of maximum MG dye consumption rate (V_{max}), decolorization rate constant (K_m) and reaction kinetics order models was investigated at 25˚C and pH 8. 40 ml of MG dye of concentrations ranging from 0.93mg/L to 9.30mg/L were placed in 100ml different conical flasks and 10 ml of crude protease added to each flask. Absorbance reading was taken in duplicates at intervals of 30 minutes for 6 hours and the mean values calculated. The results obtained were fitted to various kinetic model equations from which the appropriateness of the model determined and values for V_{max}, K_m, k_0, k_1 and k_2 calculated.

2.4 Metabolites Analysis by Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

After complete decolorization of MG, 15ml of degraded products containing the metabolites, crude enzyme and dye solution were centrifuged at 14,000 rpm for 10 minutes to remove impurities. The extracts were concentrated in vacuo to dryness then re-dissolved in 3 mL of LC−MS grade CHROMASOLV methanol (Sigma-Aldrich) before centrifuging at 14,000 rpm for 10 min; after which 0.5 µL was automatically injected into LC−QToF−MS. The chromatographic separation was achieved on a Waters ACQUITY UPLC (ultra- performance liquid chromatography) I-class system (Waters Corpo- ration, Maple Street, MA) fitted with a 2.1 mm × 100 mm, 1.7-µm particle size Waters ACQUITY UPLC BEH C18 column (Waters Corporation, Dublin, Ireland) heated to 40 °C and an auto sampler tray cooled to 15 °C. Mobile phases of water (A) and acetonitrile (B), each with 0.01% formic acid were employed. The following gradient was used: 0−1.5 min, 10% B; 1.5−2 min, 10−50% B; 2−6 min, 50−100% B; 6−9 min, 100% B; 9−10 min, 90−10% B; 10−12 min, 10% B. The flow rate was held constant at 0.4 mL/min. The UPLC system was interfaced by electrospray ionization (ESI) to a Waters Xevo QToF−MS operated in full scan MSE in positive mode. Data were acquired in resolution mode over the m/z range of 100-1200 with a scan time of 1s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature of 100 °C, and desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan

function, a collision energy ramp of 25−45 eV was applied in the T-wave collision cell using ultrahigh purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; [M + H]+ = 556.2766) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50−1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 2-propanol/ water (v/v). The elemental composition was generated for every analyte. Potential assignments were calculated using monoisotopic masses with specifications of a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The empirical formula generated was used to predict structures that were proposed based on the online database (ChemSpider, Metlin), fragmentation pattern, and literature.

2.5 Metabolites Analysis by Gas Chromatography/Mass Spectrometry (GC/MS)

Degraded metabolites of MG dye were extracted into organic phase using toluene. Two 100 ml conical flasks; one containing 25ml of 1.0 x 10^{-4} MG and another containing 25ml of degraded MG metabolite were each mixed with 5mls of toluene. The mixture was stirred at 25˚C before adding 3ml of 50 percent calcium carbonate. The mixture was stirred for one hour at 140 rpm at 25˚C and then allowed to settle for one hour after which the lower aqueous layer was drawn off and discarded, and the toluene layer containing MG dye and metabolite was removed for analysis. The GC-MS analyses of the MG dye and its degraded metabolites were carried out analysed by split/splitless injection using a model using Hewlett Packard Agilent GC/Mass Spec, Model 6890 coupled to a 5975C inert XL EI/CI mass spectrometer (Agilent Technologies, Palo Alto, CA) (GC−MS), equipped with integrated gas chromatograph with a DB-5 column (30 m long, 0.25 mm internal diameter). Helium was used as carrier gas at a flow rate of 1.3 mL/min. The injector temperature was maintained at 300°C with oven conditions. The initial column temperature was held at 40°C for 3 min, then increased linearly at 10°C/min to 325°C, and held for 2 min. The ionization was carried out in the electron impact mode. The electron multiplier voltage (El 70 eV) and automatic gain control target were set automatically. Degraded products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the National Institute of Standards and Technology (NIST) spectral library stored in the computer software of the GC-MS.

3. Results and Discussion

3.1 Effect of contact time on MG dye decolorization

Effect of contact time on decolorization of MG dye by crude enzyme *Bacillus cereus* KM201428 was investigated and the results show that MG was completely decolorization after 12 hours with over 98% decolorization. Fig. 2 illustrates a typical UV–Vis spectrum of the control MG at time zero and MG dye sample mixed with the enzyme at various time periods. The peaks were observed at 428 nm and 617 nm at initial time (0 minute). During enzymatic degradation, the peaks gradually decreased without a shift in λ_{max} before disappearing. The complete disappearance of main peak at 617 nm after 12 hours as well as the minor peak at 428 nm clearly suggests that crude protease effectively decolorized MG dye. This observation are consistent with previous findings reported by Du [10] who investigated biodegradation of malachite green by *Pseudomonas sp*. strain DY1 under aerobic conditions.

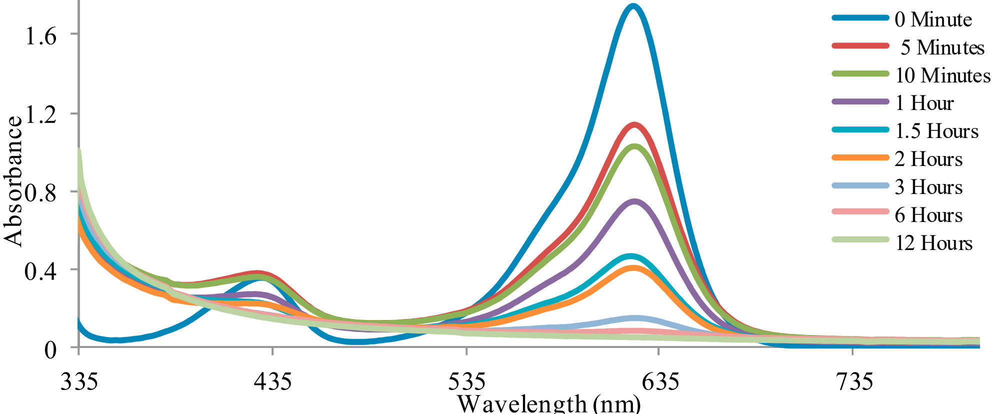


Fig. 2. UV–Vis spectra scan of MG dye (1.0x 10^{-5}M) biodegraded by crude protease from *Bacillus cereus* strains KM201428 at different time period, pH 8 and 25˚C

3.2 Effect of initial MG dye concentration on decolorization

Concentration of the substrate present in the aqueous phase has significant influence on any enzyme-mediated reaction [11]. Effect of initial MG dye concentration was investigated at different concentrations (0.93-9.30mgl^{-1}), while keeping all the other parameters constant. The amount of dye removed increases with increasing initial MG dye concentration (Fig. 3). For example, the amount of MG dye removed at equilibrium increased from 0.77 to 8.26mgL^{-1} with an increase in MG dye concentration from 0.93 to 9.3 mg L^{-1}. This could be attributed to more dye molecules available for decolorization and subsequent degradation when the dye concentration is increased. However, decolorization efficiency decreased with increasing MG dye concentration. The observed trend is consistent with published literature [12].

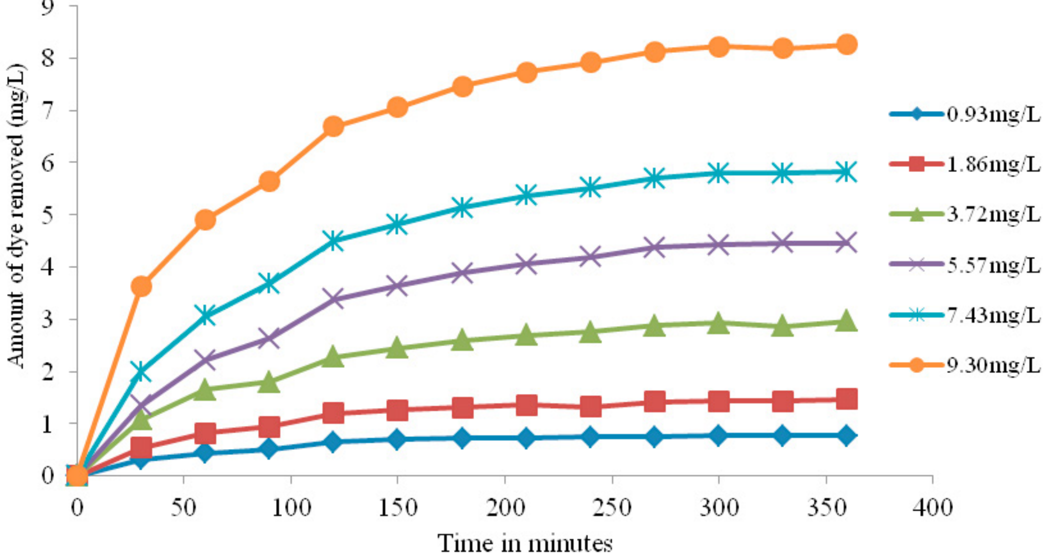


Fig.3. Effect of MG dye concentration on enzyme decolorization.

3.3 Effect of Temperature on MG dye decolorization

The influence of temperature on dye decolorization was evaluated by incubating a mixture of dye and enzyme at temperatures between 25 - 70˚C and results presented in Fig. 4. The result clearly demonstrated that decolorization increased with increase in temperature up to 40˚C, beyond which decolorization decreased gradually. The optimal temperature for decolorization was found to be 40°C. At elevated temperature beyond 40°C, there was a decrease in decolorization rate. This observation can be attributed to thermal denaturation of the enzyme molecules. In addition, extremely high temperature distorts the structure of the enzyme thus diminishing the binding capacity of its active sites for substrate molecules. Similar results have been previously reported [13].

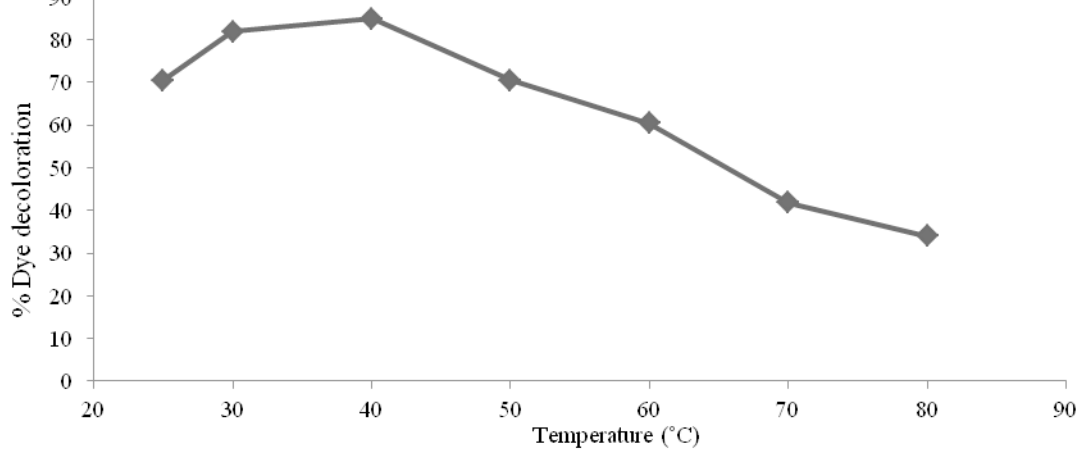


Fig. 4. Effect of temperature on MG dye decolorization.

3.4 Kinetic study

3.4.1 Order of Decolorization

The kinetics of the decolorization processes provides useful information regarding the efficiency of enzymatic degradation and feasibility of scale-up operations. The decolorization data obtained from the experimental results were analyzed by Zero, first and second order kinetic model equations given in Table 1.

Table 1. Zero, first and second order kinetic models

Kinetic models	Linear form	Plot	Rate constant
Zero order	$C_t = C_0 - k_0 t$	(C_t) vs time	k_0($mgl^{-1}min^{-1}$)
First order	$\ln(C_t) = k_1 t + \ln(C_0)$	$\ln C_t$ vs time	k_1(min^{-1})
Second order	$\frac{1}{C_t} = \frac{1}{C_0} + k_2 t$	($1/C_t$) vs time	k_2(mg/l min)

Where C_t is dye concentration in the solution (mgl^{-1}) at any time t and C_0 is the initial concentration of the dye in the solution (mgl^{-1}). Results obtained at different initial MG dye concentration were used to determine the order of dye decolorization. The rate constants of degradation reaction and coefficients of least square method analysis are tabulated in Table 2. The correlation coefficients (R^2) was highest in the first-order model in the range of 0.95 - 0.99, as compared to Zero and second order kinetic models which was below 0.83. The results show that enzymatic biodegradation of MG dye follows first-order kinetics model. Fig.5 shows a plot for the First-order kinetic model for different initial concentration of MG dye. Experimental data showed good fit to the first order kinetic model confirming that it was the best applicable for describing the kinetics of the enzymatic degradation of MG dye using crude protease from *Bacillus cereus* strain KM201428. The results show that decolorization process depends on MG dye concentration. This finding is consistent with earlier results reported in literature [14].

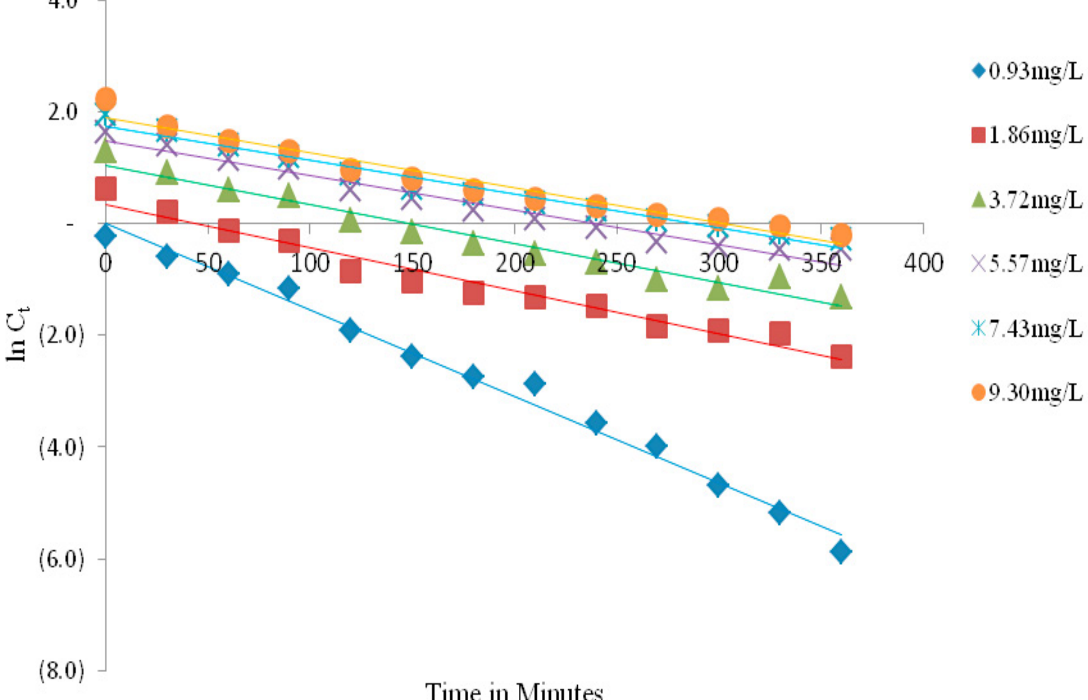


Fig 5. First order kinetic at different initial concentration of MG dye

Table. 2. Zero, first and second order kinetic constants and correlation coefficients obtained in enzymatic degradation of MG dye

Kinetics model	Constant	0.93mg/L	1.86mg/L	3.72mg/L	5.57mg/L	7.43mg/L	9.30mg/L
Zero order	k_0(mgl^{-1}min^{-1})	0.0019	0.0039	0.0076	0.0114	0.0147	0.0179
	C_o(cal)	0.5281	1.218	2.4898	3.9144	5.0876	6.0427
	R^2	0.7574	0.7444	0.780	0.8295	0.8132	0.744
First order	k_1(min^{-1})	0.0152	0.0077	0.007	0.0062	0.0060	0.0061
	C_o(cal)	1.0072	1.392	2.826	4.387	5.646	6.491
	R^2	0.9814	0.9591	0.9626	0.9664	0.9694	0.9507
Second order	k_2 (1 mg−1min^{-1})	0.0069	0.0004	0.0212	0.0133	0.0138	0.033
	C_o(cal)	14.66	1.3381	0.1341	0.3729	0.4832	0.5240
	R^2	0.8053	0.0002	0.611	0.0677	0.5247	0.6385

3.4.2 Decolorization rate constant (K_m) and Maximum Substrate Consumption Rate (V_{max})

The maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m) were determined using three different approaches namely Michaelis -Menten, Lineweaver Burk and Eadie-Hofstee.

i) Michaelis -Menten kinetics

The experimental results at various MG dye concentration were analyzed based on the interpretation of the Michaelis–Menten kinetics Equation. Michaelis-Menten equation is given as follows [15].

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

Where V_{max} is the maximum substrate (MG) consumption rate in mg l^{-1} h^{-1}; V is the substrate consumption rate in mg l^{-1} h^{-1}; S is the substrate concentration in mgl^{-1}; K_m is the Michaelis -Menten constant in mg l^{-1}. K_m is equal to the concentration of the substrate when the reaction rate is half of the maximum velocity. Fig.6 shows plots of S (MG dye concentration in mgl^{-1}) verses V (mgl^{-1}h^{-1}) for the enzymatic decoloration of MG dye. The experimental data fitted quite well to the Michaelis–Menten equation with a high R^2 value of 0.991 indicating that decoloration of MG dye obeyed Michaelis–Menten kinetics equation and also suggesting a first-order reaction order.

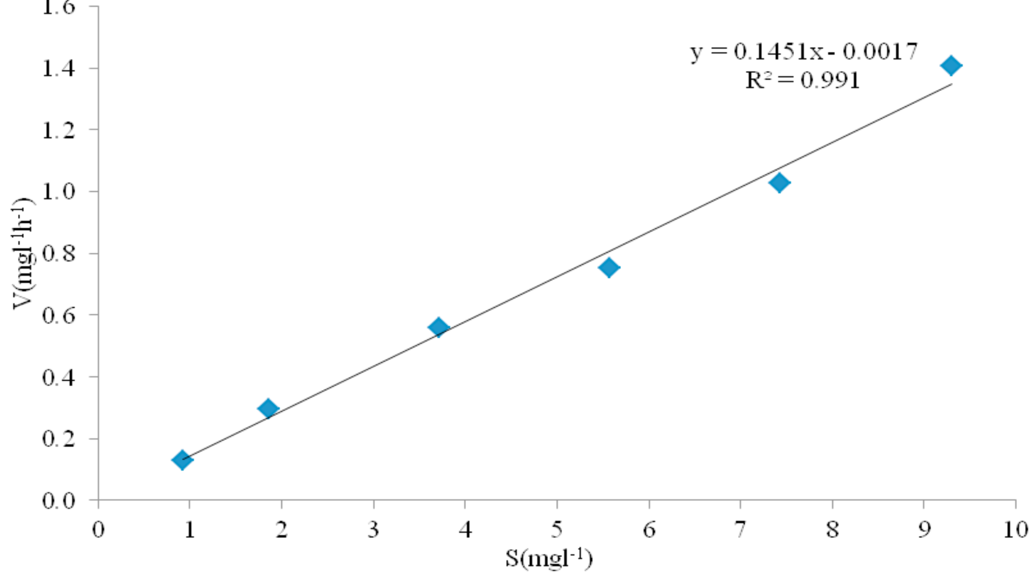


Fig.6. Michaelis–Menten plot for the enzymatic decoloration of MG dye

ii) Lineweaver-Burk plot

When Michaelis-Menten equation is transformed by a double reciprocal we obtain Lineweaver-Burk [16] equation below:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

A plot of 1/V versus 1/S gives a straight line with $1/V_{max}$ as intercept on the ordinate when 1/S approaches zero, and an intercept of ($1/K_m$) on the abscissa as V approaches zero. The Lineweaver-Burk plot is shown in Fig.7. The data

had a good fit with R^2 value of 0.996 indicating that Lineweaver-Burk plot model was also best applicable for describing the biodegradation of MG dye by crude protease from *Bacillus cereus* strain KM201428.

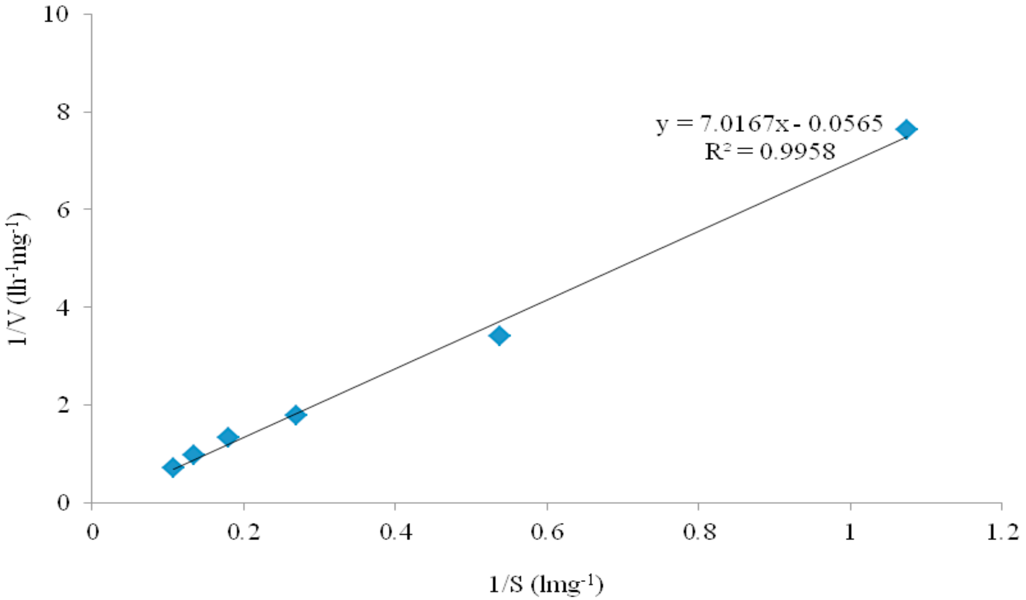


Fig. 7. Lineweaver-Burk plot for the enzymatic decoloration of MG dye

iii) Eadie-Hofstee plot

Another way to calculate K_m and V_{max} was the use of Eadie-Hofstee Plot. Solving Michaelis-Menten equation for V_{max} and rearranging, results to Eadie-Hofstee equation below:

$$V = K_m \left(\frac{V}{S}\right) + V_{max} \quad (4)$$

A plot of V against (V/S) gives a straight line with a slope of K_m and intercept V_{max}..The Eadie-Hofstee plot is shown in Fig. 8. The correlation coefficients (R^2) were not close to 1.0 (R^2 = 0.0104) indicating that Eadie-Hofstee plot was not appropriate in describing the biodegradation of MG dye by crude protease enzyme.

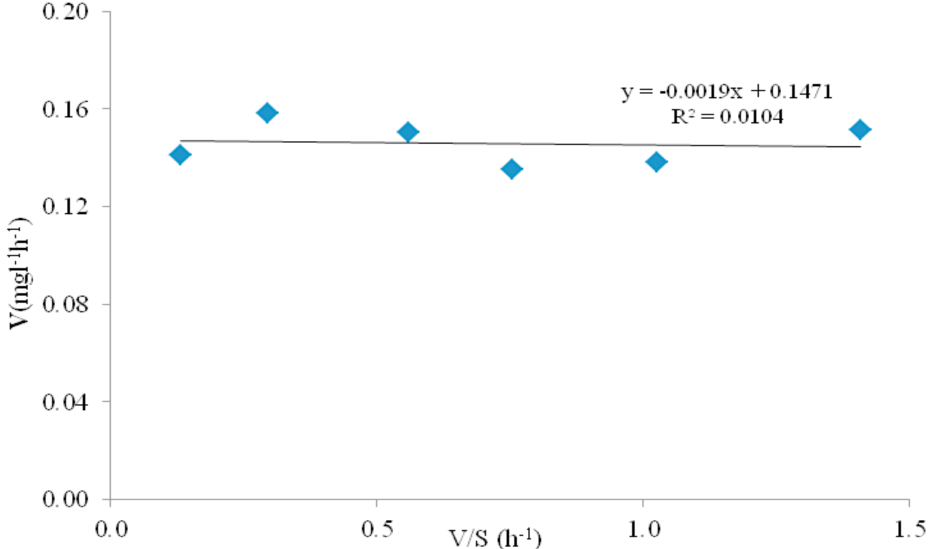


Fig.8. Eadie-Hofstee plot for the enzymatic decoloration of MG dye

A comparison of K_m and V_{max} values obtained from above three approaches is summarized in Table 3. Lineweaver-Burk plot and Michaelis-Menten equation had the highest R^2 values. It can therefore be concluded that decolorization of MG by crude protease enzyme can be best described by the Lineweaver-Burk plot and Michaelis-

Menten equation. These results further confirm that, decolorization of MG by crude protease enzyme is a first-order irreversible process. Similar observations were also made by Shah [14] who investigated enzymatic degradation of textile dye Reactive Orange 13 by newly isolated bacterial strain *Alcaligenes faecalis* PMS-1

Table.3. Comparison of K_m and V_{max} values for the enzymatic decoloration of MG dye

Plot type	V_{max} (mg l^{-1} h^{-1})	K_m (mg l^{-1})	R^2
Michaelis–Menten kinetics	0.1451	7.7017	0.9910
Lineweaver-Burk plot	17.70	124.1955	0.9958
Eadie-Hofstee plot	0.1471	0.0019	0.0104

3.5 GC–MS analysis

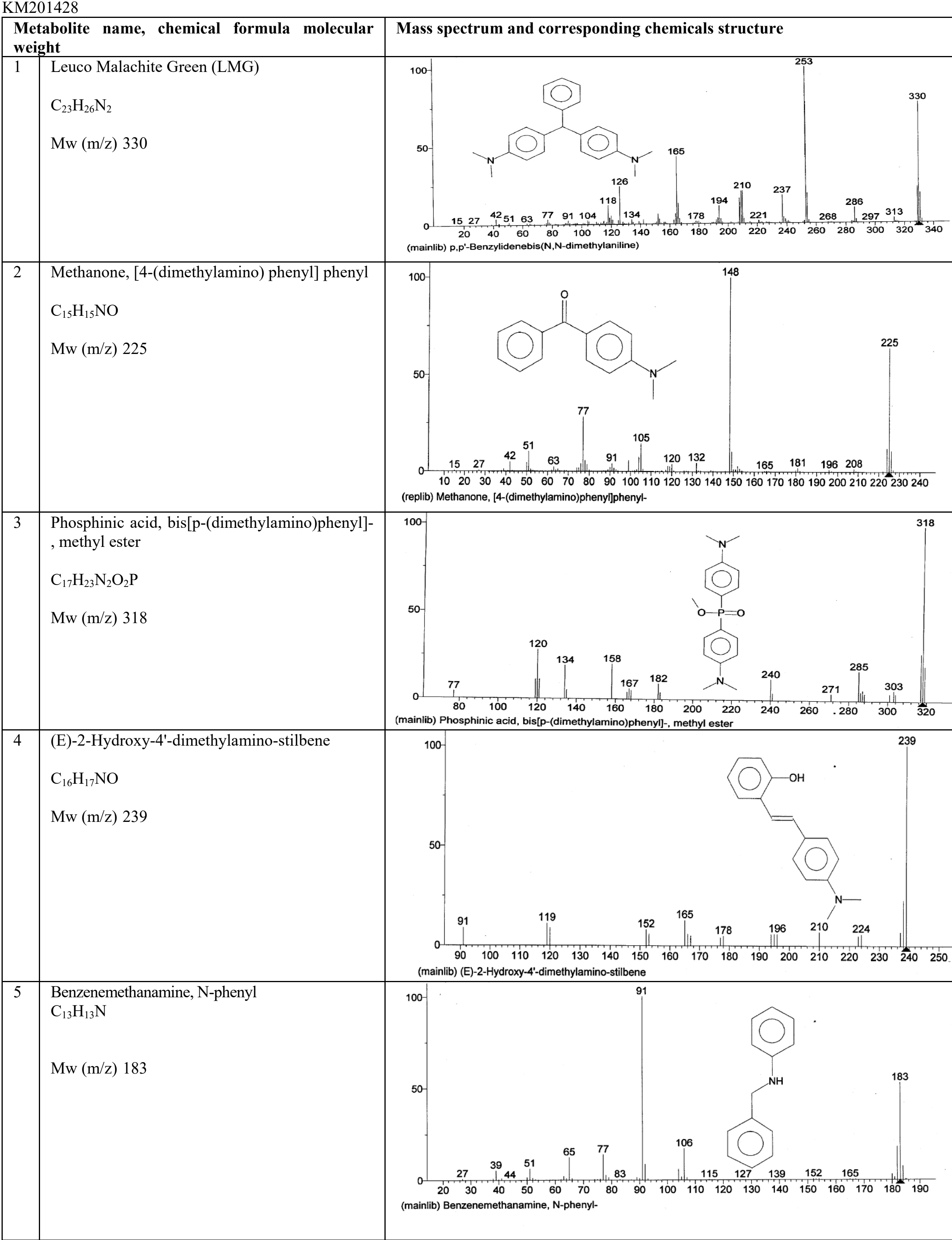
GC-MS analysis was carried out to investigate the metabolites formed during the biodegradation process. Table 4 summarizes various metabolite obtained at different retention times and corresponding chemicals structure as identified by matching their spectra with those recorded in the National Institute of Standards and Technology (NIST) spectral library stored in the computer software of the GC-MS. The results revealed that when MG dye is dissolved in water not treated with enzyme (positive control), it dissociate to Leuco Malachite Green (LMG). It is widely known that LMG is very toxic to aquatic organisms as it is deposited in fatty tissues and remain for more than ten months after treatment [17]. Hence its degradation is very desirable and critical. After enzymatic treatment, LMG was degraded to Methanone [4-(dimethylamino) phenyl] phenyl (m/z 225); Phosphinic acid, bis[p-(dimethylamino)phenyl], methyl ester (m/z 318); (E)-2-Hydroxy-4'-dimethylamino-stilbene (m/z 239) and Benzylaniline (m/z 183). The result implied crude protease from *Bacillus cereus* strain KM201428 effectively degraded MG dye. Similar results were reported by Du [10].

3.6 Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS) Analysis

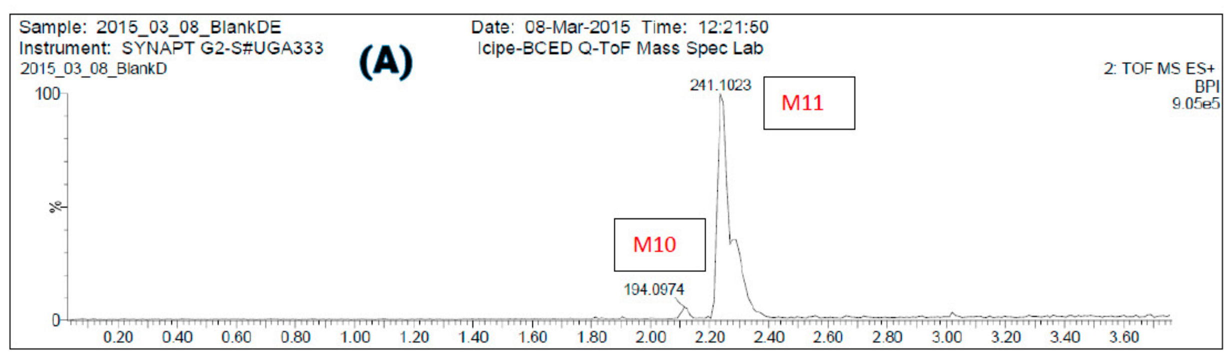
LC–QTOF-MS analysis was carried out to investigate the metabolites formed during the biodegradation process that could not be extracted and measured by GC-MS. Structures of the metabolites resulting from the enzymatic degradation of MG dye were successfully identified. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), identification and quantification were accomplished. From the LC-QToF-MS result analysis, twelve peaks of specific intermediate products that were clearly distinguished compared with the controls of MG without crude alkaline protease enzyme or only alkaline protease enzyme without MG (Fig.9). Based on the time for decolorizing, the parent compound and intermediate compounds formed were identified. The structure of MG dye could not be detected by LC−QToF−MS suggesting that the dye split into Leuco Malachite Green structure (LMG) (mw 330) before fragmentation. Similar results have been previously reported in malachite green biodegradation by *Exiguobacterium sp.* MG2 [18]. From the LC−QToF−MS results, it was possible to assign unique elemental compositions to each peak observed during the course of the analysis and probable molecular structure drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com) as shown in Fig. 10.

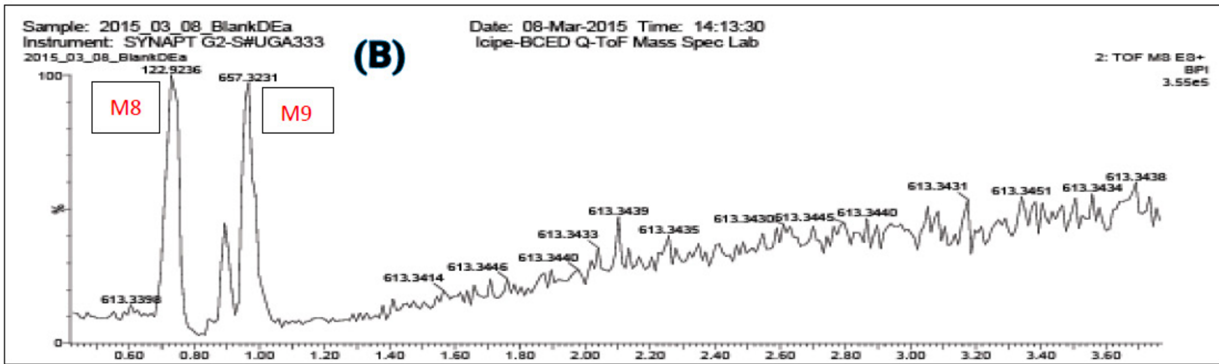
Analysis of metabolite showed that some metabolites detected were of higher molecular weight than LMG and MG. Since biodegradation of MG dye was performed at ambient conditions which could favor polymerization, the above observation could be attributed to polymerization of the resultant metabolite. The end products in the degradation of MG or LMG were found to be Cyclohexanamine, 4-(cyclohexylmethyl) cyclohexanamine and 6-amino-6-oxohexanoic acid. Due to the limitation of the analysis method, it was not possible to ascertain if these products were further degraded into CO, H_2O and NH_3. However, it is clear from the degraded metabolites that the triphenylmethane structure was cleaved off followed by benzene ring-removal together into cyclohexane. These observation differ with earlier results reported by Cha [19] which indicated that either tridesmethyl MG or LMG, the end-products in MG degradation by the *Fungus Cunninghamella elegans*, keeps the intact triphenylmethane structure. From these results, it can inferred that the enzymatic degradation of MG using protease from *Bacillus cereus* strain KM201428 comprised not only the decoloration reaction but also the more significant mineralization and benzene ring-removal which are very important for the efficient removal of organic toxic pollutants.

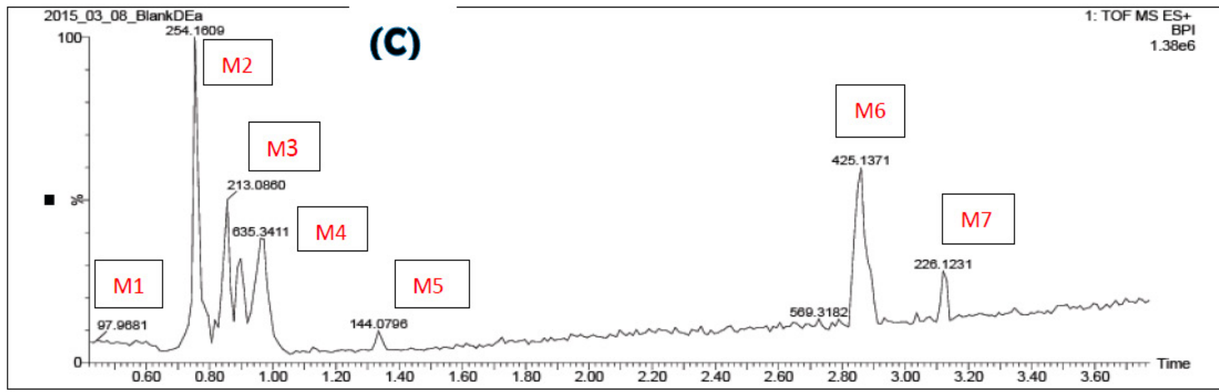
Table 4. GC-MS Scan chromatogram of metabolite obtained after MG dye degradation by crude protease from *Bacillus cereus* strain KM201428



	Metabolite name, chemical formula molecular weight	Mass spectrum and corresponding chemicals structure
1	Leuco Malachite Green (LMG) $C_{23}H_{26}N_2$ Mw (m/z) 330	
2	Methanone, [4-(dimethylamino) phenyl] phenyl $C_{15}H_{15}NO$ Mw (m/z) 225	
3	Phosphinic acid, bis[p-(dimethylamino)phenyl]-, methyl ester $C_{17}H_{23}N_2O_2P$ Mw (m/z) 318	
4	(E)-2-Hydroxy-4'-dimethylamino-stilbene $C_{16}H_{17}NO$ Mw (m/z) 239	
5	Benzenemethanamine, N-phenyl $C_{13}H_{13}N$ Mw (m/z) 183	







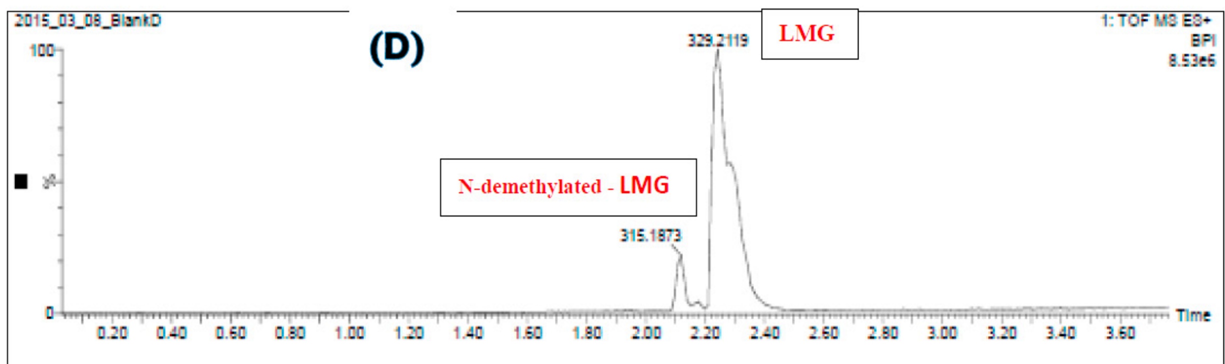


Fig.9. Extracted ion chromatograms of MG dye metabolites detected by LC–QTOF-MS in crude protease enzyme supernatant. (A), (B) & (C) represent chromatograms of degraded MG metabolites numbered M1-M12 and (D) represent chromatograms of leuco malachite green (LMG) resonance structure detected when MG dye dissolved in water without treatment with crude alkaline protease.

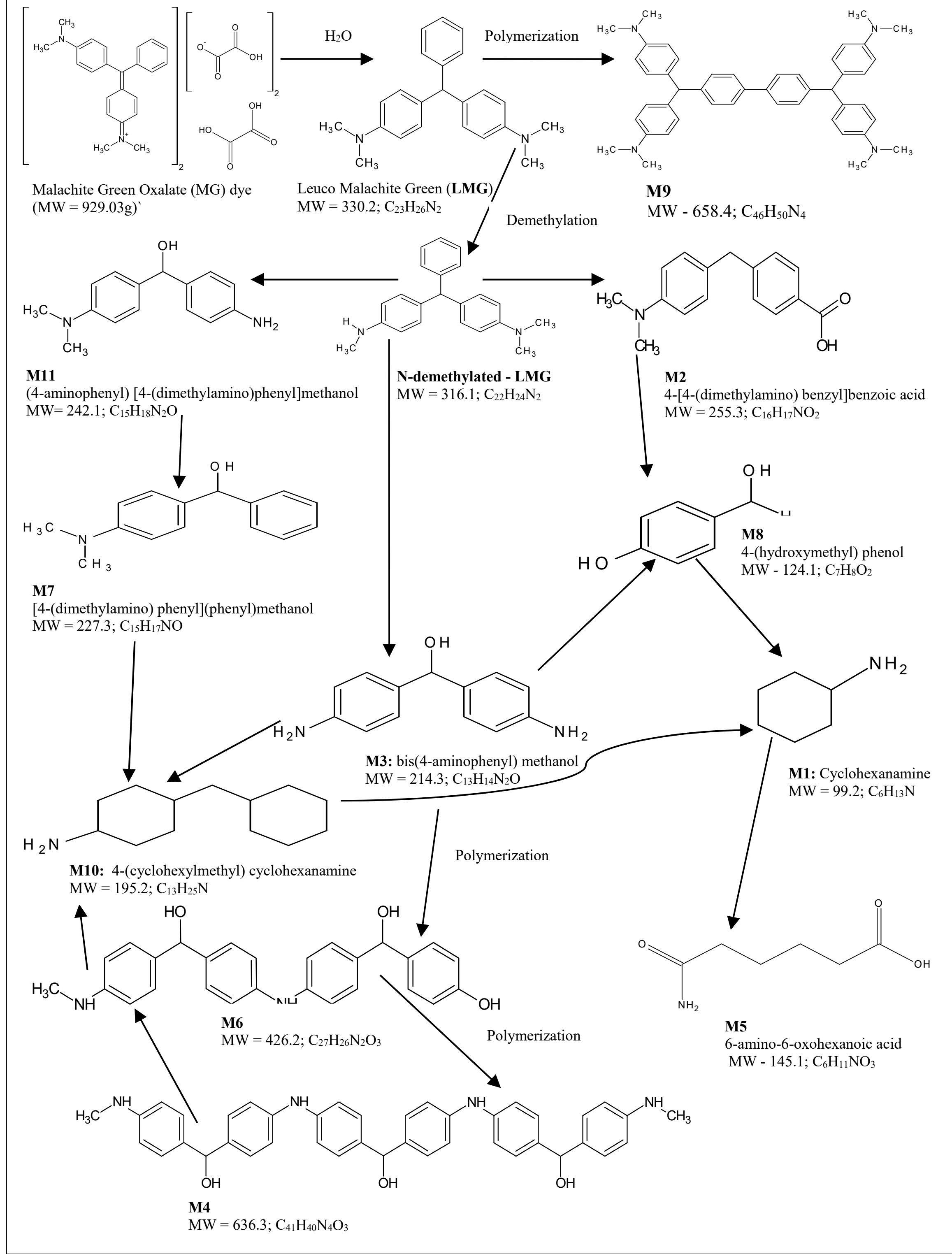


Fig.10. LC–QTOF-MS analysis of Intermediate product after degradation of the MG dye (Probable Intermediates molecular structure drawn using ACD/ChemSketch ver. 12.0)

4. Conclusions

The study was conducted to explore the applicability of using crude protease from *Bacillus cereus* strain KM201428 to decolorize and biodegrade MG dye. The experimental results obtained revealed the effectiveness of crude protease in the treatment MG dye. Operating parameters affecting dye decolorization rate such as time, temperature and initial dye concentration were extensively investigated. The ability of the enzyme to degraded triphenylmethane structure of MG dye under a broad range of temperature and different initial dye concentration suggested that the enzyme could be useful in treatment of industrial wastewater. Kinetic study of decolorization experiments approximate first-order reaction at different initial dye concentration. Decolorization kinetic was carried out by three different approaches namely Michaelis -Menten, Lineweavere Burk and Eadie-Hofstee. Lineweaver-Burk plot and Michaelis-Menten equation best described the MG dye biodegradation. The results clearly demonstrated that crude protease enzyme isolate from *Bacillus Cereus* Strain KM201428 exhibit a novel alkaline protease properties with ability to decolorize and degrade MG dye. UV-visible spectroscopy analysis confirmed decolorization while GC-MS and LC–QTOF-MS analysis confirmed degradation of MG dye into numerous non-toxic metabolites. Taking these results into consideration, it can be concluded that crude protease isolate from *Bacillus Cereus* Strain KM201428 can be exploited in bioremediation and detoxification of triphenyl methane group of dyes as a cost effective and ecofriendly alternative technology for treatment of wastewater polluted with organic dye.

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