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# Effective biotransformation of Reactive Black 5 Dye Using Crude Protease from *Bacillus Cereus* Strain KM201428

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**Abstract**

Effective effluent treatment is a paramount step towards conserving the dwindling clean water resources. The present study describes the use of crude protease extract from *Bacillus Cereus* Strain KM201428 biotransformation of azo dye Reactive Black 5 (RB5). Batch experimental results displayed over 97% decolorization efficiency with initial dye concentration of 1.0 x $10^{-4}$M. The decolorization process was highly dependent on contact time, dye concentration and pH. The optimum contact time and pH for decolorization were 120 hours and pH 9 respectively at 25˚C. Biotransformation of RB5 dye was monitored using UV-Vis spectrophotometer and formed metabolites characterized by LC–QTOF-MS. Comparison of resultant LC–QTOF-MS chromatograms after decolorization confirmed complete cleavage of RB5 dye. First order kinetic fitted well with experimental data for different RB5 dye concentrations. Lineweaver-Burk plot was used to describe the apparent relationship between the decolorization rate and the dye concentration at optimized condition with the coefficient of determination $R^2$ =1. This study comprehensively illustrates the potential of crude protease from *Bacillus Cereus* Strain KM201428 as an effective and environmentally friendly bacterial isolate in biodegradation of RB5 and opens a new approach in the treatment of wastewaters contaminated with high load of azo dyes.



*Keywords:* Biotransformation; Kinetic; Reactive Black 5 dye; Decolorization; Protease

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## 1. Introduction

Dyestuff usage has been increasing worldwide day by day because of tremendous increase of industrialization and man's urge for color variety. As a result huge volumes of wastewater contaminated with organic dyes and chemicals are discharged into environment. Water pollution is increasingly becoming one of the major environmental concerns across the globe. Azo dyes are the most common synthetic colorants preferred for coating, dying leather, paper printing and textile dyeing because of their (i) wide variety of colour spectrum, (ii) better firmness profile, (iii) simple application, (iv) inexpensive synthesis, (v) ease in structural modification, and (vi) capacity to bind to most synthetic fibers [1]. However, due to the poor exhaustion properties, as much as 20–40% of the initial dye remains unfixed and ultimately ends up in the dye effluent [2]. The continuous release of unutilized dyes into water bodies introduces hazardous chemical compounds that are toxic to aquatic life, humans and animals [3]. Discharge of colored wastewater from industries into natural streams has negative environmental effects, such as increased toxicity, turbidity, BOD, COD, aesthetic damage to sites and reduced light penetration, which has adverse effects on photosynthetic phenomena [4,5]. In addition, dyes and their intermediates are not only recalcitrant and refractory pollutants that constitute a significant burden on the environment, but are also toxic, mutagenic and carcinogenic [6]. There is an urgent need to maintain a clean environment for the survival of both aquatic and terrestrial lives. This has forced many governments to establish environmental restrictions and laws with regard to the quality of wastewaters discharged into the natural streams from industries.

In recent years, a number of studies have focused on various methods including adsorption, chemical precipitation and flocculation, photolysis, chemical oxidation and reduction, electrochemical treatment, and ion pair extraction in wastewater treatment [7,8,9]. However, most of these methods are expensive, have limited applicability, require additional chemicals which results in accumulation of sludge and generation of secondary pollutants which require further treatment [10,11]. Treatment of reactive azo dyes such as Reactive Black 5 dye (RB5) using common chemical or physical treatments methods is relatively difficult due to its high water solubility. About 90% of reactive azo dyes could remain unaffected after activated sludge treatment and then enter our aqueous environments[12]. Effective treatment and removal of color requires breakage of the dye chromophore. The most important chromophores in dyes are azo (-N=N-), carbonyl (-C=O), nitro (-N=O) and quinoid groups [13].

Biological methods have attracted more attention because of their strong adaptability, easy operation, low cost and mild reaction condition [9]. Several studies have demonstrated a variety of different types of microorganism, including fungi, yeasts, algae and bacteria, able to bio-decolorize and biodegrade a broad range of industrial dyes [14,15,16]. However, the effectiveness of microbial decolorization depends on the adaptability and the activity of the selected microorganisms [17]. High dye concentrations may be toxic to the microorganisms affecting their growth and activity. This challenge in the use of micro-organisms in effluents treatment has motivated scientist to look for new and more advanced technologies such as enzymatic methods. Compared to physicochemical treatment methods, enzymatic treatments of dyes is less costly, energy efficient, ecofriendly and easy to use although not commonly used in the textile industries [18]. In addition, cell-free or isolated enzymes is preferred over the intact organisms, especially when the effluent to be treated contains pollutants that inhibit microbial growth [19]. The present study evaluates the efficacy of crude protease from native *Bacillus Cereus* Strain KM201428 in biodegradation of RB5 dye. Factors affecting decolorization such as contact time, initial pH, and dye concentration were examined. The findings in this study will provide a rational and scalable solution for enzymatic biodegradation of organic dyes in wastewaters which is relevant to the broader environmental, food security and health impacts soil.

## 2. Materials and Methods

### 2.1 Chemicals

All chemicals, culture media, organic and inorganic compounds were obtained from Sigma-Aldrich and used without further purification. Stock solutions were prepared in double distilled water and pH adjusted by adding either 0.1 M HCl or 0.1 M NaOH. Crude protease extract was produced by *Bacillus cereus* strain KM201428 under submerged fermentation as described by Wanyonyi [20]

### 2.2 Dye decolorization under different conditions

Crude protease extract was used to study the decolorization of RB5 dye. Batch experiments were conducted in 100ml conical flask on a Thermolyne Orbital shaker at 150 rpm running at different time intervals at 25˚C. UV–Vis

Spectrophotometer (U-2810 Hitachi High-Technologies Co., Tokyo, Japan) was set at maximum wavelength value ($\lambda_{max}$= 597 nm) and used to measure the rate of RB5 dye decolorization. Effect of contact time was investigated using 10ml of crude protease extract mixed with 40 ml of 1.0 x $10^{-4}$M RB5 dye solution at pH 9 in 100 ml conical flasks. After 12 hour interval, aliquot from the reaction mixture were analyzed to determine concentrations of residual RB5 using a UV–Vis Spectrophotometer. Effect of pH was investigated over a pH range of 4-12 using 10 ml of free crude protease extract mixed with 40 ml of 1.0 x $10^{-4}$M RB5 dye solution. Initial dye concentration effect was investigated at 36˚C and pH 9 using 40 ml of RB5 dye concentration ranging from 1.0 x $10^{-5}$M to 1.0 x $10^{-4}$M and 10 ml of crude protease extract. All experiments were conducted in triplicate and mean value reported. The absorbance readings were taken at an interval of 10 minutes till attainment of equilibrium. The percentage of decolorization was calculated with reference to the control samples that were not treated with the enzyme:

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

Where $A_0$ is the initial absorbance and $A$ is the absorbance of medium after dye decolorization

### 2.3 UV–Vis spectrophotometric analysis

The decolorization of RB5 dye was monitored by UV–Vis Spectrophotometer. 10ml of crude protease extract at pH 9 was mixed with 40 ml of 1.0 x $10^{-4}$M RB5 dye solution at 36˚C in 100 ml conical flask with constant agitation. After an interval time of 12 hours, aliquot amounts from the reaction mixture were analyzed to determine residual RB5 concentrations at $\lambda_{max}$ 597 nm. Change in absorption spectrum of the reaction solution was recorded to determine the rate of dye decolorization with time.

### 2.4 Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS) analysis

After complete decolorization, the supernatant was extracted using ethyl acetate in 1:1 proportion and evaporated to dryness using rotary evaporator. The extracts were 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 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 Determination of Maximum Dye Consumption Rate ($V_{max}$), Decolorization Rate Constant ($K_m$) and Reaction Kinetics Order Models

Determination of maximum RB5 dye, consumption rate ($V_{max}$), decolorization rate constant ($K_m$) and reaction kinetics order models were investigated at 36˚C and pH 9. 40 ml of RB5 dye of concentrations ranging from 9.92mg/L to 99.18 mg/L were placed in separate 100ml conical flasks and 10 ml of crude protease at pH 9 added to each flask. Absorbance readings were taken in duplicates at intervals of 12 hours for 120 hours and the mean value 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 $k_2$ were calculated.

## 3. Results and Discussions

### 3.1 Effect of Contact Time on Reactive Black 5 Dye Decolorization

Effect of contact time on RB5 dye decolorization by crude protease extract was examined by varying the time of incubation and the results presented in Fig.1. It is evident that the % decolorization of RB5 increased with an increase in time and almost 87% of decolorization was achieved within 60 hours. The average time required to decolorize RB5 dye was about 120 hours with over 97% decolorization efficiency. The findings confirm that crude protease extract can be used to effectively treat the textile effluent containing RB5 within five days at pH 9. The results obtained are comparable with Vajnhandl and Marechal [21], who achieved about 96.5% decolorization for reactive black 5 with sonolysis as sole degradation technique.

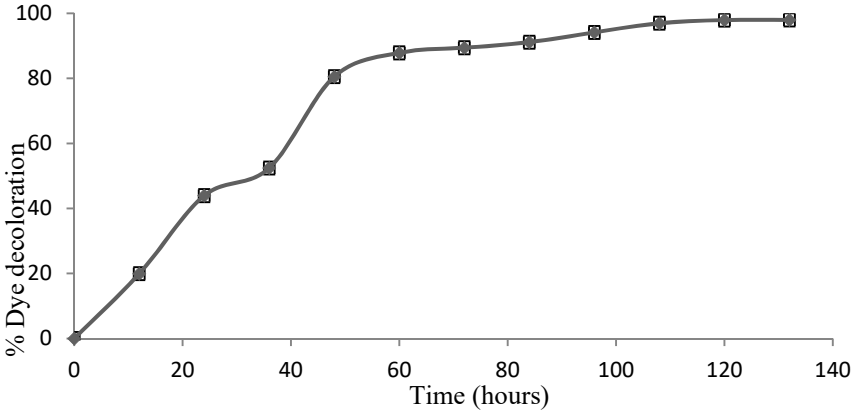


Fig.1. Effect of contact time on decolorization of Reactive Black 5 dye.

### 3.2 Effect of pH on Reactive Black 5 Dye Decolorization

The pH tolerance of decolorizing enzyme is important because RB5 dye binds to cotton fibers under alkaline conditions and hence the effluent is usually alkaline with pH greater than eight [22]. The effect of pH on decolorization of RB5 dye was investigated over the pH range of 4 to 12. Crude protease extract effectively decolorized RB5 dye in the pH range of 5.0 -10.5 with maximum decolorization of 98 % observed at pH 9 (Fig. 2). Optimum decolorization activity was observed in a broad pH range of 7 to 10. These findings present an advantage for the industrial application since most dye wastewater discharged from textile factories usually have a pH ranged between 8 and 9 [23]. Therefore, it could be concluded that crude protease from *Bacillus Cereus* Strain KM201428 is highly effective in weak alkaline conditions. Similar results have been previously reported by researchers where maximum decolorization of various textile dyes were achieved at neutral-to- slightly alkaline pH using enzyme extract from different bacterial species [3,24].

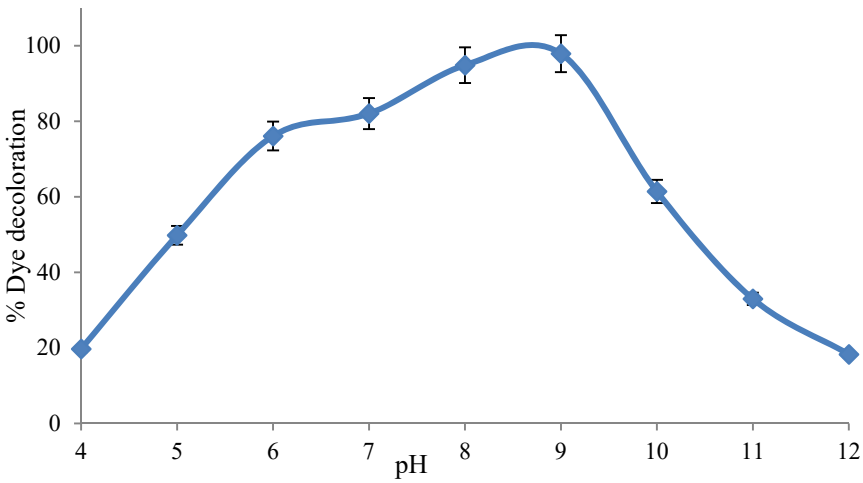


Fig.2. Effect of pH on decolorization Reactive Black 5 dye at equilibrium.

### 3.3 Effect of Initial Reactive Black 5 Dye Concentration on Decolorization

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 (dye) efficiently at very low concentrations [13]. The decolorization of RB5 was studied at various dye concentrations and the results presented in Fig.3. It is evident that for all the initial concentrations studied, the degree of decolorization was rapid over the initial period of contact time (40 hours) after which the rate of decolorization gradually decreased with increase in contact time till equilibrium was established. This can be attributed to the fact that at lower concentration of dye, the efficiency of enzymes to recognize the dye molecules as substrate decreases which in turn lowers the reaction rate. It was also observed that the amount of dye decolorized in mg/l increased with increasing initial RB5 dye concentration. For instance the amount of RB5 dye decolorized at equilibrium increased from 8.51 mg/L to 93.52mg/L with an increase in RB5 dye concentration from 9.918 mg/l to 99.182 mg/l. Textile effluent vary in dye concentration and hence the ability of the enzymes to degrade the dye at wide range of concentration is an important factor for effective treatment. In the present study the crude protease extract showed good performance with all concentrations of RB5

### 3.4 Determination of reaction order for decolorization of RB5 dye

The kinetics of RB5 dye decolorization using crude protease extract was modeled using zero, first and second -order kinetic models at different concentrations of RB5 dye. The linear forms of zero, first and second-order kinetic equations are given in Eq 2, 3 and 4 respectively.

$$C_t = C_0 - k_0 t \quad (2)$$

$$\ln(C_t) = k_1 t + \ln(C_0) \quad (3)$$

$$\frac{1}{C_t} = \frac{1}{C_0} + k_2 t \quad (4)$$

Where $k_0$ is the zero order decolorization rate constant ($mgl^{-1}min^{-1}$), $k_1$ is the first order decolorization rate constant ($min^{-1}$), $k_2$ is the second order decolorization rate constant ($mgl^{-1}$ min), $C_t$ 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 ($mgl^{-1}$). Zero order kinetic plots were obtained by plotting dye concentration ($C_t$) versus time while first-order kinetic plot was obtained by plotting $\ln C_t$ against time. Second-order kinetic model was obtained by plotting ($1/C_t$) versus time (graphs not shown). The rate constants of decolorization reaction and coefficients of least square method analysis are tabulated in Table 1. It is evident from Table.1 that first and second order reaction had a relatively high of linearity with high correlation coefficient ($R^2$). However, when experimental data were compared with calculated data ($C_{o(cal)}$), first-order model showed good compliance as compared to second order kinetic model. Zero-order kinetic model had low correlation coefficients ($R^2$) in the range of 0.67-0.83 indicating that it cannot be applied in describing the decolorization of RB5 dye. It can therefore be concluded that first-order kinetic model was best applicable in describing enzymatic degradation of RB5 dye. The result implies that rate of RB5 dye decolorization is inversely proportional to the initial dye concentration depicting that an increase in dye concentration result in a decrease in decolorization rate. The finding is consistent with earlier studies reported in the literature [25].

Table.1. Zero, first and second order kinetic constants obtained in enzymatic degradation of RB5 dye

| Kinetics model | Constant | 19.83mg/l | 39.67mg/l | 49.591mg/l | 59.50mg/l | 79.34mg/l | 99.18mg/l |
|---|---|---|---|---|---|---|---|
| **Zero order** | $k_0(mgl^{-1}min^{-1})$ | 0.1502 | 0.3168 | 0.4048 | 0.4535 | 0.6388 | 0.7416 |
| | $C_o$(cal) | 16.211 | 31.585 | 40.718 | 45.800 | 63.997 | 75.171 |
| | $R^2$ | 0.8277 | 0.7277 | 0.7380 | 0.7249 | 0.7563 | 0.7208 |
| **First order** | $k_1(min^{-1})$ | 0.0215 | 0.0254 | 0.0246 | 0.0247 | 0.0255 | 0.0242 |
| | $C_o$(cal) | 18.100 | 32.424 | 41.306 | 48.896 | 69.131 | 79.020 |
| | $R^2$ | 0.9610 | 0.8868 | 0.8704 | 0.9373 | 0.9244 | 0.9142 |
| **Second order** | $k_2(l\ mg^{-1}min^{-1})$ | 0.0045 | 0.0036 | 0.0025 | 0.0024 | 0.0018 | 0.0014 |
| | $C_o$(cal) | 277.78 | 232.56 | 192.31 | 112.36 | 138.89 | 227.27 |
| | $R^2$ | 0.9568 | 0.9257 | 0.9755 | 0.9455 | 0.9385 | 0.8806 |

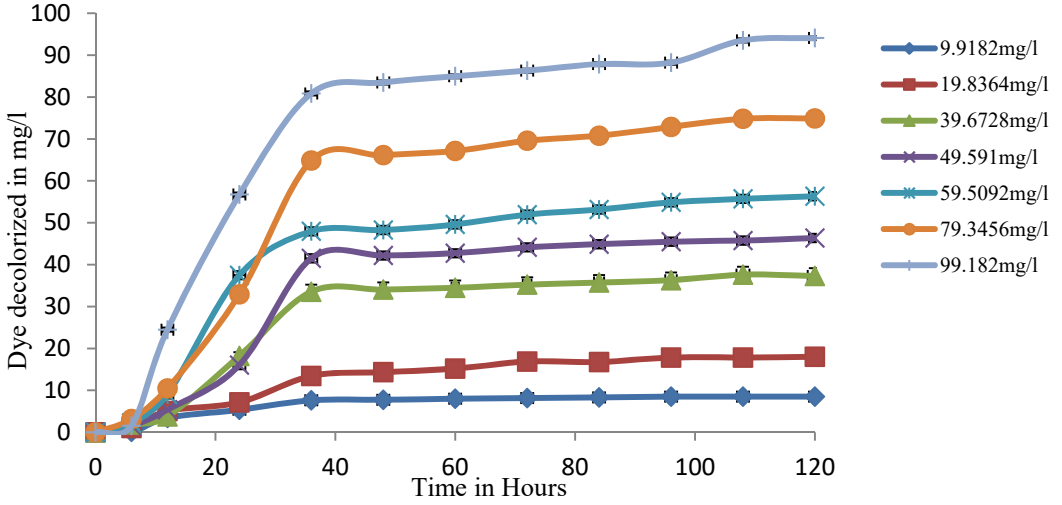


Fig.3. Effect of initial Reactive Black 5 dye concentration on enzyme decolorization.

### 3.5 Kinetics of the dye decolorization processes

Michaelis-Menten type rate model has been widely used for the kinetics study of substrate conversion using enzymes and or by living cells [13]. Decolorization of RB5 dye via enzymatic degradation in this study was analyzed based on the interpretation of the Michaelis-Menten kinetics Eq. (5).

$$V = \frac{V_{max}[S]}{K_m+[S]} \quad (5)$$

Where, $K_m$ is the Michaelis-Menten constant in mg $l^{-1}$; $V_{max}$ is the maximum decolorization rate in mg $l^{-1}$ $h^{-1}$; V is the substrate consumption rate in mg $l^{-1}$ $h^{-1}$ and S is the substrate concentration in $mgl^{-1}$. When Michaelis-Menten equation is transformed by a double reciprocal we obtain Lineweaver-Burk Eq. (6).

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (6)$$

A plot of (1/V) versus (1/S) yields a straight line with $1/V_m$ as the intercept and $K_m/V_m$ as the slope. Lineweaver-Burk plot of decolorization rate against concentration was used to calculate $V_m$ and $K_m$ (Fig. 4). The plot demonstrates a perfect fit with $R^2$ value of 0.9994 indicating that the model was best applicable in describing the biodegradation of RB5 dye by crude protease. The values obtained were $V_{max}$= 3.875$h^{-1}$ and $K_m$= 548.06 mg $l^{-1}$. The maximum decolorization rate, $V_{max}$ of 3.875$h^{-1}$ was reached, when all the active sites on the enzyme had been saturated with the dye. This would only happen, when RB5 dye concentration is greater than $K_m$ (548.06 mg $l^{-1}$).

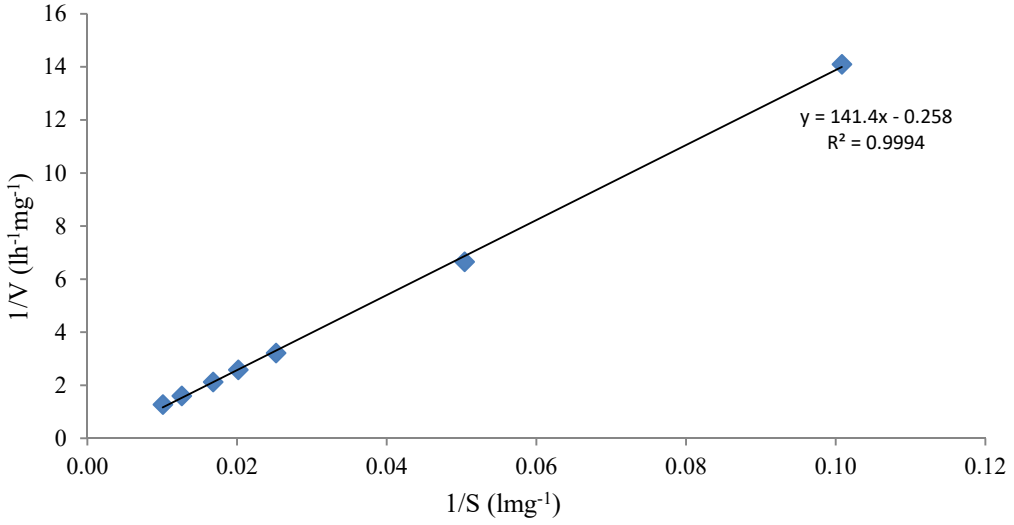


Fig.4. Lineweaver-Burk plot for the enzymatic decoloration of RB5 dye

### 3.6 Analysis of Dye Degradation Metabolites

Biodegradation of synthetic dyes not only results in decolorization of the dyes but also in fragmentation and breakdown of the dye molecules into smaller and simpler parts [14]. Biodegradation of the RB5 dye was monitored by a UV–Vis Spectrophotometer. UV–Vis spectral scan (380-800 nm) data of the control RB5 (0 minute); biodegraded RB5 sample mixture at various time periods and crude enzyme as negative control is shown in Fig. 5. The overall spectra and the peaks in the spectra decreased gradually with increase in time indicating decolorization and decrease in RB5 dye concentration in the batch solution. It can be clearly observed that peak at zero hour decreased with shift of $\lambda_{max}$ value of RB5 (597 nm) to lower wavelength (552 nm) indicating that new metabolite were being formed in the media. The absorbance peak in the UV–Vis spectra disappeared completely at the end of decolorization (120 hours). The intense black color of RB5 changed to colorless in the samples treated with the crude protease extract indicated that RB5 was completely mineralized with formation of new metabolites. This observation is consistent with previous reports by Younes and Sayadi [26] who investigated the detoxification of Indigo carmine using a combined treatment via a novel trimeric thermo stable *laccase* and microbial consortium.

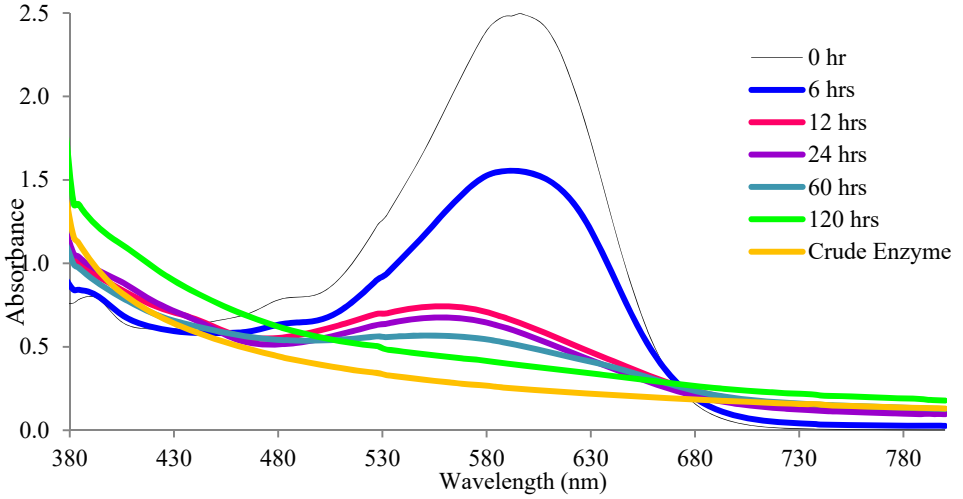


Fig.5. UV–Vis spectra scan of decolorization of Reactive Black 5 dye at different time period

### 3.7 Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS) analysis

LC–QTOF-MS analysis was carried out to identify the resultant metabolites formed during the biodegradation process. LC-QToF-MS analysis showed 9 peaks of specific intermediate products that were clearly distinguished compared with the controls of RB5 without crude protease extract or only crude protease extract without RB5 dye. Fig. 6 shows extracted chromatogram of a specific ion obtained from LC-QToF-MS. Based on the parent compound structure, intermediate compounds formed were positively identified. RB5 dye molecular structure could not be detected by LC−QToF−MS; which suggest that the dye immediately dissociate when dissolved in water to form 4-amino-3, 6-dihydrazinyl-5-hydroperoxynaphthalene-2, 7-disulfonic acid (m/z = 393.29) before fragmentation. It can be noted that RB5 dye has several functional groups and the process of biodegradation is a complicated one resulting in the formation of multiple intermediates. Analysis of the identified metabolites after degradation confirms the production of low molecular weight compounds through the asymmetric cleavage of RB5 dye molecule (Fig.7). However, there are possibilities that some intermediates were not detected by LC−QToF−MS.

Based on the metabolite analysis obtained and the time at which these intermediates appeared, a degradation pathway adopted by the crude protease extract for RB5 dye was proposed (Fig. 7). The proposed post degradation pathway of RB5 suggests the formation of Cyclohexanamine ,6-amino-6-oxohexanoic acid and 1,2,3,4,5,6,7,8-octahydro naphthalene-2, 7-disulfonic acid. This result should be of great importance because RB5 diazo dye decolorization and mineralization was achieved in a single step raising hopes of using crude enzyme from *Bacillus Cereus* Strain KM201428 for industrial wastewater treatment. The results obtained are consistent with the earlier results reported by Xingzu [27] who investigated the bio-decolorization and partial mineralization of reactive black 5 dye by a strain of *rhodopseudomonas palustris.*

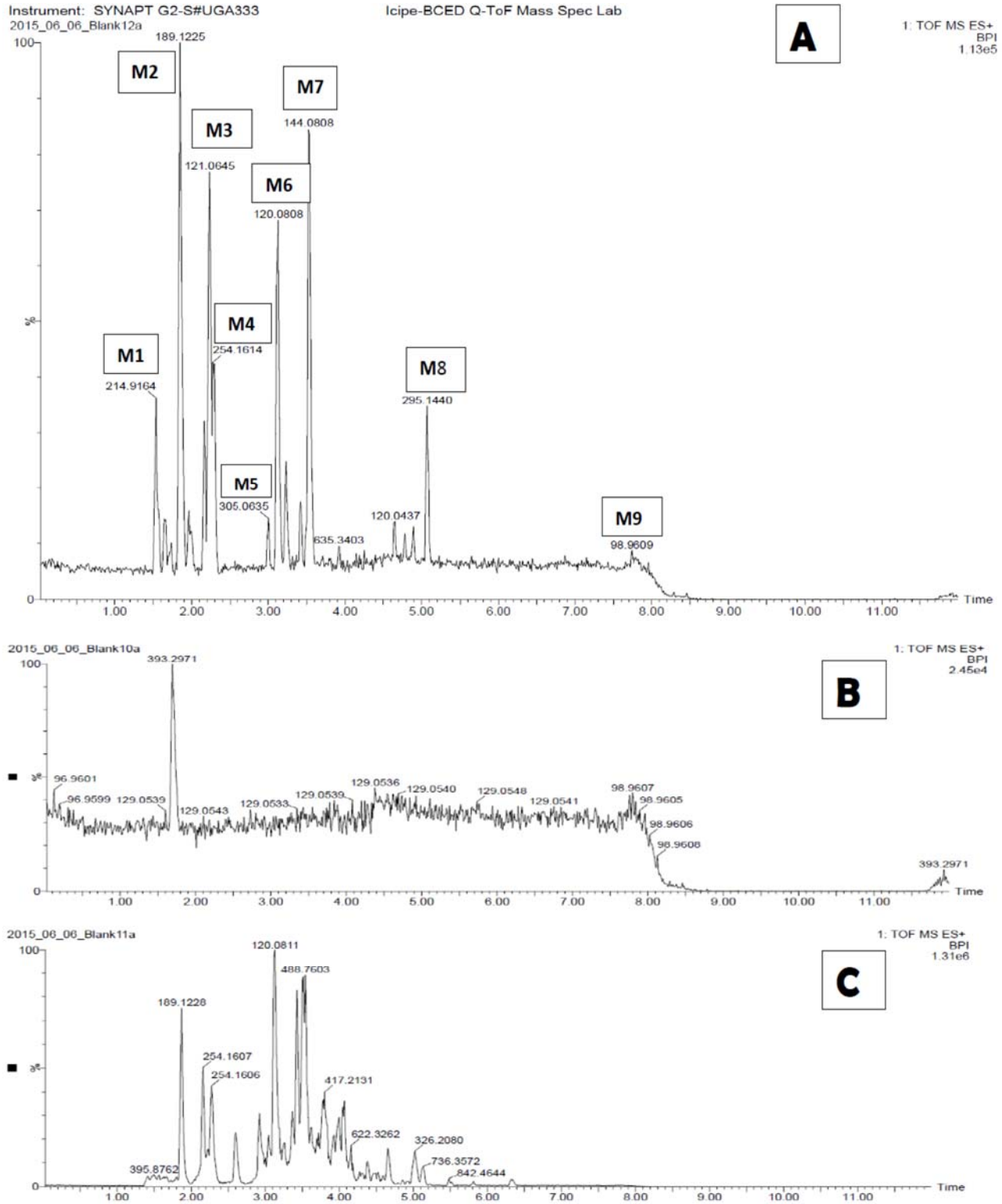


Fig.6. Extracted ion chromatograms of RB5 dye metabolites detected by LC–QTOF-MS in crude protease extract. (A) Represents chromatogram of degraded RB5 dye metabolites numbered M1-M9; (B) chromatogram of non-degraded RB5 dissolved in distilled water and (C) chromatogram of only crude protease extract.

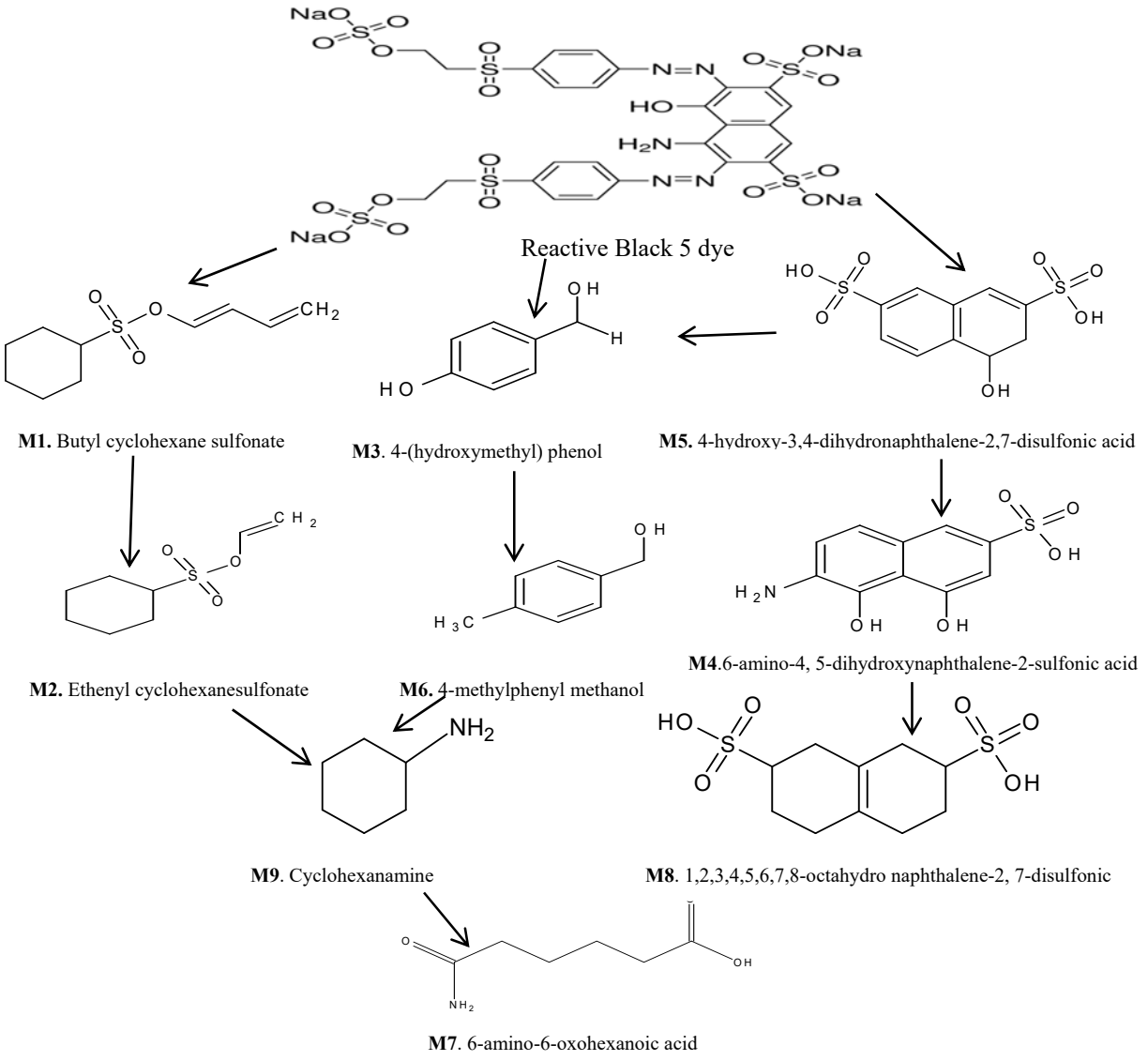


Fig.7. Proposed pathway for biodegradation of Reactive black 5 dye using crude protease from *Bacillus Cereus* Strain KM201428. The metabolites are numbered M1-M9 as identified by LC–QTOF-MS

## 4. Conclusions

The current study has demonstrated that crude protease from indigenous *Bacillus Cereus* Strain KM201428 can effectively biodegrade and detoxifying toxic azo RB5 dye under a wide range of pH and dye concentrations. UV-Vis and LC–QTOF-MS analytical techniques were used to authenticate and confirm biodegradation of RB5 dye into intermediate metabolites. Analysis of data obtained from LC–QTOF-MS revealed that biodegradation of RB5 by crude protease led to the formation of cyclohexanamine and 6-amino-6-oxohexanoic acid compound that are less toxic than the parent dye molecule. The rate of RB5 dye decolorization followed first-order kinetics at different initial dye concentrations. The double reciprocal plot for kinetics yield maximum decolorization rate, $V_m$= 3.875$h^{-1}$and half saturation constant, $K_m$= 548.06 $mgL^{-1}$. The unique ability of the enzyme to degraded RB5 dye under a broad range of pH and dye concentration suggested that the enzyme could be useful in bioremediation of textile effluent contaminated with organic dyes.

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