

# Microaerobic Biodegradation of Direct Black 38 by the Isolated Bacterial Strain *CSMB5*

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**ABSTRACT:** Direct Black 38 (DB38) is a sulphonated azo dye, with its constituent benzidine, a carcinogenic amine. Simultaneous decolorization and degradation of azo dye Direct black (DB38) was evaluated by the isolated bacterium *Bacillus sp. KMSII-3strain CSMB5* in a single microaerobic reactor. 95% decolorization was observed within 24h at a concentration of 50 mg/L of dye DB38 as sole carbon source. The steady growth of the isolated strain was observed from 12h. Dye degradation was confirmed by TOC reduction and amine degradation was confirmed by ammonia liberation. UV spectrum confirmed the phenyl ring cleavage. The strain *CSMB5* was also able to decolorize 25 mg/L of Direct, Reactive and Acid dyes within 48h. With the results obtained through FT-IR and GC-MS, a biodegradative pathway was constructed.

**KEYWORDS:** Direct black 38, Sulfonated Azodyes, Microaerobic, Biodegradation, *Bacillus*.

## I. INTRODUCTION

Azo dyes are used in textiles, leather, plastics, cosmetics, paper and food industries. Most of the direct dyes are azo compounds, containing two or three azo (N=N) groups. Sulfur dyes are inexpensive and exhibit good light fastness (1). Direct and reactive dyes are synthesized with sulfonic acid groups to give them solubility in water. Benzidine, is a precursor in the synthesis of certain azo dyes (2). The National Institute for Occupational Safety and Health as well as the International Agency for Research on Cancer (IARC) has declared it as carcinogenic (3). These dyes are used in the leather industry to render the finished leather resistant to sun light, washing, and microbial attack. The dyes enter into the waste stream, which eventually are recalcitrant to conventional treatment processes (4). Direct Black 38 (DB38) is a benzidine-based sulphonated azo dye, now a banned product due to its carcinogenic property.

The present study aims to evaluate the simultaneous decolorisation and degradation of the azo dye Direct black (DB38) as sole carbon by the isolated bacterial strain in a single microaerobic reactor. Metabolic versatility of the isolate will be investigated with structurally different dyes by utilizing the respective dyes as sole carbon source. A microaerobic biodegradative pathway was postulated.

## II. RELATED WORK

According to citations, it has been reported that the azo compounds with methyl, methoxy, sulfo or nitro groups are difficult to degrade than those with hydroxyl or amino functional groups (5; 6). Sulfonated aromatic amines remained as recalcitrant pollutant and constituted a COD fraction in wastewater after biological treatment (7). At present advanced oxidation process (AOPs) is proposed as an efficient method (8). These treatment methods are however associated with high costs. Microbial decolorisation of dyes is a cost effective method employed in wastewater treatment (9). Sequential anaerobic/microaerobic process followed by aerobic treatment of the dye or combined

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anaerobic-aerobic treatment are some of the current methods practiced for degradation of azo dyes from wastewater (10). According to Isik and Sponza, (11), decolorization was achieved with DB38 dye with the addition of glucose under both anaerobic and microaerobic conditions after 9 days of incubation period. *Bacillus thuringiensis* and *Bacillus cereus* exhibited decolorization ability to Acid Red119 (12) and cibacron red P4B dye with sucrose and ammonium nitrate as carbon/nitrogen source (13).

### III. MATERIALS AND METHODS

1. The Chemicals for the study was either procured from Sigma-Aldrich, / E.Merck, India or provided by a tannery
2. The bacterial strain *Bacillus sp. KMSII-3 strain CSMB5* used in this study was isolated from the phenol degrading microaerobic reactor in our laboratory (14). This strain was selected based on its degradability potential on DB38 and its stability even after repeated cycles. It is one among the six bacterial isolates capable of degrading a mixture of heterocyclic compounds in consortium.
3. Batch experiments on decolorization with DB 38 were carried out at 30°C in an orbital shaker at 50 rpm. Aliquot of 50 mg/L of dye was added in 40 ml mineral broth with yeast extract inoculated with 10% of exponential culture in 50 mL capacity screw capped Erlenmeyer flasks. 10% head space was provided to maintain initial dissolved oxygen concentration of 1 mg/L. Aliquots of 3 mL samples were withdrawn at regular time intervals and centrifuged (12,000×g) at 4°C for 10 min. The supernatants were monitored at 624 nm and 427 nm in a UV-Vis Spectrophotometer (Shimadzu UV2450). The decolorization activity was calculated by using the formula: Decolorization (%) = [(Ao-A)/ Ao]\*100, where Ao is the initial dye absorbance before decolorization and A is the absorbance after decolorization. To estimate growth, the centrifuged pellets of *Bacillus sp. KMSII-3 strain CSMB5* were dried at 60°C overnight for 24 h, until a constant weight was obtained.
4. In order to examine the effect of initial dye concentration on the decolorization of DB 38, experiments were conducted by inoculating the cells of *Bacillus sp. KMSII-3 strain CSMB5* to initial dye concentrations of 100, 150, 200 and 250 mg/L in screw capped flasks. The percentage decolorization was measured at different time intervals.
5. Studies on the metabolic versatility of *Bacillus sp. KMSII-3 strain CSMB5* was evaluated on structurally different azo dyes such as C.I. Direct Black (DB 22), C.I. Direct Brown (DB2), C.I. Dianil Brown (3GN), C.I. Acid Black (BG31), C.I. Acid green (AG25), C.I. Acid Yellow (AY194), C.I. Acid red (AR88), C.I. Remazol blue (RGB203), C.I. Remazol red (RR180), C.I. Reactive orange 16 (RO16) and C.I. Reactive Black (RB5). The mineral medium was amended with the respective dye at a concentration of 25 mg/L.
6. The parameters Total Organic Carbon (TOC) (5310-B), Nitrogen Ammonia, (4500-NH3-C) were analyzed using Standard Methods (15).
7. FT-IR analysis was carried out in order to assess the functional groups present in pure dye powder (DB 38) and after its biodegradation by *Bacillus sp. KMSII-3 strain CSMB5*. The samples were filtered through 0.45 µm filter, lyophilized and pelletized with potassium bromide (KBr) in the ratio of 1:50. The pellets were subjected to FT-IR analysis using transmission mode. The measurements were carried out with ABB make MB 3000 model FT-IR in the mid-infrared range from 4,000 to 500 cm<sup>-1</sup> with 16 scan speed.
8. The ethyl acetate extracts of cell-free medium were analyzed for the degraded metabolites of the dye DB38 by Gas Chromatography –Mass Spectrum analysis. The GCMS analysis was carried out using JEOL-GC-mate-II benchtop double-focusing GC mass spectrometer operating in electron impact ionization (EI) mode. Helium was used as carrier with a flow rate of 25 ml/min. The injector temperature was maintained at 220 °C (temperature range 70 - 250 °C) and the rate of increase in temperature was set to 15 °C/min. The ethyl acetate extract of the products were dissolved in methanol and subjected to GC/MS analysis. The compounds were identified on the basis of retention time and mass fractions.

### IV. EXPERIMENTAL RESULTS

#### 1. Isolation

Isolate *CSMB5* was selected based on its ability to produce large zones of decolorization around colonies growing on mineral agar containing sulfonated diazodye DB38. It is a long rod, Gram-positive, endospore forming and motile in

nature. It is positive to catalase, oxidase, urease, casein hydrolysis and nitrate reduction and negative to Voges-Proskauer test.

## 2. Decolourization

Batch reactor studies were performed to evaluate the decolourization of the azo dye DB38 by *Bacillus sp. KMSII-3* strain *CSMB5* (Fig. 1).



Fig. 1. Decolourization of the dye DB38 by *Bacillus sp. KMSII-3* strain *CSMB5*

10% of exponential culture (36 mg/L as dry weight) was inoculated in mineral medium amended with (50 mg/L) dye DB38 and yeast extract (0.1 g/L). The initial dissolved oxygen (DO) concentration observed was 1.5 mg/L. At the end of 48h, the DO concentration was 0.03 mg/L and 91% decolourization was obtained.

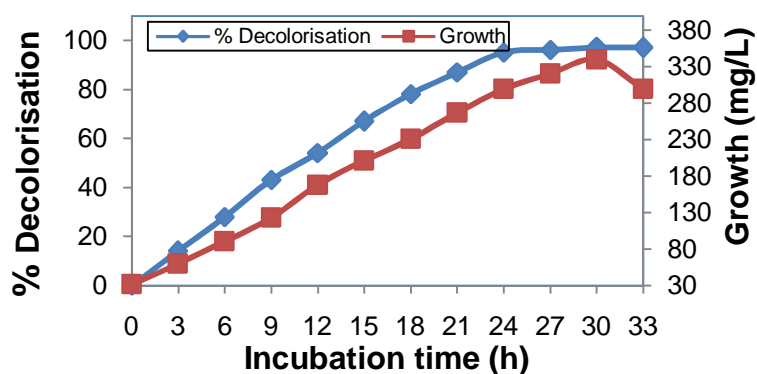


Fig. 2: Growth and percentage Decolourisation of dye DB38

However, when studies were conducted (16) in a 3.5 litre microaerobic reactor under optimized condition (DO of 0.9 mg/L, pH of 7 and temperature of 30°C), 95% decolourization of DB38 was observed within 24h (Fig. 2). Maximum decolourization of 97% was obtained at 30h with the absorbance of 1.051 at 600 nm. Biomass production was directly proportional to decolourization of dye confirming the utilization of DB38 as the sole source of carbon for the growth of the isolate.

## 3 Effect of initial substrate concentration

The potential of *Bacillus sp. KMSII-3* strain *CSMB5* to tolerate the high dye concentrations was assessed by carrying out experiment with varied dye concentration. Maximum decolourization of 80% was obtained with 100 mg/L of the dye within 36h. Though the bacterial strain tolerated 250 mg/L of dye concentration, only 36% decolourization was achieved (Fig. 3). Increase in dye concentration might have resulted in increased production of amines, which becomes toxic to bacterial strain.

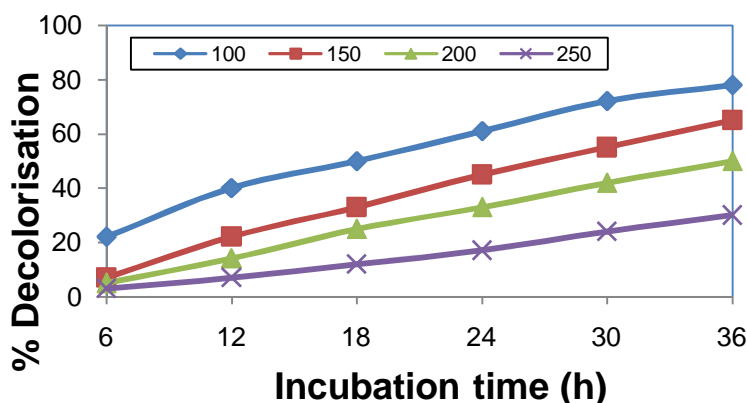


Fig.3 Effect of initial concentration of dye DB38

#### 4 Metabolic Versatility

The decolorization capacity of *Bacillus sp. KMSII-3* strain *CSMB5* to degrade eleven structurally different direct, reactive and acid dyes at a concentration of 25 mg/L was tested (Table 1).

Table. 1: Metabolic diversity shown by *CSMB5 - Bacillus sp. KMSII-3* after 48h incubation

Azodyes	% Decolorisation	Wave length ( $\lambda_{max}$ )
C.I. Direct Black (DB 22) (Diazo sulfonate dye)	89	632
C.I. Direct Brown (DB2) (Diazo sulfonate dye)	80	479.5
C.I. Dianil Brown (3GN) Mono azo sulfonate dye	86	403.5
C.I. Acid Black (BG31) (Diazo sulfonate dye)	82	603.5
C.I. Acid green (AG25) (Anthracene sulfonated dye)	52	640.5
C.I. Acid Yellow (AY194) (Triphenylmethane dye)	45	458.5
C.I. Acid red (AR88) (diazo, sulfonated dye )	79	388
C.I. Reactive Blue (RB203) (anthraquinone sulfonated dye)	90	600.5
C.I. Reactive Red (RR180) (diazo sulfonated dye)	70	533.5
C.I. Reactive orange (RO16) (diazo sulfonated dye)	89	489
C.I. Reactive Black (RB5) ( diazo sulfonate dye)	86	595

The decolorization tests were conducted in a mineral medium with the respective dye, supplemented with 0.1 g/L yeast at 30°C under microaerobic condition. As shown in the Table, the decolorization was significantly higher (70-80%) with direct and reactive dyes when compared to acid dyes. The rate of decolorization of azo dyes is affected by their molecular weight, substitution groups in the dye molecules, and the intramolecular hydrogen bond between the azo and hydroxyl groups. The dyes tested here are generally (direct and reactive dyes) used in the leather dyeing process. Since the *Bacillus sp. KMSII-3* strain *CSMB5* was already exposed to leather industrial wastewater, it was possibly able to decolorize more than 80% within 24 to 48h. However in leather industrial wastewater, the expected maximum dye concentration is between 25-50 mg/L. In the present study the isolated strain was able to degrade well above that concentration and thereby able to reduce the residual COD in treatment plant.

### 5 Biodegradation

To confirm the biodegradation efficacy of *Bacillus sp. KMSII-3* strain CSMB5, TOC and ammonia contents were monitored. In parallel to decolorization of DB38 dye and increase in biomass as given above, TOC reduction was observed from 12h of incubation as shown in Fig. 4 indicating deamination of benzidine to 4-aminobiphenyl.

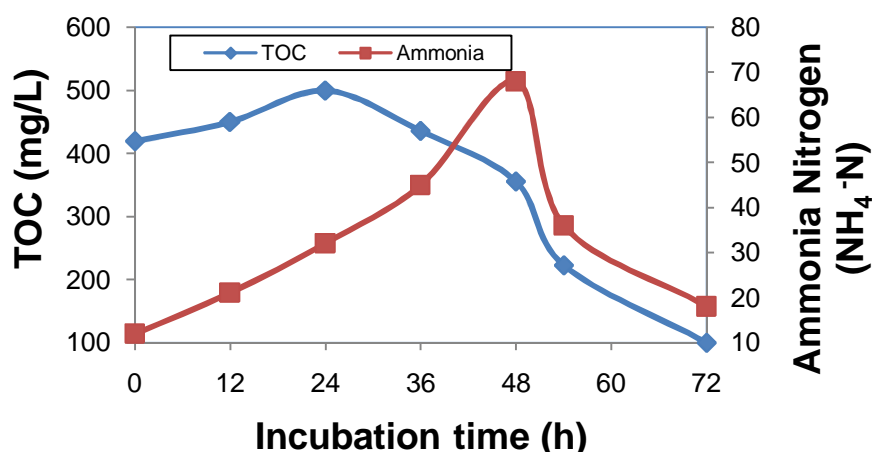


Fig. 4: Reduction in TOC content of dye DB38 and liberation of ammonia

With the azo bond cleavage, primary amine (benzidine) may be accumulating. TOC reduction observed was 77% with the release of ammonia by 81mg/L after 48 h. It may be due to further deamination to secondary amines.

### 6 UV-Visible Spectroscopy

Samples were withdrawn from the dye culture medium at regular intervals and spectral changes are recorded using UV-Vis Spectrophotometer (Fig. 4). Pure dye showed maximum peak height at ( $\lambda_{max}$ ) 624 and 472 nm.

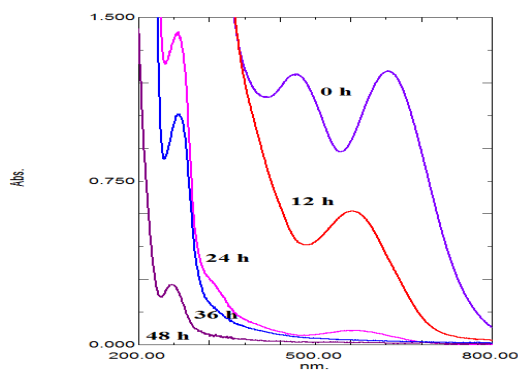


Fig. 5: Decolourization and degradation spectrum of Dye DB38

The spectra obtained on the culture supernatant drawn at different incubation times clearly showed step wise reduction in these peak heights confirming the decolorization of the dye. By 12h about 43 % of the dye was found to be decolorized. The decrease in the absorbance of the peak in the visible region and the formation of a new peak at 260 nm suggest that there were other transformational changes to the aromatic structure in correlation with azo bond cleavage (17) Similar to aerobic condition, appearance of peak at 260 nm was observed in the present study at microaerobic

condition confirming the aromatic ring cleavage of the dye DB38 and possible formation of naphthoquinone or diketones.

### 8 FT-IR Spectroscopy

By observing the FT-IR spectrum of pure dye and its degraded metabolites, a considerable change in positions of peaks were observed in samples drawn after 24, 36 and 48h extracted samples and the results are shown in Fig.6 (b) to (d) and compared to control dye (Fig. 6a) (18).

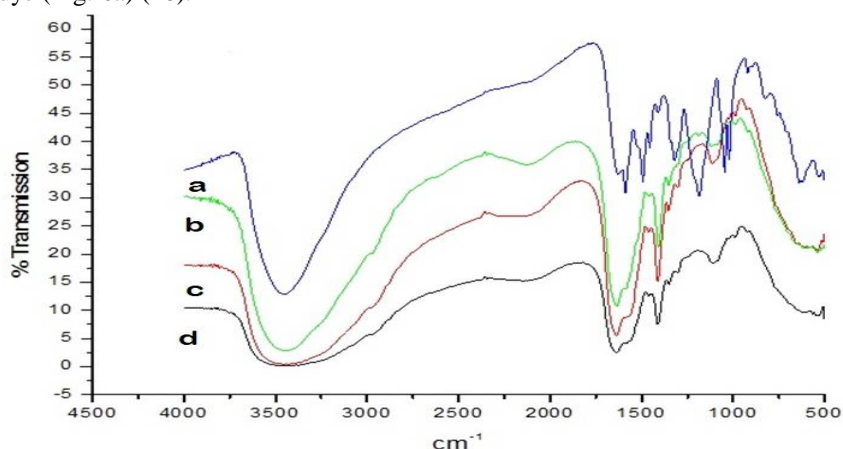


Fig. 6: FT-IR spectrum of DB 38 (a) 6h (b) 12h (c) 24 h and (d) 48h

The FT-IR spectrum of DB38 has a peak at  $3446\text{ cm}^{-1}$  for N-H stretching, a peak at  $2102\text{ cm}^{-1}$  for asymmetric  $\text{CH}_3$  stretch and a band for C=C stretching at  $1628\text{ cm}^{-1}$  are due to amino and carbonyl groups, while a peak at  $1588\text{ cm}^{-1}$  represented -N=N- stretching of azo group. Peak at  $1493\text{ cm}^{-1}$  showed -OH stretching vibration. In addition, a peak at  $1186\text{ cm}^{-1}$  represented primary aromatic amines with -C-N vibration. A peak at  $1046\text{ cm}^{-1}$  for -S=O stretch and a peak at  $831\text{ cm}^{-1}$  for -C-S stretch support the presence of sulphur containing group in control dye. A significant reduction in 24 and 36h of incubation confirmed that the concentration of dye reduced with time. The 48h extracted sample showed a significant changes with the appearance of band at  $1636\text{ cm}^{-1}$  representing the formation of aromatic amines. A peak at  $1411\text{ cm}^{-1}$  due to stretching of -CH<sub>2</sub> deformation, a peak at  $3374\text{ cm}^{-1}$  showing -NH stretching and a peak at  $1350\text{ cm}^{-1}$  representing C-N vibration which indicate the formation of secondary aromatic amines. The absence of peak representing the azo bonds indicated the cleavage of azo linkage. A peak at  $703\text{ cm}^{-1}$  represents aromatic C-H bending. Disappearance of peak at  $831\text{ cm}^{-1}$  proved the removal of sulfur containing group from the biodegraded product.

### 9 Gas Chromatography –Mass Spectrum:

GC-MS analysis was carried out to find out the metabolites formed during the biodegradation of DB38 by the *Bacillus sp. KMSII-3 strain CSMB5*. The metabolites of the dye were isolated, identified and compared with authentic standards (19). The ethyl acetate extract obtained after 24 h and 48 h incubation are shown in Fig 7a, b. The culture after 24 h incubation showed two amines at a retention time of 16.09 min indicating reduction of the two azo bonds of DB38. The compound observed at 100% peak level was found to correspond to benzidine (m/z 180) and with 75% peak level was 4-amino-5-hydroxynaphthalene-1-sulfonic acid (m/z 241). For the culture supernatant collected after 48h of incubation, the peak was observed at the retention time of 18.07 minutes. The predominant metabolite observed with 100% peak level was 2, 4-diaminophenol (m/z 126) and acetyl sulfanilic acid (m/z 209) along with an unknown metabolite at m/z 252. Another peak with a peak height of 50% was 4-aminobiphenyl (m/z 169).

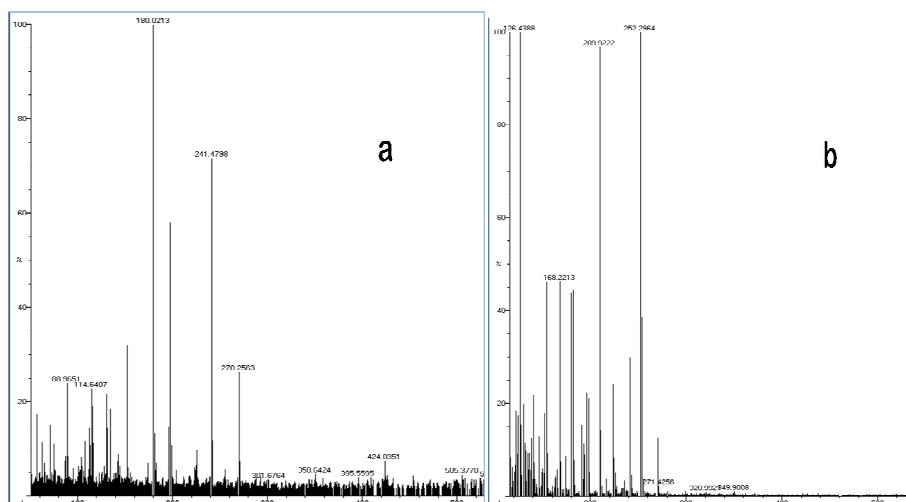


Fig. 7: GC-MS profile of degraded DB 38 dye metabolites after (a) 24h and (b) 48 h

### 3.10 Biodegradation pathway

With the results obtained through UV-Visible spectrum, FTIR and GCMS, a biodegradation pathway is proposed for the dye DB 38 by *Bacillus sp. KMSII-3* strain *CSMB5* at microaerobic condition and is shown in Fig. 8.

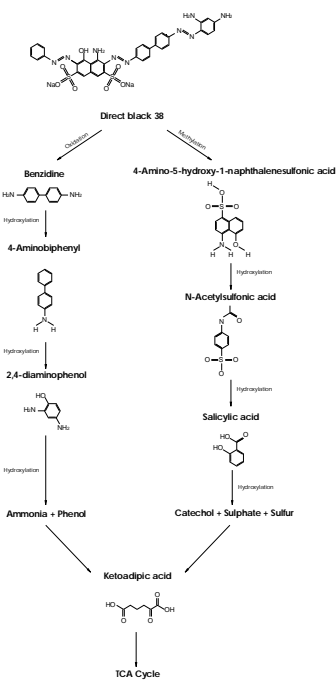


Fig.8: Microaerobic Biodegradative pathway of DB38 by *Bacillus sp. KMSII-3* strain *CSMB5*

The metabolism of DB38 is initiated with cleavage of azo bond by the action of azoreductase with the corresponding release of benzidine. By deamination, acetylation and hydroxylation of benzedine, intermediary metabolites are formed, such as 4-aminobiphenyl, 4-amino-5-hydroxynaphthalene-1-sulfonic acid, 2, 4-diaminophenol, Acetyl sulfanilic acid

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and an unknown metabolite. Ortho cleavage of benzyl ring was achieved by the activities of catechol 1,2 oxygenase and finally the metabolites may enter the TCA cycle.

## V. CONCLUSION

The present study demonstrated that DB 38 was degraded to non toxic end products under microaerobic condition by *Bacillus sp. KMSII-3* strain *CSMB5*. The microaerobic process is an energy-efficient with minimum production of settleable sludge when compared to the conventional treatment. Simultaneous decolourization and degradation of azodye Direct black (DB38) was evaluated by the isolated bacterium in a single microaerobic reactor. 95% decolourization was observed within 24h at a concentration of 50 mg/L of dye DB38 as sole carbon source. Biodegradation efficacy was shown by TOC reduction and release of ammonia was observed in parallel. There was a steady increase in the growth of biomass from 12h of incubation. Disappearance of peak at 260 nm in UV spectrum, confirm the cleavage of phenyl rings by 48h. The strain *CSMB5* was also able to decolorize 25 mg/L of direct, reactive and acid dyes within 48h. By observing the changes in functional groups by FT-IR and identification of mass spectrum in GCMS, a biodegradative pathway was constructed. The microaerophilic *Bacillus sp. KMSII-3* strain *CSMB5* may be considered as one of the promising bacterium for commercial application.

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