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Decolorization and biodegradation of Indigo carmine by a textile soil isolate *Paenibacillus larvae*

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Abstract The potential of recently isolated bacteria *Paenibacillus larvae* for the effective decolorization of Indigo carmine was evaluated. The effects of operational parameters (temperature, pH, dye concentration, shaking/non shaking) were tested. Maximum extent of decolorization was observed when the medium was incorporated with 10 g/l of yeast extract and peptone. Decolorization was strongly inhibited at non-shaken conditions as well as incorporation of inorganic sources (sodium nitrite and ammonium chloride) in the medium. Maximum decolorization was observed at 30°C (100%) and 40°C (92%) at 8 h of incubation. The LC-MS and NMR analysis confirms the oxidation of Indigo carmine .The primary degradation products were found to be Isatin sulfonic acid and anthranilicacid.

Keywords Paenibacillus *larvae* · Decolorization · Degradation · Indigo carmine · Isatin sulfonic acid

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Introduction

Approximately 10–15% of the dyes are released into the environment during manufacturing and usage (Spadarry et al. 1994). The majority of these dyes are either toxic to flora and fauna or mutagenic and carcinogenic (Nilsson et al. 1993), and therefore pose a potential health hazard to all forms of life (Sharma et al. 2000). The elimination of such dye containing effluents is mostly based on physico-chemical procedures (e.g. absorption, concentration, chemical transformation and incineration). These methods are rather costly and sometimes produce hazardous bye-products and therefore other alternatives such as biodegradation attract attention (Shaul et al. 1991; Kandelbauer et al. 2004). One potentially attractive strategy is bioremediation, which is becoming an important issue in both developed and developing countries (Waffa et al. 2003) it is aimed at providing a low cost environmentally acceptable treatment solution to this waste problem.

The major classes of dyes have anthroquinoid, indigoid, and azo aromatic structures. The main drawback of this class of dyes is that they are not easily degraded by aerobic bacteria, and with the action of anaerobic or microaerobic reductive bacteria, they can form toxic and/or mutagenic compounds such as aromatic amines (Chung et al. 1992; Wong et al. 1996).

Indigo dyes are widely used for dyeing and printing protein and cellulose fibers (Reife et al.

1995). Biodegradation of indigo dyes was carried out by a variety of microorganisms including alkalophilic bacteria, (Nakajima et al. 2005), thermophilic bacteria, (Nicholson et al. 2005), anaerobic bacteria, (Nicholson et al. 2004). Enzymatic degradation of Indigo carmine with laccases from the fungi *Trametes hisuta* and *Sclerotium rolfsia* was reported (Campos et al. 2001). The degradation of textile dye indigo by bacteria provide a basis for the development of a sustainable alternative to the present chemical methods used to reduce indigo for denim dyeing.

Hence an attempt was made to isolate and identify a bacterium to decolorize Indigo carmine effectively and to study effect of operation parameters (medium composition, temperature, pH, dye concentration) on the decolorization of Indigo carmine. The degradation product was analyzed and confirmed using LC-MS &NMR analysis. However, there has been no previous study on *Paenibacillus larvae* on dye decolorization.

Materials and methods

Chemicals

Indigo carmine (Indigo-5,5′-disufonic acid disodium salt) and Isatin sulfonic acid were purchased from Sigma Aldrich, USA. All other chemicals and media were purchased from Himedia, Bombay, India. The stock solution of Indigo carmine was filter sterilized and added to growth medium in the required concentration (Fig 1).

Screening and selection of microorganisms

Sludge samples from textile industry near Kumbakonam, Tamilnadu, India, were collected and inoculated in liquid minimal medium of the following composition (g/l), NaCl (2.00), Casein enzymatic hydroxylate (5.0), $C_6H_{12}O_6$ (1.00), K_2HPO_4 (7.00), KH_2PO_4 (2.00), $Na_3C_6H_5O_7$ (0.50), $MnSO_4$ (0.10), $(NH_4)_2SO_4$ (1.00) with 100 mg/l Indigo carmine. The pH of the medium adjusted to 7.0. After incubation at 37°C for 48 h in a rotatory shaker at 150 rpm, 100 µl of each liquid culture was separated on to solid medium with 100 mg/l of Indigo carmine to isolate the dye decolorizing strains. From that the best decolorizing organism was isolated and preserved in nutrient agar slants at 15°C for further use.

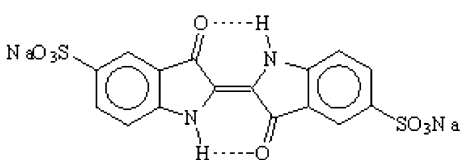


Fig. 1 Chemical structure of Indigo carmine (Indigo-5,5′-disufonic acid disodium salt)-λmax 610 nm

Strain Identification

Strain with higher decolourization ability in solid minimal medium with 100 mg/l Indigo carmine were subjected to Gram Stain and identified by Biolog Inc., at Microbial Type Culture Collection Center (MTCC), Chandigarh, India.

PCR amplification of 16s ribosomal DNA

Genomic DNA from the bacteria was isolated from the 24-h-old liquid cultures by a standard phenol-chloroform procedure. A pair of universal primers 8f(5′-GAGTTTGATYMTGGCTCAG-3′) and 1495r(5′-CTACGGCTACCTTGTTACG-3′) (Grifoni et al. 1995) was used for amplification of a 1.5 kbp region of the 16SrDNA.The PCR was performed in 25 µl reaction mixture containing 200 µmol of dNTPs, 200 ng of each primer, 10× buffer solution, $MgCl_2$, DMSO, 1 U of Taq DNA polymerase, 100 ng template DNA, and sterile water. Amplification was carried out in thermal cycler (Master cycler personal, Eppendorf, Germany). The initial denaturation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min and the final extension at 72°C for 5 min. The PCR products were loaded on to 0.8% agarose gel, and DNA fragments were separated at field strength of 4.4 V/cm for 1.5 h, then stained with ethidium bromide and documented using Quantity One Software (GelDocumentation System, BIORAD, Australia)

Cloning and sequencing of amplified 16srDNA

PCR products were cloned into vector PDK 101 vector (modified pGEM-2T) within XcmI sites. Using an Applied Biosystems 3100 DNA automatic

sequencer, 16srDNA sequencing was carried out and deposited to the Genbank.

Decolorization study

Dye decolorization was detected by UV–Vis spectrophotometer (Labomed, USA) at the Indigo carmine λmax (610 nm) using the supernatant from the liquid culture medium after centrifugation at 10,000 rpm for 10 min in a refrigerated centrifuge (Eppendorf 5804 R). The removal of color was reported as % decolourization ($\% = A_0 - A_t \backslash A_0 \times 100$, where A_0 and A_t are absorbance of the dye solution initially and at cultivation time (t), respectively). Each decolorization value is a mean of three parallel experiments. Abiotic controls (without microorganism) were always included.

Effects of physico-chemical factors on Indigo carmine decolorization

Aliquot of 1 ml overnight night culture (7×10^7 CFU) was inoculated into 500 ml conical flask containing 100 ml of liquid minimal medium. The culture was incubated at various temperatures (20,30,40 and 50°C), pH (2–9), with various nitrogen sources such as yeast extract, peptone, sodium nitrate, ammonium sulphate (10 g/l) and with various concentration of dye (25, 50, 100, 200 mg/l) in an orbital shaking incubator at 150 rpm. Aliquots of the samples from the culture supernatent were used for the decolourization studies.

Liquid chromatography-Mass spectrometric analyses (LC-MS)

About 100 ml of culture sample taken at various time intervals (hours 0, 12, 24, 48 and 72) centrifuged, filtered through 0.45 μm membrane filter (Millipore). The filtrate was then extracted thrice with dichloromethane and evaporated in a vacuum evaporator (Buchii R 124,Germany) with 40–50°C water bath, after which the residue was dissolved in acetonitrile (HPLC grade) and used for LC-MS analysis, performed using an Ion Trap Mass spectrometer (Agilent Technologies,USA) using C18 column from Waters. The cartridge were conditioned with pure acetonitryl, washed with deionized water (0.1% formic acid), and the elution took place with 70% acetonitrile, containing 0.1% formic acid .The flow rate was 0.8 ml/min. The ion trap detector with atmospheric pressure electro spray ionization (API-ES) source was used for quantification in negative ionization mode. Operating conditions were dry temperature 325° C, Capillary voltage 3500 V, Nebulizer 14 psi, dry gas helium 5.0 l/min. Ion trap full scan analyses were conducted from m/z 200–1400 with an upper fill time of 300 min. The nebulizer gas flow and the curtain gas flow (N_2) were set at 10 and 8 psi. The ion spray, orifice and ring voltage were set at +4800, 40, +170 V respectively. Instrument control and data acquisitions were performed with 1100 series Data analysis for MS.

NMR analysis

The cell free supernatent obtained during 0 and 24 h were filtered through 0.45 μm and extracted three times with dichloromethane to isolate the transformed product. The pooled extract was concentrated on a Vacuum Rota vapour (Buchii R124 Germany) and used for degradation experiments. The completely dried sample was dissolved in $CDCl_3$ and transferred in to NMR tubes (Wilmed, USA). The ^{1}H-NMR spectra of the before treatment and after treatment samples were recorded using a 300 MHz, Bucker NMR spectrometer, Switzerland to observe the structural transformations in the dye molecule during the process of degradation

Results and discussion

Isolation and characterization of Indigo carmine degrading bacterium

Twenty four bacterial isolates of Indigo carmine decolorizing bacteria were isolated, among these one bacterial isolate which showed highest decolorization was isolated. It was selected and tested for various biochemical and physiological reactions. The result of the identification of this Indigo carmine degrading bacterium is listed in Table 1. The organism was found to be *Paenibacillus larvae* (MTCC 7949) and the 16sRNA sequence of 1520 bp was deposited to the Genbank (ID EF381743).

Table 1 Biochemical and physiological profile of *Paenibacillus larvae*

Morphology	Rod
Motility	–
Gram Staining	+
Aerobic Growth	+
Anaerobic Growth	+
Catalase	+
Oxidase	+
Nitrate Reduction	+
Arginine dihydrolase	+
Ornithine decarboxylase	–
Methyl Red	+
Indole test	–
Voges Proskauer test	–
Citrate utilization	–
Caesin hydrolysis	+
Esqculine hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	–
H_2S production	–
Acid from glucose	–
Acid from sucrose	–
Acid from lactose	–
Utilization of	
L-arabinose	+
Beta –hydoxybutyric acid	–
Galactose	–
i-Inositol	–
D-Mannitol	–
D-Malic acid	–
Propionic acid	–
L-Rhamnose	–
D-Sorbitol	–
Trehalose	+
Xylitol	–

Effect of physico-chemical factors on decolorization

The decolorization studies were carried out at various temperatures (20, 30, 40 and 50°C). Maximum decolorization was observed at 30°C (100%) and 40°C (92%) at 8 h of incubation. Between 30 and 40°C the decolorization rate was higher when compared to 20 & 50°C (Table 2). The percentage decolorization was found to be low at 20°C (31% at the end of 12 h). The above results confirm that the organism is mesophilic in nature. Possible reason for this kind of activity may be because the enzymes responsible for decolorization have an optimum temperature requirement around 30–40°C. However, detailed studies will have to be carried out to characterize the enzyme in vitro. No dye decolorization was observed in the control flask without the inoculum.

The organism showed a very low % decolorization at pH 3. The maximum decolorization of Indigo carmine by our organism was seen at pH 7 and 8 at the end of 12 h of incubation (Table 2). When the pH increases from 3 to 8 the decolorization percentage also increased. Similar results were reported using *Aeromonas hydrophila* and mixed cultures respectively (Chen et al.2003; Fang et al. 2004). The trend in pH dependence of decolorization is similar to that observed in *P. luteola* (Knapp et al. 1995). Therefore it could be suggested for treating basic textile effluents. The percentage colour removal was high in culture maintained at shaken condition (100%) at the end of 8 h (Table 2). Similar observation has been reported for other cases in literature (Carliel et al. 1995). This may be due to sufficient aeration and increased availability of nutrients in a shaken culture. Increased decolourization in a shaken culture may be due to increased activities of the microbial enzymes. Decolorization rate was low in non-shaken culture (60% at the end of 12 h). This may arise from the insufficient mass transfer between the organism and the dye solution as well as insufficient aeration.

The decolorization efficiency is strongly affected by the medium compositions. Organic nitrogen sources (peptone, yeast extract) incorporation shows higher decolourization when compared with inorganic sources (nitrate, ammonium chloride). Efficiency of organic sources (peptone and yeast extract) in decolorization seemed to be same (100% at the end of 12 h). Table 2 shows a low degree of decolorization with inorganic sources such as nitrate (11% at the end of 12 h) and ammonium chloride (15% at the end of 12 h).

Complete decolourization of the dye was observed when we used 25 mg/l at 14 h of incubation while 50 mg/l and 100 mg/l of Indigo carmine the decolourization occurred only at 20 h of incubation (Table 2).When the concentration increases time required for the decolorization also increased.

Table 2 Effect of Physico-Chemical factors on decolorization

Effect of Various temperature (pH7.5, Indigo Carmine 100 mg/l, with shaking)							
Time in hours	0	2	4	6	8	10	12
20°C	2 ± 0.4	4 ± 0.4	8 ± 0.2	15 ± 1.2	8 ± 0.27	14 ± 1.2	31 ± 1.3
30°C	2 ± 0.4	15 ± 1.2	74 ± 1.6	90 ± 0.7	100 ± 1.1	100 ± 1.1	100 ± 1.5
40°C	2 ± 0.3	35 ± 0.9	83 ± 0.86	88 ± 1.0	92 ± 0.8	100 ± 0.98	100 ± 1.3
50°C	2 ± 0.33	25 ± 1.0	27 ± 0.74	42 ± 0.8	50 ± 0.91	56 ± 1.4	57 ± 0.63
Effect of shaking condition (pH 7.5, Indigo Carmine 100 mg/l, Temp 37°C							
Non Shaken condition	1 ± 0/54	12 ± 0.61	26 ± 0.64	33 ± 1.06	54 ± 1.32	57 ± 0.58	60 ± 1.33
Shaken Condition	1 ± 0.54	15 ± 0.39	77 ± 1.2	91 ± 1.1	100 ± 1.6	100 ± 0.9	100 ± 1.33
Effect of medium Composition (pH7.5, Indigo Carmine 100 mg/l, with shaking, Temp 37°C)							
Peptone	1 ± 0.54	4 ± 0.42	87 ± 1.0	95 ± 1.2	96 ± 0.98	100 ± 1.34	100 ± 0.97
Yeast Extract	2 ± 0.33	14 ± 0.6	87 ± .0.25	95 ± 1.2	94 ± 1.5	100 ± 2.2	100 ± 1.9
Ammonium Nitrate	1 ± 0.25	4 ± 0.28	9 ± 0.56	6 ± 0.9	8 ± 0.12	10 ± 0.42	11 ± 0.28
Ammonium Chloride	1 ± 0.36	2 ± 0.25	3 ± 0.58	2 ± 0.59	8 ± 1.45	14 ± 1.9	15 ± 0.9
Effect of pH (Temp 37°C,IndigoCarmine 100 mg/l, with shaking)							
Decolorization % at 24 h	pH3	pH4	pH5	pH6	pH7	pH8	PH10
	6 ± 0.56	15 ± 0.28	80 ± 1.5	88 ± 2.1	100 ± 1.8	100 ± 1.33	86 ± 1.36
Effect of dye Concentration (pH7.5, with shaking, Temp 37°C)							
Time in hours	4	8	12	16	20	24	26
25 mg/l	15 ± 0.6	75 ± 1.2	92 ± 1.2	100 ± 0.7	100 ± 0.95	100 ± 0.4	100 ± 1.23
50 mg/l	16 ± 1.2	70 ± 0.2	75 ± 0.56	85 ± 0.45	97 ± 0.7	100 ± 1.25	100 ± 1.65
100 mg/l	1.4 ± 0.21	52 ± 0.25	68 ± 0.56	77 ± 0.4	80 ± 0.9	86 ± 1.86	100 ± 1.95
200 mg/l	3 ± 0.12	14 ± 0.32	28 ± 0.6	40 ± 1.5	66 ± 1.8	88 ± 0.9	100 ± 1.35

However the higher concentration of dye (200 mg/l) is not toxic to cells. The above results show that faster decolorization of Indigo carmine occurs with this organism in a shorter duration and it can treat textile effluents effectively.

Chromatographic analysis of products formed

LC-MS analyses were carried out to investigate the metabolites formed during biodegradation process. As shown in Fig. 2, dye sample collected at 0 h showed a major peak with the retention time of 0.622 min (m/z 420.9). After 12 h the sample was subjected to similar studies, one new peak was observed while other peaks disappeared at a retention time of 1.433 with the m/z value of 519 which suggests that the compound is a dimerized one, when the spectrum was taken for the same sample after 24 h, it is quite interesting to note that the peak at m/z 519 disappeared instead, it showed a new peak with m/z 243.8 (Fig. 3) at the retention time of 1.4 min. At the end of 48 h the initial formation of antharnilic acid with the retention time 1.69 was observed along with isatin sulfonic acid (Table 3). At the end of 72 h anthranilic acid was found as stable oxidation compound as reported earlier (Campos et al. 2001). All the LCMS analyses lead to the conclusion that the degradation of the initial dye yielded the product as Isatin sulfonic acid, which was further, confirmed by injecting standards.

^{1}H NMR analysis

The ^{1}H NMR spectrum of the Indigocarmine (Fig. 4) showed two singlet in the downfield and an aromatic proton at 7.6δ and 7.62δ corresponding to –NH and two doublets at 6.90δ and 6.88δ, which accounts for the presence of two adjacent protons on the aromatic

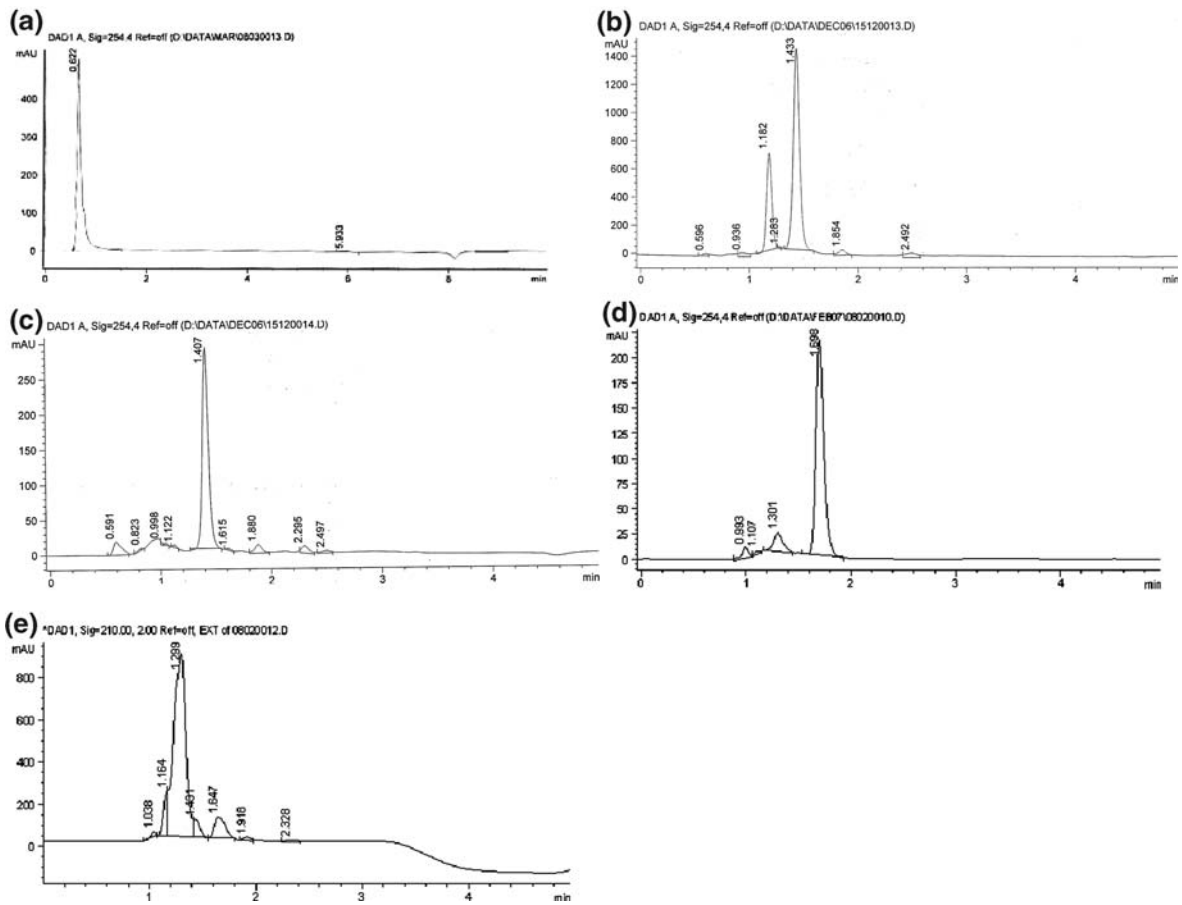


Fig. 2 Chromatogram of the degradation study of Indigo carmine (100 mg/l).(a) 0 h (b)12 h, (c) 24 h (d) 48 h (e)72 h

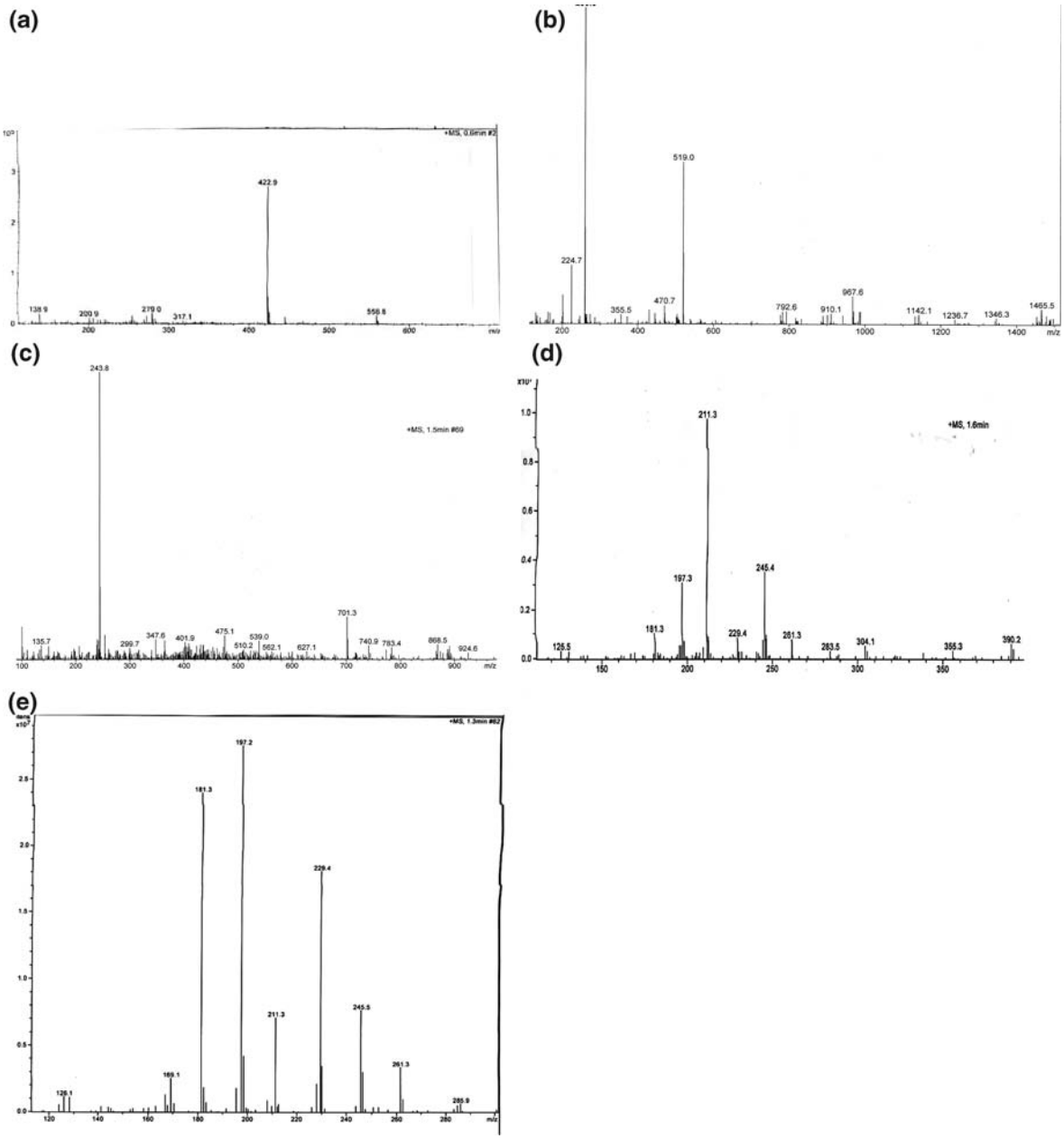


Fig. 3 Mass spectra of dichloromethane extract of the culture filtrate at different time intervals (a) O h (b) 12 h. (C) 24 h (d) 48 h (e) 72 h

ring. The over all spectrum clearly depicts the structure of Indigocarmine.

In order to understand vividly whether the indigocarmine has undergone effective degradation or not, ^{1}H NMR spectrum of the product was also recorded using the same NMR spectrotrometer. The product Isatin exhibited three singlets at 7.57δ, 6.9δ and 6.8δ which reveal the presence of the proton of $-SO_3H$ group an aromatic proton and an imino proton respectively .In addition to these signals, the product also gave two doublets at closer chemical shifts (6.9δ and 6.8δ) which indicate the presence of two aromatic protons located adjacently. Thus, from the ^{1}H NMR spectrum of the product (Isatin),

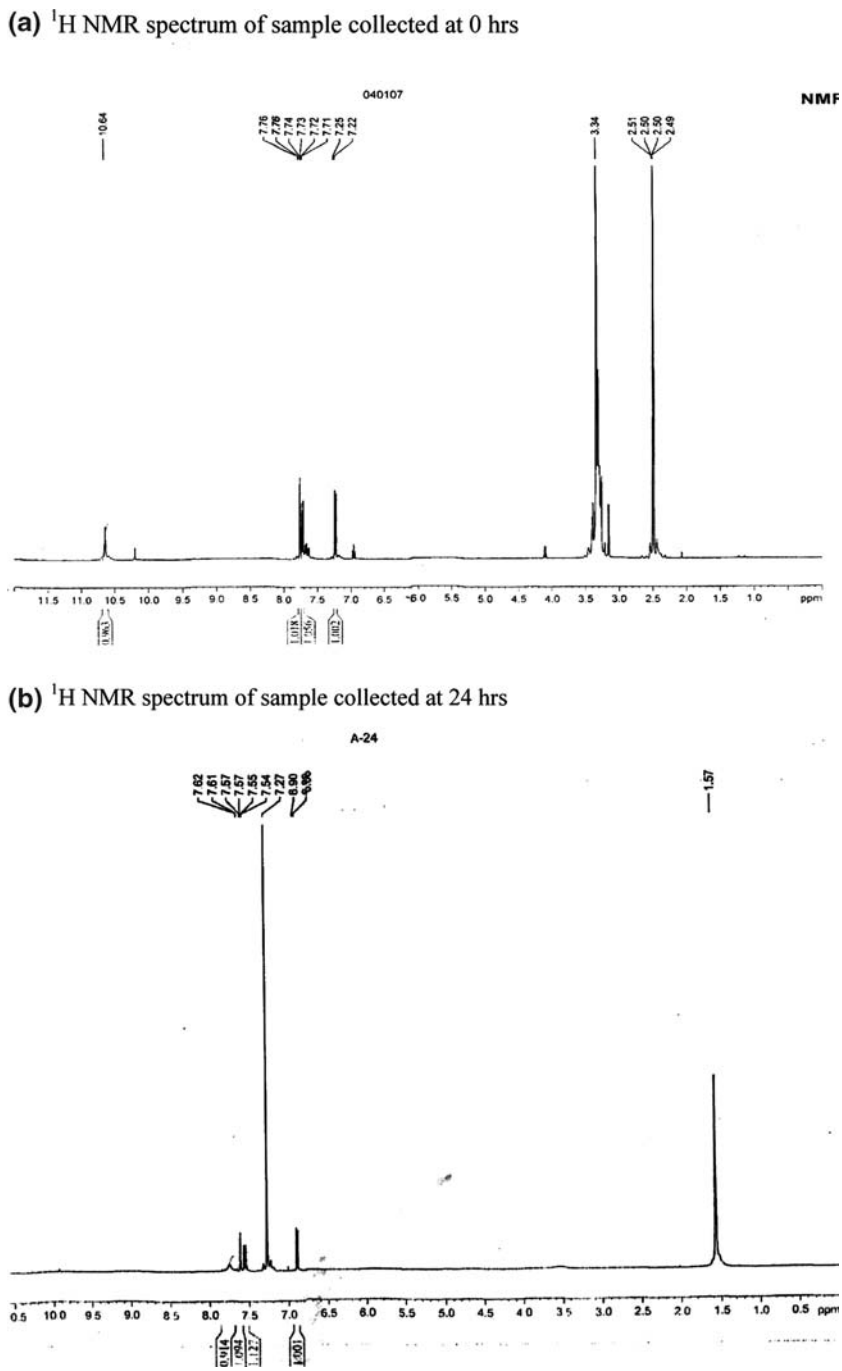


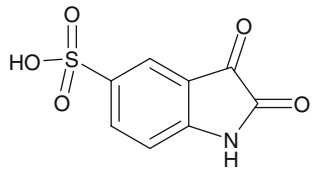
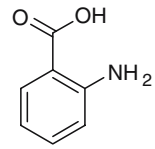
Fig. 4 [1]H NMR spectrum of sample collected at (a) 0 h, (b) 24 h

it is clear that Indigo carmine has undergone degradation

Conclusion

Indigo is the most important dye used in the manufacturing of blue jeans and whose degradation is challenging, we have isolated and identified *Paenibacillus larvae* from the soil flora. It was found to be effective in the degradation of Indigo carmine in shorter duration. As the organism utilizes moderate temperature and slight basic pH, is best suited for decolorization of textile effluents. Agitation is an essential condition to produce best results. Organic

Table 3 Analysis of degradation products using LCMS

S.No	Dye	λmax(nm)	Primary degradation Products	Mass obtained	Retention time (R_T)
1	Indigocarmine	610 nm	Isatin sulfonic acid 	243.8 m/z	1.4 min
			 Anthranilate	181 m/z	1.2 min

nitrogen sources like yeast extract and peptone were better than inorganic sources. Isatin sulfonic acid and anthranilic acid were found to be degradation products. Since the anthranilic acid is an intermediate in biosynthesis of tryptophan, it may enter into the tryptophan biosynthetic pathway.

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