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Biological decolourization of C.I. Direct Black 38 by *E. gallinarum*

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Abstract

In the present study, an *Enterococcus gallinarum* strain was isolated from effluent treatment plant of a textile industry based on its ability to decolourize C.I. Direct Black 38 (DB38), a benzidine-based azo dye. Effects of dye concentration and medium composition on dye decolourization were studied. The strain was found to decolourize DB38 even under aerobic conditions. Kinetics of DB38 decolourization was also examined, and V_{max} and K_s of decolourization were found to be higher in Luria broth (12.8 mg l^{-1} h^{-1} and 490.6 mg l^{-1}) than in minimal medium (4.09 mg l^{-1} h^{-1} and 161.84 mg l^{-1}). However, decolourization rate/biomass was found to be higher in minimal medium than in Luria broth, indicating greater decolourization efficiency of biomass in the former. The study also revealed biodegradation of DB38 to benzidine and its further deamination to 4-aminobiphenyl (4-ABP) by the culture. Ammonia released during this process was used as nitrogen source for growth of the culture.
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Keywords: Azo dye; Benzidine; 4-Aminobiphenyl; Biodegradation; Aerobic

1. Introduction

Azo dyes represent the largest class of dyes applied in textile processing, constituting up to 70% of all the known commercial dyes produced [1]. In textile dye baths, the degree of fixation of dyes to fabrics is never complete, resulting in dye-containing effluents [2]. Azo dyes are characterized by presence of one or more azo bonds (–N=N–) and are prepared by coupling a diazotized aromatic amine with a phenol or aromatic amine. However, these dyes are split back to the constituent aromatic amines in animal body by liver enzymes and intestinal microflora [3,4]. Out of the 2000 dyes synthesized so far, more than 500 are based on carcinogenic amines. In fact, several of these aromatic amines including benzidine and 4-aminobiphenyl (4-ABP) have been classified by the International Agency for Research on Cancer (IARC) as human carcinogens [5]. Hence, majority of azo dyes are also mutagenic and carcinogenic to humans as well as other animals [6], and removal of these dyes from the effluents is necessary.

Different physical, chemical and biological techniques have been applied for this purpose [7–10]. Most physico-chemical methods are expensive, are greatly affected by other wastewater constituents, or generate waste products that must be handled [11]. Also, azo dyes are recalcitrant to conventional aerobic wastewater treatment processes [12]. Their persistence is mainly due to presence of sulpho and azo groups, as these groups do not occur naturally [13]. However, under anaerobic conditions, bacteria can reduce azo bonds by azoreductase enzyme [14] and release constituent amines that are susceptible to aerobic biodegradation [15].

Most of the enterococci are normally associated with gastrointestinal tract of humans and other animals and can cause variety of infections and nosocomial diseases due to their increasing resistance to antibiotics. So far, azoreductase activity has been characterized only in *Enterococcus faecalis* in this group [16]. Present study describes isolation of another enterococcal strain, *Enterococcus gallinarum* capable of decolourizing azo dye C.I. Direct Black 38 (DB38). DB38, although banned in several countries, is still widely used for various applications like dyeing of fabric, leather, cotton, cellulose materials and plastic, and hence, it was selected for the study. *E. gallinarum*, used in this study, differs from other enterococci (including *E. faecalis*) in that it is not a true faecal species and is normally present in the environment. It is a motile vancomycin-resistant species implicated in several cases of nosocomial infections like bacteraemia [17]. This is the first report showing dye decolourizing activity in *E. gallinarum*.

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Effect of medium composition on DB38 decolourization was also investigated along with monitoring of decolourization kinetics. DB38 was found to be degraded to benzidine, which was further biotransformed to 4-ABP by the culture. Although there are several reports about release of 4-ABP from benzidine-based dyes by mixed microbial communities [18,19], only Gnanamani et al. [20] have demonstrated this in a pure culture of *Streptomyces* species. In our knowledge, this is not only the first report demonstrating conversion of benzidine to 4-ABP by *E. gallinarum*, but also the first report of utilization of benzidine as sole source of nitrogen for growth by any pure culture.

2. Materials and methods

2.1. Chemicals

DB38, a benzidine-based azo dye, was readily available in the local market and was used as the model azo dye for characterizing dye decolourization activity of *E. gallinarum* (Fig. 1). The absorption maxima (λ_{max}) of DB38 was found to be 550 nm by scanning dye solution in spectrophotometer. Benzidine and 4-ABP were obtained from Sigma–Aldrich Chemicals, USA.

2.2. Isolation of the culture

The culture used in the study was isolated from effluent treatment plant of a textile industry located in Nagpur, India, based on its ability to decolourize DB38. It was cultivated on Luria agar (composition in $g\,l^{-1}$: casein enzymic hydrolysate, 10; yeast extract, 5; NaCl, 5; agar, 15) and identified by 16S rRNA sequencing.

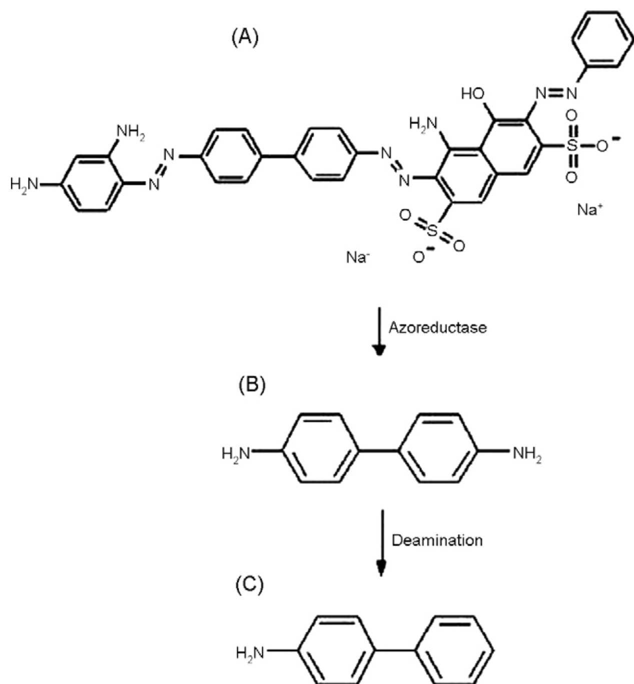

Fig. 1. Proposed mechanism of DB38 degradation by *E. gallinarum*. Structure of (A) Direct Black 38 (M.W. 781, C.I. no. 30235, CAS no. 1937-37-7), having three azo bonds, which are the sites of action for azoreductase enzyme, and its degradation products, (B) benzidine, and (C) 4-aminobiphenyl.

2.3. Decolourization of DB38

To study the effect of nutritional value of the medium on decolourization, experiments were performed in two different media: a nutritionally rich complex medium and a nutritionally poor minimal medium. Luria broth (composition in $g\,l^{-1}$: casein enzymic hydrolysate, 10; yeast extract, 5; NaCl, 5) and a minimal medium (Composition in $g\,l^{-1}$: Na_2HPO_4, 1.264; KH_2PO_4, 0.326; NH_4Cl, 1; $MgSO_4$, 0.098; $CaCl_2$, 0.044; glucose, 1) were selected for this purpose. Dye concentrations used in the study were 25, 50, 75, and 100 $mg\,l^{-1}$ in minimal medium and 20, 50, 100, 200, and 250 $mg\,l^{-1}$ in Luria broth. To set up the experiment, culture was grown to $O.D._{600}$ of 0.1 before adding the dye. Experiments were carried out under ambient conditions and samples were removed at different time intervals up to 72 h for minimal medium and up to 48 h for Luria broth, as decolourization was slower in minimal medium.

2.4. Degradation of benzidine

To check whether *E. gallinarum* can degrade benzidine, it was inoculated in a nitrogen-free medium (Composition in $g\,l^{-1}$: Na_2HPO_4, 1.264; KH_2PO_4, 0.326; $MgSO_4$, 0.098; $CaCl_2$, 0.044; glucose, 1) containing 25 $mg\,l^{-1}$ benzidine. A flask containing benzidine, but not the culture, was kept as control. Both flasks were incubated at ambient temperature on shaker and samples were removed at different time intervals. Growth of the culture was measured in terms of $O.D._{600}$, while ammonia release was measured by distillation followed by titration with H_2SO_4 according to the Standard Method 4500-NH_3-E [21].

2.5. Analytical methods

2.5.1. Decolourization

Samples were centrifuged to remove the bacterial cells and O.D. of the supernatant was determined at 550 nm (λ_{max} of DB38) using Lambda 900 UV–vis–NIR spectrophotometer (PerkinElmer). The concentration of dye was determined from the O.D. by plotting a standard curve of dye concentration versus O.D. Decolourization results were expressed as:

$$\%\,\text{decolourization} = \left(-\frac{d[DB38]}{[DB38]_0}\right) \times 100$$

$$\frac{\text{Fractional decolourization rate}}{\text{biomass}}\,(h^{-1}\,g^{-1}) = -\frac{d[DB38]}{dt \times [DB38]_0 \times C}$$

where d[DB38] = change in DB38 concentration ($mg\,l^{-1}$), $[DB38]_0$ = initial DB38 concentration ($mg\,l^{-1}$), dt = time (h), and C = biomass (g).

Fractional decolourization rate is an important parameter as it is independent of initial dye concentration and remains constant for a dye-culture pair under a given set of operating condi-

tions. Thus, it can be used to compare the effects of operating conditions on dye decolourization.

2.5.2. Adsorption

Dye decolourization by bacteria can be a biological process or occur by physical adsorption. To determine the extent of bioadsorption, cells were treated with desorbing agents like NaOH [22], tween80, acetone, methanol and ethanol [23,24]. NaOH was found to be more effective and was selected for further study. Briefly, pellets from centrifuged samples were homogenized with 0.1N NaOH for 5 min using a vortex mixer. Cell debris was removed by centrifugation and supernatant was neutralized with HCl before measuring O.D. at 550 nm.

2.5.3. Biomass

Samples were filtered through 0.22 μm filter (Millipore) and dried in oven. Biomass was calculated from the dry weight of cells collected on the filter paper.

2.5.4. Oxidation/reduction potential (ORP)

In situ ORP values were measured with Orion 420A+ instrument equipped with combo redox/ORP electrode (Thermo Electron Corporation).

2.5.5. HPLC analysis

Samples were centrifuged and supernatant clarified by passing through 0.45 μm filter. The filtrate was extracted with diethyl ether, organic phase was dried in an evaporator and the resulting residue was dissolved in methanol. Extracted samples were analyzed on Waters HPLC system using mobile phase of methanol:water (50:50) at a flow rate of 0.8 ml min^{-1}. RP18 column (Spheri-10, 250 mm × 4.6 mm, 10 μm particle size, Biosystems) was used for the separation and the separated components were detected using Lambda-max 481 UV/vis detector set at 280 nm.

2.5.6. GC–MS analysis

Samples were processed as in case of HPLC, except that dichloromethane was used as the solvent for final dissolution of samples. GC–MS analysis was carried out on Varian CP-3800 gas chromatograph equipped with Saturn 2200 GC/MS/MS and CP-SIL-8CB capillary column (30 m × 0.25 mm i.d.). The GC operating temperatures were: injector temperature 300 °C, oven temperature programmed initially at 40 °C for 4 min and then increased to 270 °C at 10 °C min^{-1}. Helium was used as carrier gas at a flow rate of 1.1 ml min^{-1} and the analysis was carried out as per US EPA method 8270. Compounds were identified using NIST library.

3. Results and discussion

3.1. Decolourization of DB38

A culture was isolated from effluent treatment plant of a textile industry based on its ability to decolourize dye and was identified as *E. gallinarum* by 16S rRNA sequencing (GenBank accession number DQ864487; Fig. 2). Dye decolourization activity has not been reported in this culture so far. It was tested for its ability to decolourize DB38 dye in a minimal medium. To determine the effect of dye concentration on decolourization, 4 different concentrations of DB38 (25, 50, 75, and 100 mg l^{-1}) were studied. Decolourization was found to be due to both biological azo dye reduction and adsorption (Fig. 3). Net biological dye decolourization was estimated by subtracting dye adsorption from total dye removal in the medium (Fig. 4). From figures, it is clear that *E. gallinarum* showed good dye decolourization activ-

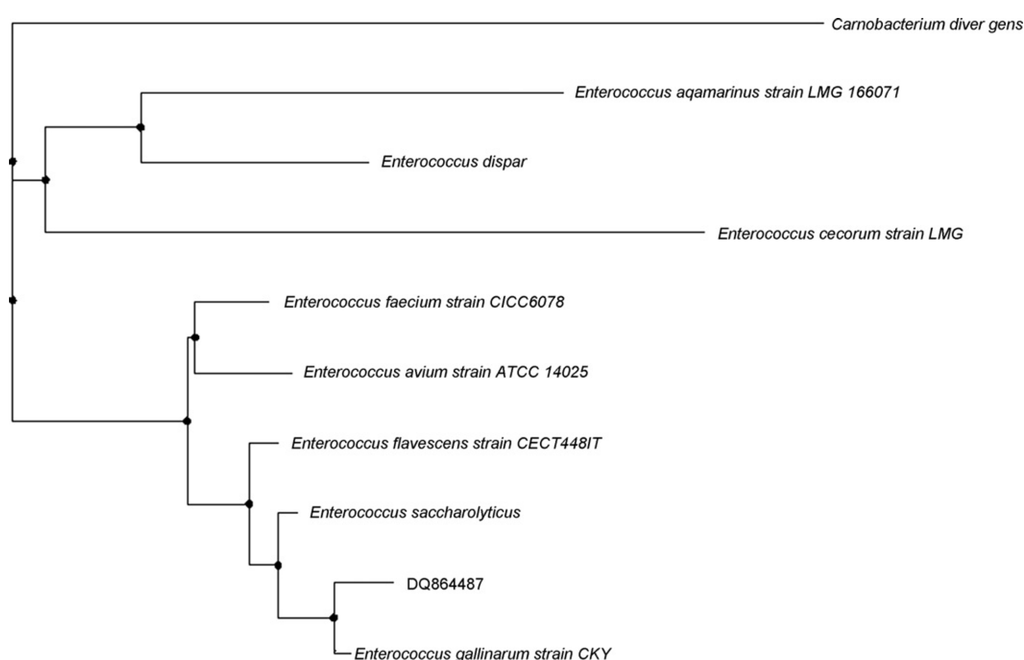

Fig. 2. Neighbour-joining tree showing phylogenetic placement of the *E. gallinarum* culture isolated in this study (GenBank accession no. DQ864487).

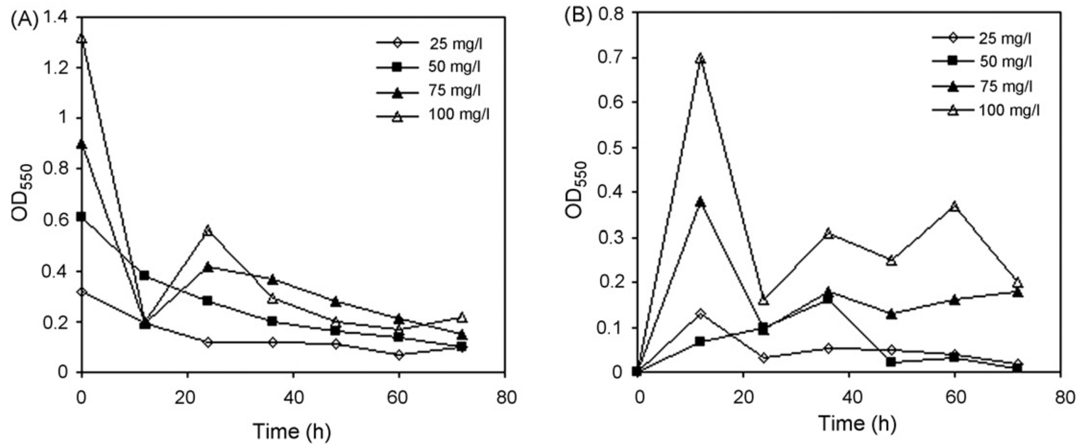

Fig. 3. (A) Total DB38 removal and (B) adsorption of DB38 by *E. gallinarum* in minimal medium. Legends show the concentrations of DB38 used.

ity and could remove 53–63% of DB38 within 24 h in minimal medium. Decolourization was also tested in Luria broth and dye removal was found to be 71–85% (Fig. 4). Thus, decolourization was better in nutritionally richer Luria broth than minimal medium.

3.2. Biomass

Dry weight was used as the measure of biomass because dye adsorbed to cells interfered with spectrophotometric estimation in terms of O.D. Biomass was determined in both minimal medium and Luria broth at various dye concentrations and growth in minimal medium was found to be slower than that in Luria broth (Fig. 5). This might be the reason why decolourization in Luria broth was better than that in minimal medium, as higher biomass will decolourize the dye faster [25]. However, if biomass is present in far excess than required, although it decolourizes at faster rate, it results in lower efficiency of biomass as biocatalyst. This can be checked by normalizing the decolourization rate with biomass. Indeed, the fractional decolourization rate/biomass was found to be higher in minimal medium ($0.17\,h^{-1}\,g^{-1}$) than Luria broth ($0.1\,h^{-1}\,g^{-1}$), indicating more efficient use of biomass for dye decolourization in minimal medium.

Further, growth of *E. gallinarum* was found to be inhibited by dye and/or its degradation products in both the media (Fig. 5). Maximum biomass achieved in the presence of dye was found to be only 30.4 and 37.5% of that obtained in the absence of dye in minimal medium and Luria broth respectively.

3.3. ORP

There are several reports about decolourization of azo dyes under anaerobic conditions [26]. On checking ORP of experimental systems in the present investigation, all the values were found to fall in aerobic range in both minimal medium (175.2–190.8 mV) as well as Luria broth (109.2–136.7 mV). Thus, *E. gallinarum* seems to be able to decolourize azo dyes even under aerobic conditions.

3.4. Degradation mechanism

It is very well known that decolourization of DB38 involves reduction of its azo bonds, resulting in release of benzidine (Fig. 1). This was clearly evident from spectrophotometric analysis of DB38 before and after decolourization. While DB38 exhibited maximum absorbance at 550 nm, upon decolourization, absorbance at 550 nm disappeared and a new peak

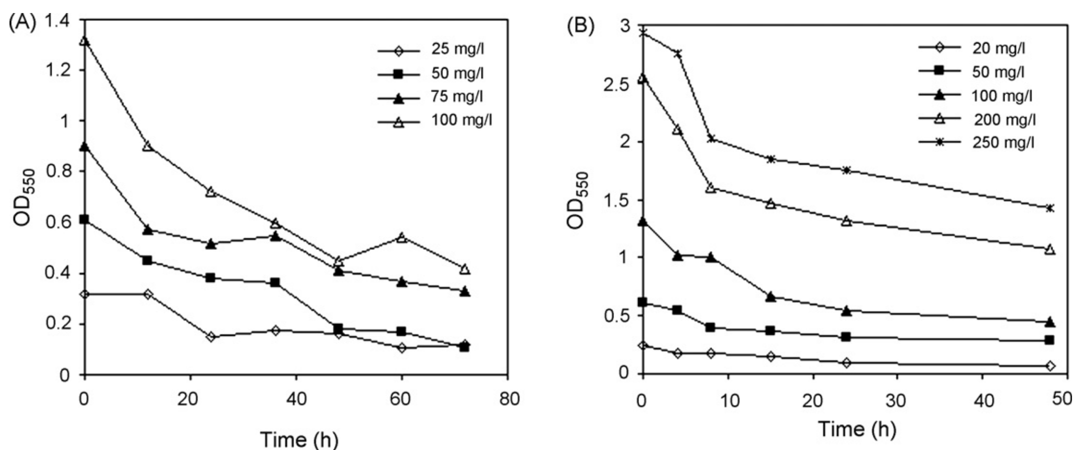

Fig. 4. Net biological decolourization of DB38 by *E. gallinarum* in (A) minimal medium and (B) Luria broth. Legends show the concentrations of DB38 used.

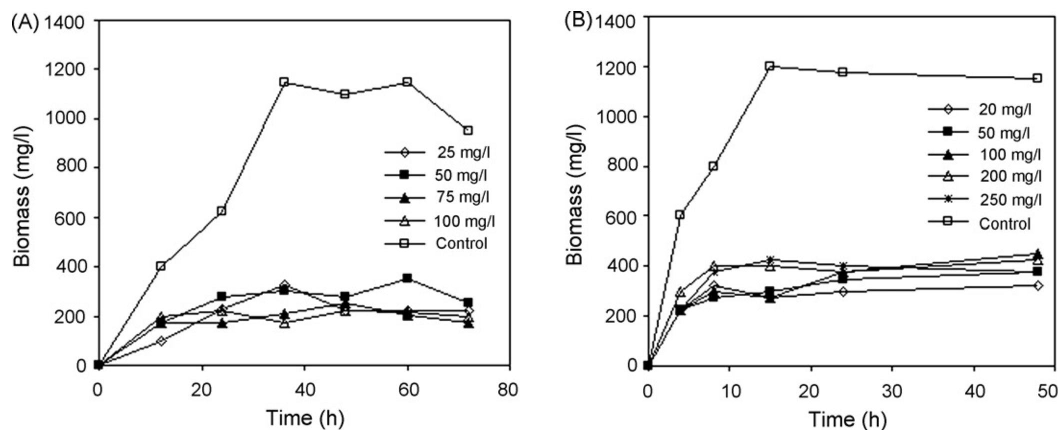

Fig. 5. Growth of *E. gallinarum* in presence or absence (control) of DB38 in (A) minimal medium and (B) Luria broth. Legends show the concentrations of DB38 used.

emerged at 284 nm, giving the typical spectrum of benzidine (Fig. 6).

Since benzidine is the major mutagenic moiety of DB38, it was necessary to check whether it can be degraded further or not. Also, the decolourized dye would contain other dye components apart form benzidine and hence, it was reasonable to study the degradability of benzidine explicitly to avoid any confusion. For this *E. gallinarum* was inoculated in a nitrogen-free medium containing 25 mg l^{-1} benzidine. Samples were withdrawn at different time intervals and analyzed by HPLC. Fig. 7 shows the chromatograms of 0th day and 10th day samples along with standard 4-ABP. 0th day sample shows the peak of benzidine only, while 10th day sample shows a peak of benzidine along with another peak corresponding to 4-ABP. This clearly demonstrates degradation of benzidine to 4-ABP. Identification of 4-ABP was further confirmed by GC–MS analysis (Fig. 7). Thus, DB38 was not only decolourized by reduction of azo bonds to release benzidine, but benzidine was also further degraded to 4-ABP. No other intermediates of benzidine degradation could be identified in the analysis. Although there are several reports about release of 4-ABP from benzidine-based dyes by mixed microbial communities [18,19], only Gnanamani et al. [20] have demonstrated this in a pure culture of *Streptomyces* sp. This is the first report demonstrating conversion of benzidine to 4-ABP with a pure bacterial culture of enterococcal group.

Since degradation of benzidine to 4-ABP is a deamination reaction, it must involve release of NH_3 (Fig. 1), which can be used as a source of nitrogen for growth. Hence, we also measured release of NH_3 from benzidine in the nitrogen-free medium. NH_3 level peaked at 4.2 mg l^{-1} on 5th day followed by gradual

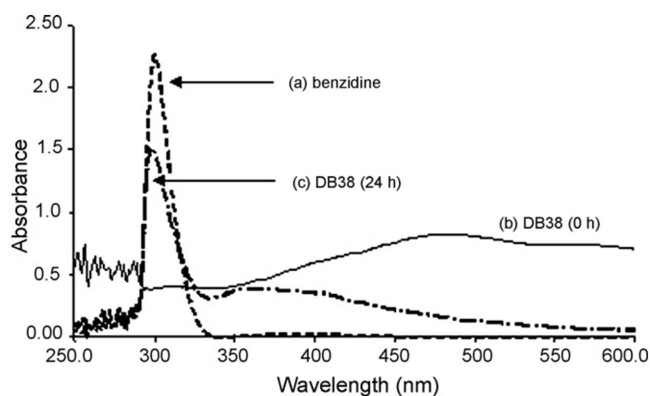

Fig. 6. UV–vis spectra of (a) benzidine, (b) DB38, and (c) decolourized DB38 after 24 h.

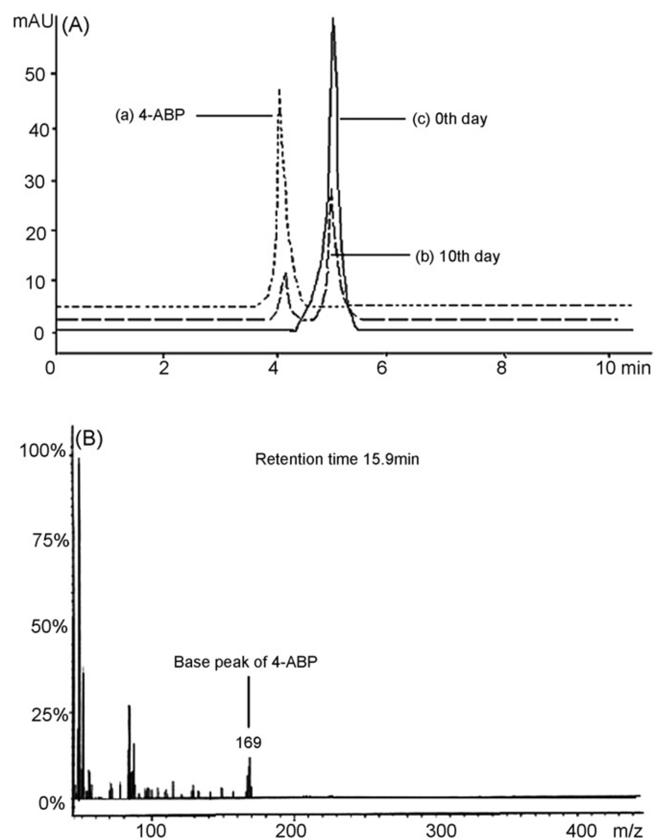

Fig. 7. Identification of intermediates of DB38 degradation by *E. gallinarum*. (A) HPLC chromatograms of (a) 4-ABP, and samples of benzidine degradation on (b) 10th day and (c) 0th day. Benzidine peak has clearly reduced on 10th day as compared to 0th day sample. A peak corresponding to 4-ABP is also seen in the 10th day sample, but not in 0th day sample. (B) GC–MS spectrum of sample of benzidine degradation on 10th day showing presence of 4-ABP.

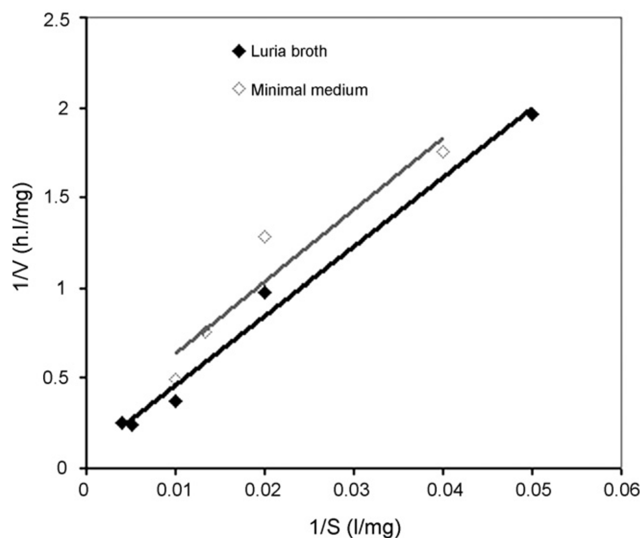

Fig. 8. Reciprocal plot showing effect of DB38 concentration on rate of decolourization by *E. gallinarum*.

reduction to 1.4 $mg\,l^{-1}$ on 10th day. Control flask showed no NH_3, indicating that NH_3 release in the experimental flask was not abiotic. Also, $O.D._{600}$ of the culture in the experimental flask increased from 0 to 0.12 on 10th day. This conclusively proves that *E. gallinarum* can degrade benzidine to release NH_3 and use it as nitrogen source for growth.

3.5. Kinetic analysis of DB38 decolourization

Dye decolourization by *E. gallinarum* was studied at different DB38 concentrations in both minimal medium and Luria broth. Dye decolourization rates at various DB38 concentrations were found to follow Monod kinetics. Fig. 8 shows the reciprocal plot of decolourization rate ($mg\,l^{-1}\,h^{-1}$) against dye concentration ($mg\,l^{-1}$). V_{max} and K_s for dye decolourization could be determined from the graph as:

In minimal medium, $V_{max} = 4.09\,mg\,l^{-1}\,h^{-1}$ and $K_s = 161.84\,mg\,l^{-1}$
In Luria broth, $V_{max} = 12.8\,mg\,l^{-1}\,h^{-1}$ and $K_s = 490.6\,mg\,l^{-1}$

Thus, V_{max} as well as K_s of decolourization are higher in Luria broth. This must be due to relatively higher biomass values achieved in Luria broth than in minimal medium (Fig. 5; Section 3.2).

4. Conclusions

An *E. gallinarum* strain capable of decolourizing azo dye DB38 was isolated from effluent treatment plant of a textile industry. The dependence of dye decolourization rate on concentration of DB38 could be described in terms of Monod kinetics. The culture was found to exhibit versatile activities of decolourizing azo dyes under aerobic conditions, degrading resulting benzidine by deamination and utilizing the NH_3 released as sole source of nitrogen for growth. Hence, it seems to be a good candidate for further research for bioaugmenting large-scale decolourization processes.

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