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Choline-Based Ionic Liquids-Enhanced Biodegradation of Azo Dyes

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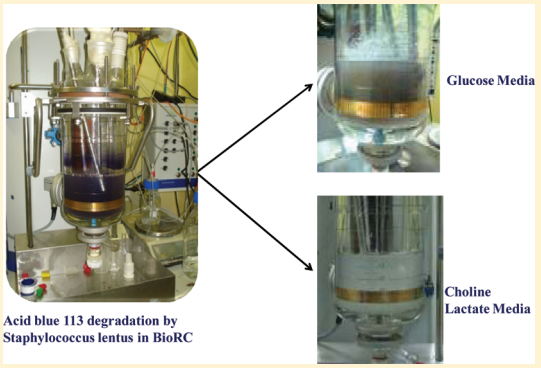
Supporting Information

ABSTRACT: Industrial wastewaters such as tannery and textile processing effluents are often characterized by a high content of dissolved organic dyes, resulting in large values of chemical and biological oxygen demand (COD and BOD) in the aquatic systems into which they are discharged. Such wastewater streams are of rapidly growing concern as a major environmental issue in developing countries. Hence there is a need to mitigate this challenge by effective approaches to degrade dye-contaminated wastewater. In this study, several choline-based salts originally developed for use as biocompatible hydrated ionic liquids (i.e., choline sacchrinate (CS), choline dihydrogen phosphate (CDP), choline lactate (CL), and choline tartarate (CT)) have been successfully employed as the cosubstrate with *S. lentus* in the biodegradation of an azo dye in aqueous solution. We also demonstrate that the azo dye has been degraded to less toxic components coupled with low biomass formation.



1. INTRODUCTION

Minimizing the environmental impact of a process often involves ultimately the removal of residual organic contaminants and unused reactants from a waste stream after all possible recovery and recycling methods have been applied. For example azo dyes are one of the most versatile classes of synthetic colorants employed in textiles, leather, and plastics industries.[1] These are xenobiotic compounds, resisting biodegradation in conventional aerobic treatment processes[2] and also in the environment. The recalcitrance of azo dyes in waste streams therefore often results in serious contamination of groundwaters.[3] In the dyeing processes, dyes are neither completely utilized nor recovered in the downstream processes and as a result the effluent stream[4] usually contains more than 10−15% of dissolved dye. Most of these dyes and their metabolites are toxic, potentially carcinogenic in nature, and their removal from the effluents is a major problem and presents a serious environment threat.[5] Hence, there is an ongoing need to develop more effective means of removal of the residual dyes from such aqueous waste streams in an eco-friendly manner.

Currently the state of the art employs several physical and chemical methods to treat the dye effluents, but the major problems in these processes are that they are time-consuming, costly, and, most importantly, generate secondary pollutants.[6] Adsorbents such as charcoals, activated carbons, clays, chitin, chitosan, chemically modified cellulose, and lignocellulose have also been used for decolorization of dye effluents.[7] But the drawback of adsorption processes is that the adsorbent needs to be regenerated and this adds to the cost of the process. In the chemical treatment processes, oxidation is the most commonly used method. In the literature there are reports on the use of ozone,[8] or Fenton's reagents, as oxidizing agents, but these methods are not ecologically acceptable in the long-term.[9] Recently, advanced oxidation processes (AOPs)[9] have been applied to the treatment of dye effluents. The limitation of AOPs is that they are susceptible to scavenging of hydroxyl radicals by nontarget substances and are not suitable for certain compounds such as perchlorinated species, because they cannot be attacked by hydroxyl radicals. Electrochemical processing is another method[10] but the implementation of this method involves a large investment in equipment. None of these methods allow recovery and reuse of the valuable dye compound. Recently we have demonstrated the use of hydrophobic ionic liquids[11] in the extraction and recovery of azo dyes, but the ionic liquid media involved are expensive components and may not be effective for low concentrations of dye solutions.

In contrast to conventional physical/chemical methods, biological treatment of dye effluents offers excellent benefits owing to its low operating cost, versatility of the active microbial species, and range of metabolic pathways for effective degradation.[12,13] However, anaerobic degradation of azo dyes tends to produce a number of aromatic amines which are carcinogenic and mutagenic.[14] During the past few years a

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number of microorganisms, including *S. lentus*, have been identified that are able to transform azo dyes to noncolored, nontoxic products under aerobic environmental conditions, or even to completely mineralize them.[15] However, the industrial application of this process has been limited by the problematically low rate of degradation and the high rate of biomass residue produced. Hence, the goal of the present study was to develop an improved process for the biodegradation of azo dyes with a particular emphasis on the rate of the degradation process.

The field of ionic liquids (liquid organic salts) has generated a very large number of novel organic salts (both room temperature liquids and solids) in recent years, offering a wide range of properties. A number of papers have described novel ionic liquids that are biocompatible and in which protein structure and enzyme function can be maintained or even enhanced.[16,17] The work of Devie et al.[18] with a variety of fungal species and ionic liquids has also shown that metabolic pathways can be altered selectively by these biocompatible ILs in aqueous conditions. This led us to hypothesize that some of these biocompatible organic salts would have beneficial effects on the action of *S. lentus* in the biodegradation of azo dyes when present as solutes in the aqueous media. (We emphasize that the ionic liquid nature of these salts is of minor importance here—other than to enhance the solubility of the compound). In this study, we use choline-based biocompatible ionic salts because they are nontoxic to the enzymes which are responsible for biodegradation of dyes. We show here that *S. lentus* is able to use the organic salt as a metabolic carbon source and thereby that the enzymatic degradative action is stimulated, producing a more rapid degradation process with lower biomass residue. The results suggest that manipulation of bioremediation processes may allow these to become an important component of approaches to greening of some industries. We also quantified the metabolic heat with dye degradation through reaction calorimetric[19−21] techniques and compared the same with conventional glucose-based carbon source.

2. MATERIALS AND METHODS

2.1. Materials. The Azo dye Acid blue 113 used here has the molecular formula $C_{32}H_{21}N_5O_6S_{22}Na$ (mol wt 681.85). It was a commercial-grade sample manufactured by M/s Clarient Industries located in Chennai, India. NADH (N6879) was purchased from Sigma Aldrich. All other chemicals and reagents were of analytical grade, manufactured and procured from M/s.SD-Fine Chemicals, Bangalore, India. The choline salts employed in this study were choline lactate, choline tartrate, choline saccharinate, choline dihydrogen phosphate, and choline citrate. They were synthesized according to the procedures reported in the literature.[22] Because these salts were made by neutralization reactions with the corresponding acids and choline hydroxide, they are generally free from impurities. However, the purity and structural identity of the choline salts were confirmed using ^1H and ^{13}C NMR.

2.2. Bacterial Strain Isolation and Culture Conditions. The organism used was *Staphylococcus lentus,* which is a salt-tolerant bacterium. One rationale behind the selection of a halobacterial strain was for possible future requirement for the treatment of industrial effluents with high salinity. The strain was isolated from seawater and was identified by both biochemical tests and 16S rRNA sequencing methods. The details are given in the Supporting Information. The strain is deposited in a public microbial type culture collection and gene bank at Institute of Microbial Technology, Chandigarh, India and has the accession number MTCC 11104.

2.3. Media for Dye Degradation. Mineral salt medium (MSM) was used in the dye degradation studies. The composition of the MSM is given elsewhere.[21] The pH of the medium was adjusted to 7.0. The 4% of inoculum (v/v) was used to inoculate 1 L of growth medium containing 100 mg/L of Acid blue 113 in the biocalorimeter. Dye degradation was monitored spectrophotometrically by withdrawing samples at different times. The samples were centrifuged at 10 000*g* (Sigma, model 3-18 k) at 4 °C for 15 min to remove the biomass (or any other sediment). The supernatant was used for determining the degradation efficiency.

The calorimetric details and the measurement procedures including the evaluation of bioenergetics are given in the Supporting Information (ESI-1).

2.4. Ionic Liquid Analysis. *2.4. Lactate Analysis.* Several attempts were made to assay the choline salts using liquid chromatographic[23] techniques and other enzymatic assay methods as suggested earlier.[24] The methods interfered with the metabolic sub products and conclusive inferences could not be drawn. Because the lactate anion was found to be preferentially utilized by the organism under study, it was decided to use a modified DNS method for quantifying the lactate. The principle behind the modified DNS method adapted for lactate estimation and the procedure of analysis are given in ESI-1. The chemical oxygen demand and dye decolorization measurement procedures are also outlined in the Supporting Information.

2.5. Cytotoxicity Testing. The cytotoxicity testing of the metabolites collected at 72nd hour was carried out according to the previously reported method.[25] The bacterial culture mediums along the degradation products were centrifuged. The supernatant was extracted with equal volume of ethyl acetate thrice. The extract was dried over Na_2SO_4 and concentrated in a rotary evaporator. The concentrated extract was used for cytotoxicity testing.

VERO South African monkey kidney cell lines were used to test the cytotoxicity of the decolorized metabolites of Acid blue 113 on the cultured cells at concentrations of 0.039 to 5 mg/mL on exposure of these samples to cells up to 48 h. The percentage of surviving cells was determined by counting the number of live and dead cells on a hemacytometer. At least 250 cells were counted for each measurement.

3. RESULTS AND DISCUSSION

3.1. Degradation of Acid Blue 113 using *Staphylococcus lentus*. The degradation of Acid blue 113 (25 ppm) by *S. lentus* was carried out in a shaker flask under optimized conditions, with various biocompatible choline salts (choline lactate, choline tartrate, choline saccharinate, choline dihydrogen phosphate, and choline citrate) as the sole carbon source. The results are shown in Figure 1. Different dye degradation profiles were observed for the various choline salts, and the best results were obtained for choline lactate (with a maximum degradation of 92% in about 72 h) under identical experimental conditions. With choline dihydrogen phosphate the dye degradation was observed to be around 60%. The effect of the anion could be due to "nutrient" effects during the growth which is responsible for dye degradation. In a recent communication elsewhere,[26] which investigated the metabolic pathways involved, we have shown the preferential utilization of lactate anion and a faster rate of growth by this organism in

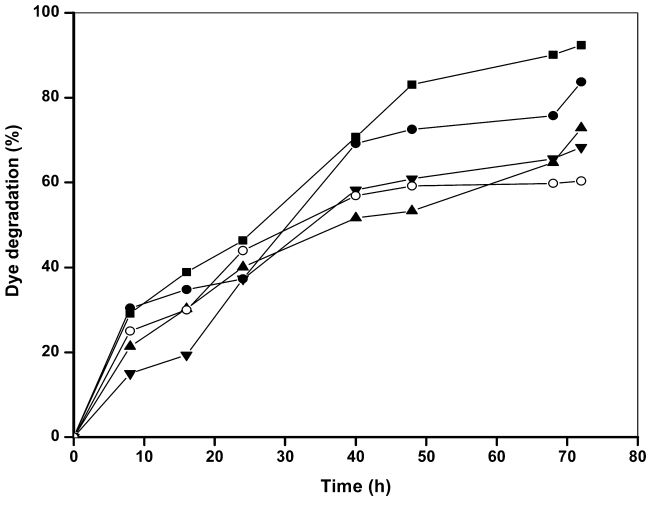

Figure 1. Different ionic liquids employed on Acid blue 113 degradation by *S. lentus* in shake flask. (Choline lactate (92%) (■), choline citrate (83%) (●), choline tartrate (73%) (▲), choline saccharinate (68%) (▼), choline dihydrogen phosphate (60%) (○)).

comparison with conventional carbon sources such as glucose (Figures S2 and S3). After screening choline lactate as the best of the choline salts, we have focused our attention on the optimization of this carbon source by varying its concentration from 1.0 to 5.0 g/L and the degradation profiles are given in Figure 2. The results show that maximum degradation occurs

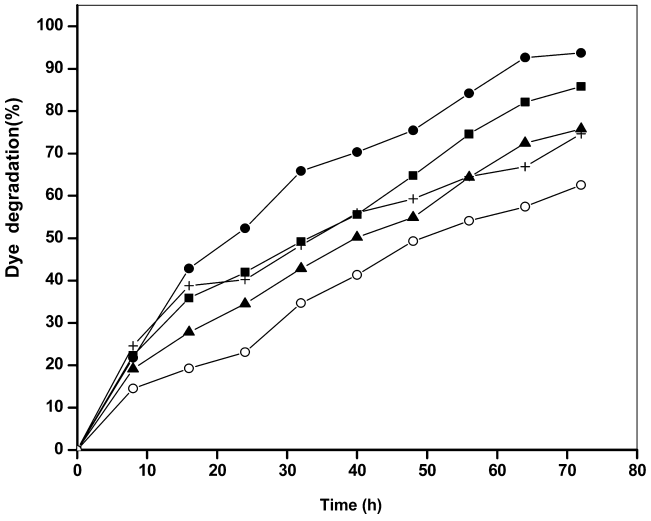

Figure 2. Effect of initial choline lactate concentration on Acid blue 113 degradation by *S. lentus* in shake flask. (1 g/L (■), 2 g/L (●), 3 g/L (+), 4 g/L (▲), 5 g/L (○)).

with a CL concentration of 2 g/L and further increase in concentration did not yield better results. It is worth mentioning here that the conventionally used carbon source, glucose, requires 5 g/L (2.5 times more compared to the ionic liquid) (Figure S3) in order to obtain similar degradation profiles. To assess the effect of initial dye concentration on the dye degradation efficiency, different initial concentrations of dye (ranging from 25 to 100 ppm) were studied. The results are given in Figure 3. It is interesting to note that the degradation was observed in all the cases, however, the best degradation result is found at 25 ppm (92% in 72 h) although

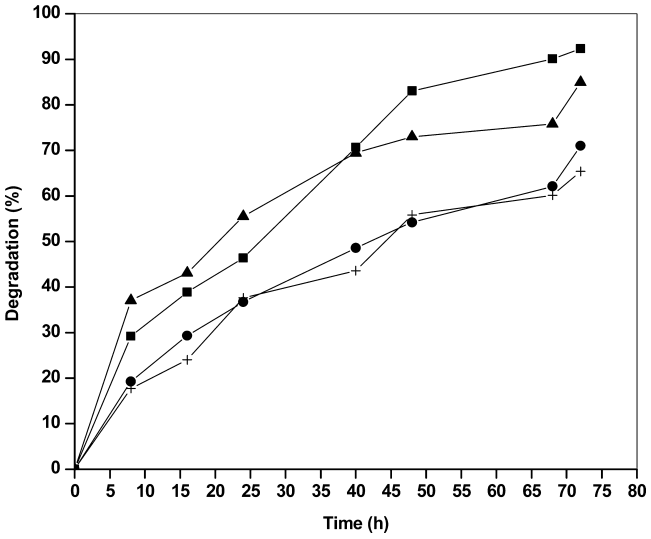

Figure 3. Effect of initial dye concentration on Acid blue 113 degradation by *S. lentus* in shake flask. (25 ppm (■), 50 ppm (▲), 75 ppm (●), 100 ppm (+)).

there is little difference up to 75 ppm (84% in 72 h). Under similar experimental conditions, the conventional glucose as carbon source produced relatively less degradation (Figure S4). Thus these results show the superior nature of choline salts (choline lactate) in effectively engineering the microbe for enhanced dye degradation and also as an effective carbon source for biological dye degradation.

3.2. Biocalorimetric Studies of Growth and Acid Blue 113 Degradation by *S. lentus* with Choline Lactate As a Nutrient Carbon Source. *3.2.1. Biocalorimetry for the Cultivation of S. lentus in CL Media.* In one of our recent communications,[21] we have shown that the bacteria *S. lentus* could be successfully grown in a choline lactate limited MS medium in a bioreaction calorimeter. From the power−time profile (Figure S5), the three phases of the growth can be distinctly identified; the bacteria grew faster, evidenced by larger microbial population. The heat yield computed for the growth metabolism confirmed the preferential affinity of choline lactate by the bacteria. Because the objective of this Article is to show the energetics of azo dye degradation aspects of the bacteria in choline lactate media, further discussion on growth metabolism will not be dealt with here. In the recent past, calorimetry's capability as a promising online tool for monitoring the growth of microorganisms has been proved adequately.[20,21]

3.2.2. Biocalorimetry for Acid Blue 113 Degradation by S. lentus in CL Media. Thermodynamic responses of *S. lentus* in the choline salt medium (2 g/L) with dye degradation were studied in the biocalorimeter. A comparative plot showing heat flux, dye degradation, choline lactate consumption, enzyme activity, COD profiles, and oxygen uptake rates (OUR) is shown in Figure 4. This figure shows three distinct phases of growth. It was observed that the organism adapts to the reactor environment quickly, and begins to consume the ionic liquid. During the biodegradation process, choline lactate consumption takes place slowly. It can also been seen from Figure 4 that the percentage of dye degradation (total COD reduction) and choline lactate consumption proceed closely to each other, thus indicating their simultaneous utilization. During this process, as observed in the previous literature studies,[27] azo reductase

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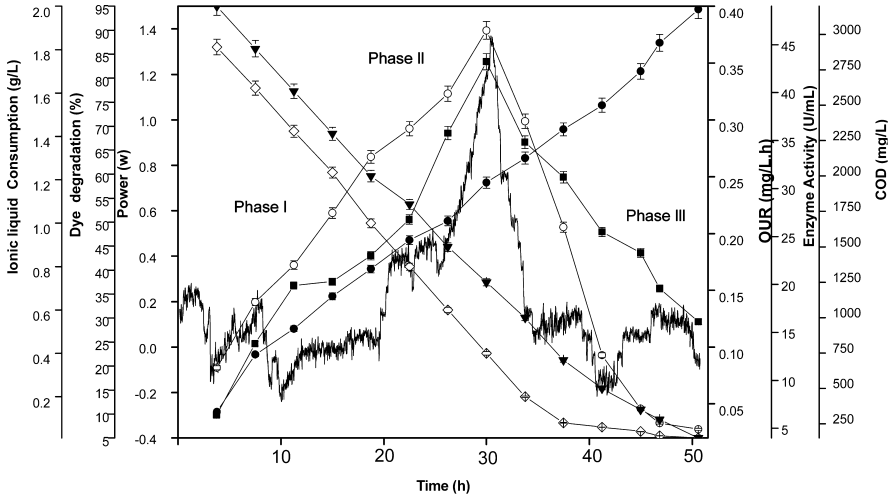

Figure 4. Comparative profiles of heat production during Acid blue 113 degradation by *S. lentus* in presence of choline lactate. (Heat production rate (-), oxygen uptake rate (■), dye degradation (●), enzyme activity (○), substrate consumption (▼), and COD (◇)).

enzyme release (shown in Figure 4) occurs from the beginning of degradation triggering the decolorization process.

The heat released during the dye degradation process is due to growth of the organism coupled with oxidative and enzymatic degradation of the dye molecule. In the calorimetric experiment it was observed that during the first phase of the activity (extended up to 21.4 h) 35% dye degradation occurred, perhaps due to primary cleavage of the chromophoric azo bond. In the second exponential phase (starting from 21.4 h with a maximum at 37.5 h) 58% of dye was degraded and a maximum enzyme (azo reductase) activity was noticed (46 U/mL) during this phase. Heat release rates were maximum during this phase, indicating rapid enzymatic oxidative reaction. In this phase the maximum consumption of choline lactate was also noticed.

However, during the third phase (37.5−55 h), there was a decline in heat release rate (indicated also by slow consumption of choline lactate, along with decline of OUR), the enzymatic and partial oxidative degradation reactions continue to occur, resulting in further dye degradation. Furthermore, in this phase the decline in growth rates is indicative of the culture reaching its death phase, due to partial utilization of the biomass (dead cells) by the surviving cells. (The toxic nature of the intermediates and secondary metabolites that may have been present may be yet another reason for the onset of declining phase.) In the degradation process, heat and OUR profiles follow each other indicating that heat profiles could be used to monitor the dye degradation process on an industrial scale. Heat yield values were calculated (based on the profiles obtained in Figure 4) for substrate, biomass, and oxygen uptake and are given in Table 1. Heat yield content is an important aspect in biothermodynamics which reflects the behavior of the organism at different environments. The degradation results were compared to the conventional glucose-meditated process and it was shown that IL-mediated degradation results are better and faster (94% degradation in 54 h) as against glucose mediated degradation (84% degradation in 64 h). $Y_{Q/O}$ for microbial dye degradation in the IL medium was found to be 443 ± 10 kJ/mol. This value is well within the range reported[28] and confirms the predominantly aerobic nature of the process. The bioenergetics data given in Table 1 are compared with the data for growth only processes (Table S1). The growth energetic data showed that the bacteria *S. lentus* has more affinity toward choline lactate than glucose as indicated in the $Y_{Q/S}$ values (9.6 and 23.4 kJ/g). However during dye degradation the $Y_{Q/S}$ values shown in Table 1 are more are less same for glucose and choline lactate. The differences in the behavior of *S. lentus* in the substrate heat yield values shows that its affinity toward the dye does not depend on the initial nature of the substrate. The data shown in Table 1 confirm that in presence of choline lactate, the degradation is efficient, which is basically related to the enhanced enzyme activity. It is also interesting to note that the $Y_{Q/COD}$ (8..97 kJ/mg) values and the ratio of BOD to TOC [29] of the final samples of IL mediated ones (0.953) are lesser than the corresponding glucose mediated samples (5.11). This shows that the final IL mediated sample is less toxic than the glucose mediated ones. To confirm this point, another set of independent experiments was carried out (Figure S6) on cell viability studies for these samples. The results show that there is reasonably more growth in IL-mediated samples compared to glucose-treated samples.

Table 1. Comparison of Heat Yields of Choline Lactate and Glucose during Acid Blue 113 Degradation by *S. lentus* in BioRTCal

substrate	$Y_{Q/X}$ (kJ/g)	$Y_{Q/S}$ (kJ/g)	$Y_{Q/O}$ (kJ/mol)	$Y_{Q/COD}$ (kJ/mg)
choline lactate	13.1	22.3	443	8.97
glucose	13.27	25.4	460	12.2

3.3. Acid Blue 113 Degradation Mechanism by *S. lentus*: FT-IR, HPLC, and GC-MS studies. The FT-IR spectrum of sample collected at the 72nd hour during the degradation of Acid blue 113 in IL-mediated *S. lentus* along with pure dye is given in Figure 5. It shows that the degraded product was not an aryl amine.

HPLC analysis was carried out for samples treated with IL withdrawn at 24 and 72 h along with pure dye (as control). The results are given in Figure S7. The results show that in the control sample a large intensity peak at around 8 min and a medium intensity peak at 9.3 min, along with a broad hump (10−15 min) were observed, whereas in the 24-h treated sample the broad hump was resolved and 3 peaks with medium intensities were shown indicating the breakdown of dyes. In the 72-h sample, a major single peak (at about 7 min) was observed

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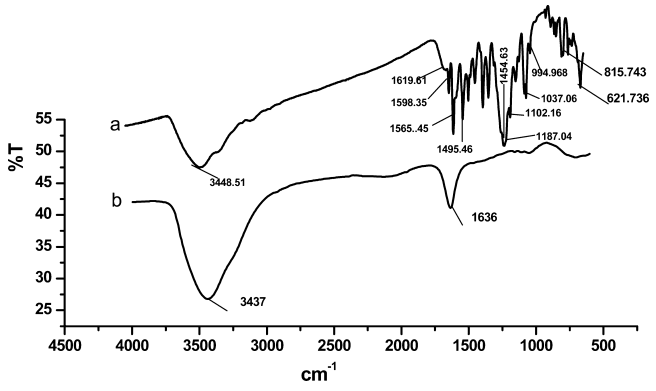

Figure 5. FT-IR spectrum of (a) pure dye, (b) degraded sample collected at 72 h.

along with small intensity peaks indicating that the dye was degraded to a single compound. Although HPLC studies support the degradation theory, for the nature of products and the mechanism, it is to be further related to the GC-MS analysis. The GC-MS analysis was also carried out for samples (shaker flask and calorimetry after 72 h) after degradation and the results are respectively given in Table 2 and 3. The

Table 2. Major Compounds Identified from GC-MS Studies during Acid Blue 113 Degradation by *S. lentus* under Aerobic Conditions

retention time (min) (72 h sample)	*m/z*	prominent compound formed
8.2	128	naphthalene
12.62	170	dodecane
12.87	212	pentadecane
19.53	166	pthalic acid
21.32	278	dibutyl phthalate
26.80	146	adipic dihydroxamic acid monohydrate
28.53	282	oleic acid
28.81	368	diisooctyl adipate
31.27	122	pyridine-3-carbaxamide

Table 3. Major Compounds Identified from GC-MS Studies during Acid Blue 113 Degradation by *S. lentus* under Aerobic Conditions

time (h)	retention time (min)	*m/z*	prominent compound formed
24	5.95	106	benzaldehyde
24	9.90	136	benzeneacetic acid
36	18.84	110	1,4-octadiene
36	16.78	99	2-piperidinone
48	15.67	135	*N*-benzyl formamide
48	16.16	154	3,5-dimethoxy phenol
48	34.41	282	oleic acid
72	34.4	144	2-naphthalenone

compound identification was based on the library of GC-MS compounds, fragmentation pattern, and predicting possible degradation compounds based on dye structure.

3.4. Degradation Pathways of Acid Blue 113 Dyes Using *S. lentus*. It has been shown in the previous sections that choline lactate has been utilized by the bacteria for effective dye degradation. In our earlier studies we have shown that the carbon atoms of the lactate ion were consumed during early stages of the growth of *S. lentus*. Based on the GC-MS studies, the degradation pathways of Acid blue 113 were deduced. The biodegradation pathways of IL-mediated samples (shaker flask and calorimetry) are respectively given in Figures 6 and 7. It is

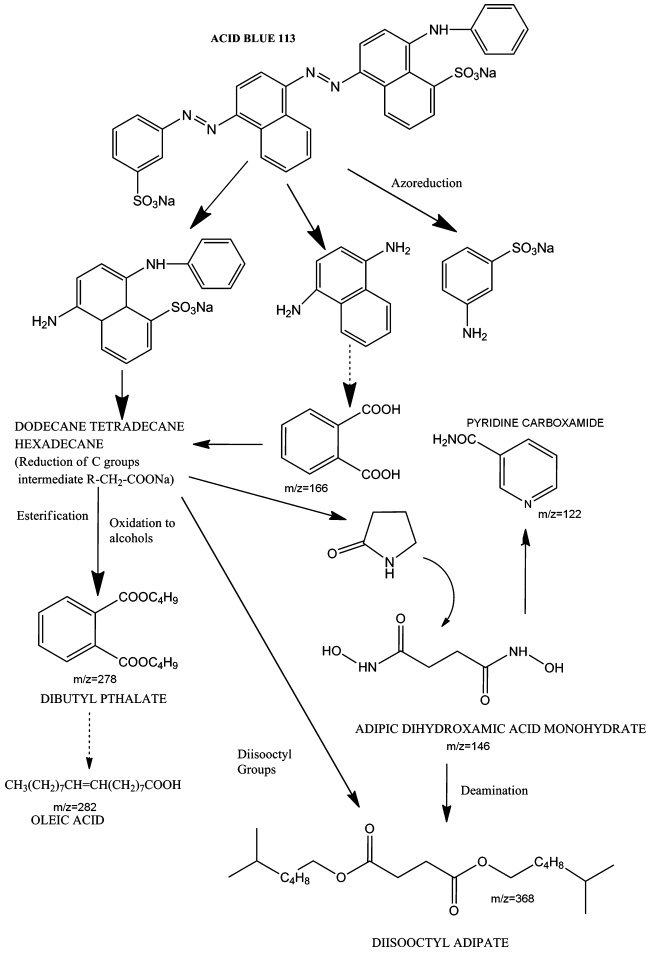

Figure 6. Degradation pathways of Acid blue 113 by *S. lentus in* shake flask.

evident that the Acid blue dye was degraded using reductive enzymes produced by the microorganisms and different products of degradation were observed for shaker flask and calorimetric experiments.

The differences in the product profiles are due to forced supply of oxygen in the biocalorimetry which contributed to enhance degree of oxidation. This was corroborated with the measurement of COD values taken for the final samples of both the glucose- and ionic liquid-mediated ones. In the case of glucose-mediated degraded sample the COD value was found to be 600 ppm whereas for the IL-degraded sample it was observed to be 483 ppm, indicating that the degree of oxidation is better in IL-degraded sample.

3.5. Cytotoxicity Assessment. Figure 8 shows the percent viability of Vero cells to the toxins. The IC50 values for the samples were found to be 1.22 mg/mL. Since the concentration levels used for cytotoxicity testing were very high in our studies in comparison to many reported studies it was thought appropriate to consider the IC90 values to assess the toxic nature. The IC85 values (39 mg/mL) show 84% cell viability and confirms less toxic nature of the degradation products obtained in our studies. Moreover, in actual effluents the concentration levels will be very much less due to dilution of

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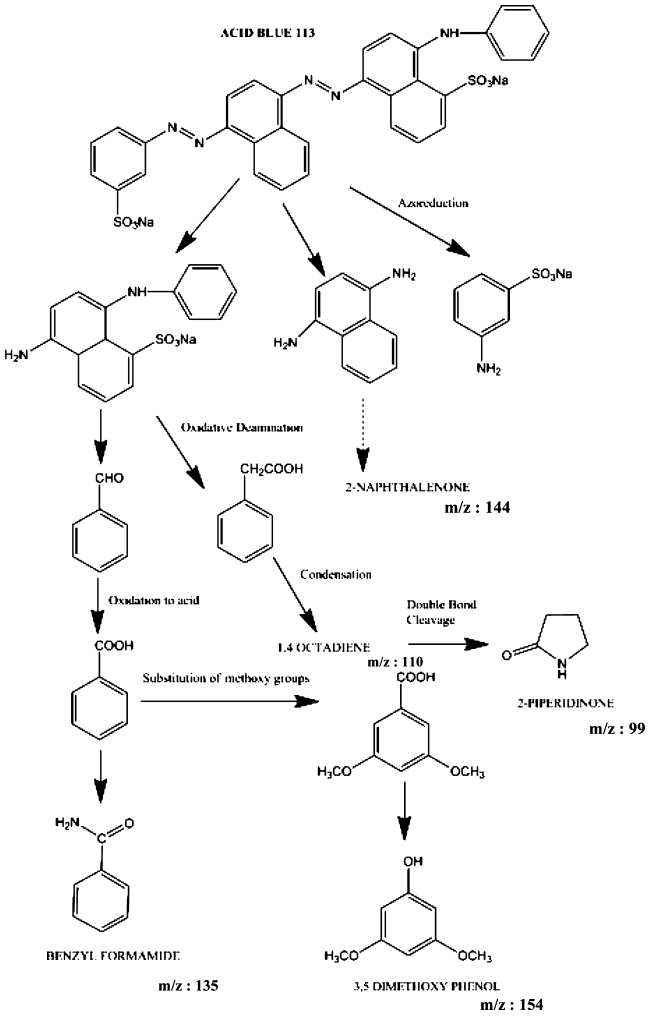

Figure 7. Degradation pathways of Acid blue 113 by *S. lentus* in BioRTCal.

large quantities of water. Oxidation of aromatic amines in the aerobic stage was found to be responsible for less toxicity of the extracts.[29]

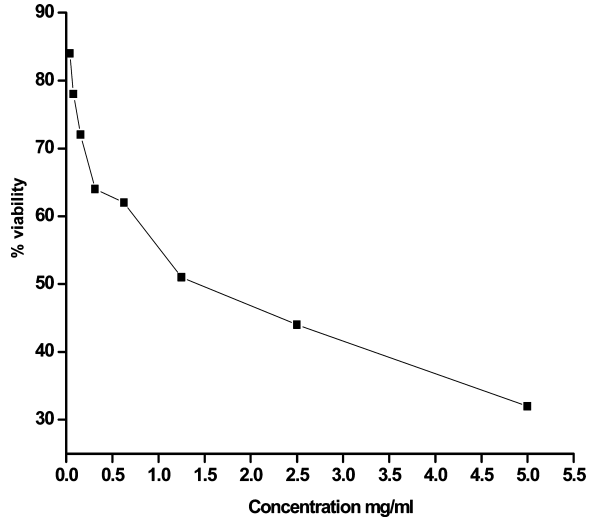

Figure 8. MTT assay of Vero cells percent of cytotoxicity levels with choline lactate cytotoxicity.

Thus the choline-based salts, in particular choline lactate ionic liquid, were successfully used as a carbon source for an effective degradation of Acid blue 113 using *S. lentus* as a bacterial source. The degradation products were characterized by HPLC and GC-MS techniques, and the IL-mediated degraded sample was (analyzed for COD and cell viability) found to be less toxic in contrast to the glucose-mediated degraded sample.

■ ASSOCIATED CONTENT

Supporting Information

Additional text and data as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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