CHAPTER 8

BIOCOMPATIBLE IONIC LIQUIDS FOR MICROBIAL
AZO DYE DEGRADATION

8.1 INTRODUCTION

Azo dyes are the most versatile class of synthetic colorants employed in textiles, leather and plastics industries (Zolinger et al 1991). They are xenobiotic compounds, resist biodegradation in conventional aerobic treatment processes (Shaul et al 1991). The recalcitrance of azo dyes results in contamination of ground water (Riu et al 1998). In the dyeing processes, dyes are neither completely utilized nor recovered in the down stream processes and as a result the discharge of dyes in the effluent stream (Hildenbrand et al 1999) usually contains more than 10-15% of the dye as such. Most of these dyes and their metabolites are toxic, potentially carcinogenic in nature and their removal from the industrial effluents is a major problem (Pearce et al 2003 and Selvam et al 2003). Hence, there is an immediate need to remove the residual dye from aqueous effluent in a more eco-friendly manner.

Current 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 more importantly secondary pollutants (Forgacs et al 2004). Adsorbents such as charcoals, activated carbons, clays, chitin, chitosan, chemically modified cellulose and lignocellulose were also used for decolorization of dye effluents (Laszlo et al 1994). But the drawback
of adsorption processes is that the adsorbent needs regeneration and this adds to the cost of the process. In the chemical treatment processes, oxidation is the most commonly used methods. There are reports on the use of ozone, (Srinivasan et al 2009) Fenton’s reagents, (Arslan et al 2001) as oxidizing agents and in some cases (the use of Fenton’s reagent) the mechanism involves flocculation, impurities are often transferred to the sludge and hence these methods are not ecologically acceptable (Arslan et al 2001). Recently, advance oxidation processes (AOP) (Lv et al 2005 and Ashraf et al 2006) have been tried for the treatment of dye effluents and involves break down of dye molecules into smaller components through the production of highly reactive hydroxyl radicals. The limitation of AOP is that they are susceptible to scavenging of hydroxyl radicals by non target substances and not suitable for certain perchlorinated compounds because they cannot be attacked by hydroxyl radicals (Pignatello et al 2006). Electrochemical processing is another method (Takayama et al 2006) used for the treatment of the dye effluents but the implementation of this method involves a large investment in equipment. Recently hydrophobic ionic liquids (Vijayraghavan et al 2006) have been used to extract and recover azo dyes but ionic liquids could be expensive 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 their low operating cost and versatility of micro organisms (Pandey et al 2007). Anaerobic degradation of azo dyes produces aromatic amines which are carcinogenic and mutagenic (Senan et al 2004) and during the last few years it has been demonstrated that under aerobic environmental conditions several microorganisms are able to transform azo dyes to non-colored products or completely mineralize them even (Hong et al 2010). Azo dyes are generally non degradable by most of the bacteria under aerobic conditions but in this study, S. lentus bacterial strain are identified, grown in an ionic liquid
medium and degrade Acid Blue 113, the most commonly used dye in textile and leather industries.

In recent years the toxicities of a number of imidazolium, pyridinium, phosphonium and pyrrolidinium ionic liquids towards enzymes, microorganisms and cell cultures have been evaluated and tabulated in a review paper recently (Ranke et al 2007). The effect of alkyl chains of imidazolium ionic liquids on the anti-microbial activities have been studied (Pernak et al 2003) and the results revealed that the shorter the chain length the lower the anti-bacterial action and vice versa. Since this study required bacteria to grow in ionic liquids and subsequently produce enzymes and participate in dye degradation without producing toxic by-products, it was envisaged to focus on bio-compatible ionic liquids. There were results reported on Choline based ionic liquids for various biological applications (Fujita et al 2006, Vijayaraghavan et al 2006 and Vijayaraghavan et al 2010), yet, to the best knowledge, there are no studies on the use of bio-compatible ionic liquids for biological dye degradation. One of the objectives of the present study was to synthesize some of the Choline based ionic liquids and employ them as nutrients for biological dye degradation studies.

An indirect technique for on-line measurement of substrate consumption or biomass formation, is reaction calorimetry and there are a few studies reported on the use of technique to correlate the heat release in terms of biomass formation (Voisard et al 2002 and Surianarayanan et al 2010). Here the use of this technique was planned to monitor metabolic activity of bacteria while simultaneously monitoring the heat release and observe dye degradation at different time intervals.
The products of degradation were analyzed by Gas Chromatographic coupled with Mass Spectroscopic techniques at different times of degradation. A mechanistic path way for the degraded products was proposed along with their toxicity by employing COD and cell viability studies.

8.2 MATERIALS AND METHODS

The present chapter deals with the biocalorimetric studies of acid blue 113 degradation using Choline based ionic liquids, and differ from the previous chapter in the selection of carbon source. Hence, most of the materials and procedures employed were similar as presented in the previous chapter. Additional information that is not covered in the previous chapter was only detailed here. The details about the Choline based salts; their purity determination and Choline lactate assay were presented in Chapter 5. The procedure for Enzyme activity, COD, FTIR, GCMS, and Choline lactate consumption cytotoxicity testing presented in Chapter 7 is followed here too.

8.3 RESULTS AND DISCUSSION

8.3.1 Degradation of Acid Blue 113 using S. lentus

The degradation of Acid blue 113 (25 ppm) by S. lentus was carried out in a shake flask under optimized conditions, with various 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 8.1. Different dye degradation profiles were observed for the various Choline salts and the best results were obtained for Choline Lactate (CL) (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 Chapter 5, the metabolic pathways involved when these salts
are present as well as the preferential utilization of lactate anion and a faster rate of growth by this organism in comparison with conventional carbon sources such as glucose were shown. The next focus is on the optimization of Choline lactate by varying its concentration from 1 g/L to 5 g/L and the degradation profiles are shown in Figure 8.2. The results show that maximum degradation occurs with a CL concentration of 2 g/L and further increase in concentration did not yield further degradation. The optimized conditions were pH 7 at 37°C with a 4% inoculum, CL at 2 g/L with dye at 100 ppm. It is worth mentioning here that the conventionally used carbon source, glucose, requires 5 g/L (2.5 times the CL used here) in order to obtain similar degradation profiles. In order 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 shown in Figure 8.3, where the best degradation result is found at 25 ppm (92% in 72 h) although there is little difference up to 75 ppm (84% in 72 h).

![Figure 8.1](image)

**Figure 8.1** Different Ionic Liquid’s 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%) (○))
Figure 8.2 Effect of initial Choline lactate concentration on acid blue 113 degradation by S. lentus in shake flask.

(0.1% (85%) (■), 0.2% (93%) (●), 0.3% (74%) (▲), 0.4% (75%) (▲), 0.5% (62%) (○))

Figure 8.3 Effect of initial dye concentration on acid blue 113 degradation by S. lentus in shake flask.

(25 ppm (92%) (■), 50 ppm (88%) (▲), 75 ppm (84%) (●), 100 ppm (80%) (▼))
Under similar experimental conditions, the conventional carbon source produced relatively lower degradation (Figure 7.8). Thus, these results show the superior nature of Choline salts in effectively stimulating the microbe for enhanced dye degradation and also as an effective carbon source for biological dye degradation.

8.3.2 Biocalorimetric Studies of Acid Blue 113 Degradation by S. lentus and Choline Lactate as a Carbon Source

Thermodynamic responses of S. lentus cultivated in the Choline salt medium (2 g/L) were studied in the biocalorimeter during dye degradation. A comparative plot showing heat profile, dye degradation, Choline lactate consumption, enzyme activity, COD profiles and OUR is shown in Figure 8.4. 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 CL. During the biodegradation process, CL

Figure 8.4  Comparative profiles of heat production during acid blue 113 degradation by S. lentus presence of Choline lactate. (Heat production rate (-), oxygen uptake rate (■), (dye degradation (●), Enzyme Activity (○), substrate consumption (▼) and COD (◇))
consumption takes place slowly. It can also seen that the percentage of dye degradation (total COD reduction) and CL consumption proceed in unison, thus indicating their simultaneous utilization. During this process, as observed in the previous literature studies (Zimmermann et al 1982), azo reductase enzyme release 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. It was observed that during the first phase of the activity (extending up to 21 h) 35 % dye degradation occurred, perhaps due to primary cleavage of the chromophoric azo bond. In the second exponential phase (starting from 21 h with a maximum at 37.5 h) 58 % of dye was degraded and a maximum enzyme (azo reductase) activity was noted (46 U/mL) during this phase. Heat release rates were at their maximum during this phase, indicating rapid enzymatic oxidative reaction. In this phase the maximum consumption of CL was also observed. Although, during the third phase (37.5 – 55 h), there was a decline in heat release rate (indicated also by slow consumption of CL, along with decline of oxygen uptake rates (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 Fig 8.4) for substrate, biomass and oxygen uptake and given in Table 8.1. \( Y_{Q/O} \) for microbial dye degradation in the IL medium was found to be 443 ± 10 kJ/mol. This value is well within the range reported (von Stockar et al 1989) and confirms the predominantly aerobic nature of the process.

The bioenergetics data given in Tables 4.4 and 5.4 are compared with the data in Table 8.1. The data shows that the bacterial S.lentus has more affinity towards 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 8.1 are more or less same for glucose and choline lactate. The differences in the behaviour of S.lentus in the substrate heat yield values show that its affinity towards the dye does not depend on the initial nature of the substrate. The data shown in Table 8.2 confirm that in presence of choline lactate, the degradation is efficient, which is basically related to the enhanced enzyme activity.

Table 8.1 A comparison of heat yields of choline lactate and glucose during acid blue 113 degradation by S. lentus in BioRTC

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( Y_{Q/X} ) (kJ/g)</th>
<th>( Y_{Q/S} ) (kJ/g)</th>
<th>( Y_{Q/O} ) (kJ/mol)</th>
<th>( Y_{Q/COD} ) (kJ/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline lactate</td>
<td>13.1</td>
<td>22.3</td>
<td>443</td>
<td>8.97</td>
</tr>
<tr>
<td>Glucose</td>
<td>13.27</td>
<td>25.4</td>
<td>460</td>
<td>12.2</td>
</tr>
</tbody>
</table>
Table 8.2 A comparison of degradation profiles between choline lactate and glucose during acid blue 113 degradation by S. lentus in BioRTCal

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity (U/mL)</th>
<th>COD (mg/L)</th>
<th>Dye degradation (%)</th>
<th>Time for maximum degradation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline lactate</td>
<td>46</td>
<td>150</td>
<td>94</td>
<td>54</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>300</td>
<td>85</td>
<td>62</td>
</tr>
</tbody>
</table>

8.3.3 Acid blue 113 Degradation Products – HPLC and GC-MS Studies

HPLC analysis was carried out for samples withdrawn at 24 and 72 hours along with pure dye (as control) (Figure 8.5). The results show in the control sample a peak with large intensity at around 8 min and a medium intensity peak at 9.3 min along with a broad hump (10-15 min), whereas in the 24 h treated sample the broad hump was resolved into 3 peaks with medium intensities indicating the breakdown of the dye. In the 72 h sample, a major single peak (at about 7 min) was observed, along with small intensity peaks indicating that the dye was degraded to a single compound.

The FT-IR spectrum of sample collected at the 72 h during the degradation of acid blue 113 in CL mediated S. lentus along with pure dye is shown in Figure 8.6. It shows that the degraded product was not an aryl amine. 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 150 ppm indicating that the degraded products of the IL treated sample was less toxic.
Figure 8.5 HPLC Chromatogram showing the progress of dye degradation (a) pure dye (b) 24\textsuperscript{th} h (c) 72\textsuperscript{nd} h
8.3.4 GC-MS Studies - Degradation Pathways of Acid Blue 113 by S. lentus

It has been shown in the previous sections that Choline lactate was utilized by the bacteria for effective dye degradation. In the earlier studies shown in Chapter 5, the carbon atoms of the lactate ion were consumed during early stages of the growth of S. lentus. Again, GC-MS analysis was also carried out for shaker flask and calorimetry samples (shown in Appendix 6 and 7) after 72 h degradation and the results are summarized in Tables 8.3 and 8.4. The compound identification was based on the library of GC-MS compounds, fragmentation pattern and prediction of possible degradation compounds based on dye structure. The poly aromatic dye underwent azoreduction via ring cleavage, to yield aromatic compounds and one sulphur – aniline derivative. While reporting the degradation products of Navitan Fast Blue by pseudomonas auroginasa (Valli Nachiyar et al 2004) by GC-MS analysis, the authors also found the ring cleavage of the aromatic dye to yield similar products as found in our study. The formation of intermediates such
as pthalic acid, long chain alkanes and diethyl pthalate indicated a similar
degradation approach for the non – nitrogen moiety in the dye. In addition,
products such as palmitic acid and its corresponding unsaturated vinyl ester
were also identified. Aerobic biodegradation of aromatic compounds have
several common features. Structurally diverse compounds are degraded
through many different peripheral pathways to a few intermediates that are
further channeled via a few central pathways to the central metabolism of the
cell. In the aerobic catabolic funnel, most peripheral pathways involve
oxygenation reactions carried out by monoxygenases and hydroxylating
dioxygenases that generate dihydroxy aromatic compounds (catechol,
protocatechuate, gentisate, homoprotocatechuate, homogentisate,
hydroquinone, hydroxyquinol). These intermediate compounds are the
substrates of ring-cleavage enzymes that use molecular oxygen to open the
aromatic ring between the two hydroxyl groups (ortho cleavage, catalyzed by
intradiol dioxygenases) or proximal to one of the two hydroxyl groups
(Eduardo Diaz 2004). Thus the formation of products such as palmitic acid
and dibutyl formate leading to oleic acid are in accordance to the previously
reported literature.

The results are shown in Figures 8.7 and 8.8. It was 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 difference in the product
profiles was due to forced supply of oxygen in the biocalorimetry which
contributed to enhanced degree of oxidation.
Table 8.3  Major components identified from GC - MS studies during acid blue 113 degradation by S. lentus in combination with choline lactate shake flask

<table>
<thead>
<tr>
<th>Retention time (min) (72h sample)</th>
<th>m/z</th>
<th>Prominent compound formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.2</td>
<td>128</td>
<td>Naphthalene</td>
</tr>
<tr>
<td>12.62</td>
<td>170</td>
<td>Dodecane</td>
</tr>
<tr>
<td>12.87</td>
<td>212</td>
<td>Pentadecane</td>
</tr>
<tr>
<td>19.53</td>
<td>166</td>
<td>Pthalic acid</td>
</tr>
<tr>
<td>21.32</td>
<td>278</td>
<td>Dibutyl phthalate</td>
</tr>
<tr>
<td>26.80</td>
<td>146</td>
<td>Adipic dihydroxamic acid monohydrate</td>
</tr>
<tr>
<td>28.53</td>
<td>282</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>28.81</td>
<td>368</td>
<td>Diisoctyl adipate</td>
</tr>
<tr>
<td>31.27</td>
<td>122</td>
<td>Pyridine-3-carboxamide</td>
</tr>
</tbody>
</table>

Table 8.4  Major components identified from GC - MS studies during acid blue 113 degradation by S. lentus in combination with choline lactate in BioRTCal

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Retention time (min)</th>
<th>m/z</th>
<th>Prominent Compound Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5.95</td>
<td>106</td>
<td>Benzaldehyde</td>
</tr>
<tr>
<td>24</td>
<td>9.90</td>
<td>136</td>
<td>Benzeneacetic acid</td>
</tr>
<tr>
<td>36</td>
<td>18.84</td>
<td>110</td>
<td>1,4 octadiene</td>
</tr>
<tr>
<td>36</td>
<td>16.78</td>
<td>99</td>
<td>2-Piperidinone</td>
</tr>
<tr>
<td>48</td>
<td>15.67</td>
<td>135</td>
<td>N-Benzyl formamide</td>
</tr>
<tr>
<td>48</td>
<td>16.16</td>
<td>154</td>
<td>3,5 Dimethoxy phenol</td>
</tr>
<tr>
<td>48</td>
<td>34.41</td>
<td>282</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>72</td>
<td>34.4</td>
<td>144</td>
<td>2-naphthalenone</td>
</tr>
</tbody>
</table>
Figure 8.7 Degradation pathways of acid blue 113 by S. lentus in shake flask
Figure 8.8  Degradation pathways of acid blue 113 by S. lentus in BioRTCAl
Also, cytotoxicity studies using cell culture were performed for both the samples (IL treated) and the results shown in Figure 8.9. That there was good growth in CL mediated degraded sample confirmed its less toxic nature.

![Figure 8.9 MTT assay (a) Vero cells % of Cytotoxicity levels with Choline Lactate cytotoxicity, (b) Vero cells treated with Choline Lactate sample with 50% cytotoxicity level seen under microscope](image)

8.4 CONCLUSIONS

- The degradation of Acid blue 113 (25 ppm) by S. lentus was carried out in a shake flask under optimized conditions, with various Choline salts (Choline lactate, Choline tartrate, Choline saccharinate, Choline dihydrogen phosphate and Choline citrate) as the sole carbon source.

- The best degradation was obtained for Choline lactate (with a maximum degradation of 92% in about 72 h) under identical experimental conditions.

- Maximum degradation occurs with a CL concentration of 2.0 g/L and further increase in concentration did not yield any improvement.
Thus the superior nature of Choline salts (CL) in effectively stimulating the microbe for enhanced dye degradation and also as an effective carbon source for biological dye degradation is proved through these studies.

Biocalorimetric studies showed that the percentage of dye degradation (total COD reduction) and Choline lactate consumption proceed in unison, thus indicating their simultaneous utilization.

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 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.

The degradation products were characterized by HPLC, GC-MS techniques and the IL mediated degraded sample (analyzed for COD and cell viability) was found to be less toxic in contrast to the glucose mediated degraded sample.

The Acid blue dye was degraded using reductive enzymes produced by the microorganisms and different products of degradation were observed for shake flask and calorimetric experiments. The difference in the product profiles was due to forced supply of oxygen in the biocalorimetry which contributed to enhanced degree of oxidation.

Choline based salts, in particular Choline lactate ionic liquid, was successfully used as a carbon source for an effective degradation of Acid blue 113 using S. lentus as a bacterial source.