6.1 Introduction

Dyes removal from wastewaters has been a big challenge over the last few decades and till date there are limited treatment strategies that can effectively decolorize dyes. In recent years, notable achievements were made in the use of biotechnological applications in the area of wastewater treatment, not only for colour removal but also for the complete mineralization of dyes. The discharge of highly coloured wastewaters is currently an important environmental problem. Among the many different groups of dyes, tryphenylmethane dyes are the most common synthetic compounds used in the textile industries. The uses is around 30–40% of total consumption of dyes (Carliell et al. 1995) belonging to this class of dye and which implies their wide occurrence in the wastewaters.

The triphenylmethane dye (crystal violet) is extensively used as human and veterinary medicine, as a biological stain, and as a textile dye (John et al. 1988). Unfortunately, wastewater treatment facilities are often unable to completely remove the commercial dyestuffs, including triphenylmethane dyes (such as crystal violet) from contaminated wastewater thus contributing to the pollution and problem of aquatic life. This class of dye has been suggested as promoter of tumor growth in some species of fish (Bangert et al. 1977).

The various physico-chemical methods, such as adsorption, coagulation-flocculation and advanced oxidation could be very effective for the removal of the colour in wastewaters. However, oxidative degradation by chlorine and ozone are the most common chemical processes for colour removal, but chlorination has the disadvantage of producing organochloride by-products. Advanced Oxidative Processes (like ozone or hydrogen peroxide) can attack organic compounds 106–109 times faster than normal oxidizing agents (Sarasa et al. 1998).

On the other hand, biological processes provide a low-cost and an efficient alternative for simultaneous colour and organic matter removal. However, complete dye degradation in wastewater treatment plants based only on aerobic processes is difficult to achieve since the main mechanism responsible for colour removal is adsorption onto the sludges (Pagga and Brown. 1986). Anaerobic treatment may be a feasible alternative to treat textile wastewaters, especially tryphenylmethane class of dyes and in most of the cases they are easily reduced under anaerobic conditions (Van der Zee et al. 2000 and 2001).
Anaerobic reactors are truly accepted for the degradation and destruction of various dyes in wastewaters because of their low initial operational costs, smaller space requirements, high degradation efficiency and low sludge production, combined with a net energy benefit through the production of biogas. The upflow anaerobic sludge blanket reactor (UASB) and anaerobic filters (AF) are the most frequently used high-rate anaerobic reactors, but both types suffer from technical problems (Jhung & Choi. 1995). However, upflow anaerobic sludge-filter bed (UASFB) reactor hybridizes the advantages of both UASB and upflow anaerobic filter (UAF) processes. The use of packing media only in the top portion of the reactor minimizes channeling problem associated with UAF and loss of biomass due to flotation associated with poorly performing UASB reactors.

Therefore, the present research work has been carried out to study the biodegradation of crystal violet in a hybrid upflow anaerobic sludge-filter bed (UASFB) reactor.

6.2 Materials and methods

6.2.1 Experimental procedure

The schematic diagram of the laboratory scale hybrid up flow anaerobic sludge-filter bed (UASFB) reactor used in this study is shown in Fig 6.1. The diameter of the (UASFB) reactor was 50 mm and height 100 cm. The reactor was made of Pyrex-glass with an effective volume of 1.6 L. The reactor column constituted of two compartments viz. bottom part was operated as a UASB reactor; whereas the top part was operated as an anaerobic filter (AF). The top portion of the UASFB reactor was randomly packed with 30 pieces of small PVC packing media, which are cylindrical in shape and having rough surface. The reactor was operated for a total period of 150 days at 30-35°C. Continuous feeding of the reactor started with an initial conc. 1 mg L⁻¹ and then it was increased step wise to 100 mg L⁻¹ by increasing the feed CV, while maintaining a constant HRT (24 hr). The influent consisted of a synthetic wastewater containing CV (1-100 mg L⁻¹) and CH₃COONa (1 g chemical oxygen demand (COD)L⁻¹) was added as the co-substrate for the degradation of this dye. The methanogenic sludge was activated by acetic acid and the experiment started when the removal efficiency reached 90%.
Fig. 6.1 Schematic diagram of a hybrid UASFB reactor

6.2.2 Biomass sources and basal medium

Methanogenic granular sludge from a full-scale upflow anaerobic sludge bed (UASB) reactor treating effluent of Delhi municipal wastewater treatment plant (Delhi) was used for this experiment. Granular sludge source was washed and sieved to remove the adhered particles before use in this experiment and all biomass source was stored at 4 °C before use. The Lab scale UASFB reactors (liquid volume 1.6 L) were initiated with 16 g of volatile suspended solids (VSS) L⁻¹ of the anaerobic granular sludge with a neutralized acetic acid mixture. The hydraulic retention times of the reactors were kept constant up to 24 hr.

The basal medium (Tan et al. 2000) used in this experiment contained (mg L⁻¹):

Co-substrate: CH₃COONa (1000),
Macro nutrient: NH₄Cl (280), CaCl₂·2H₂O (10), K₂HPO₄ (250), MgSO₄·7H₂O (100), KH₂PO₄ (250)
Micro nutrient: H₃BO₃ (0.05), FeCl₂·4H₂O (2), ZnCl₂ (0.05), MnCl₂·4H₂O (0.05), CuCl₂·2H₂O (0.03), NH₄SeO₃·5H₂O (0.05), AlCl₃·6H₂O (2), NiCl·6H₂O (0.05), NaSeO₃·5H₂O (0.1),
6.2.3 Analyses

The performance of this reactor was evaluated by monitoring chemical oxygen demand (COD), suspended solids (SS), volatile suspended solids (VSS), and alkalinity in influent and effluent samples according to the Standard Methods for Examination of Water and Wastewater (APHA-2002). The percentages of biogases (oxygen, carbon dioxide, nitrogen and methane) in the headspace of the batches were measured with liquid displacement method. Methane gas was detected by using 3 % NaOH (wv⁻¹) containing liquid solution (Razo Flores et al. 1997). The concentration of sodium acetate is expressed in terms of chemical oxygen demand (COD), commonly used in wastewater treatment and the conversion factor used was 780 mg COD g⁻¹ sodium acetate. The tryphenylmethane dye (Crystal Violet) was measured by UV-Visible spectrophotometer (Elico sl-164, Elico, India) at its maximum absorbance of 588 nm. The samples were centrifuged at 10,000 rpm for 10 minutes (Remi, India) and diluted in a 0.1 M sodium phosphate buffer (pH 7.0) solution and then measured in a quartz cuvet. A calibration plot (absorbance versus concentration of CV) was drawn and used for estimating the concentration of unknown dye solutions. The plot was linear (R² =0.98) between 0–100 mg L⁻¹ CV solution. The test samples drawn from experiments with higher concentrations of CV were adequately diluted and then the absorbance was determined.

The degradation products were also analyzed by UV-Visible spectrophotometer and high performance liquid chromatography (HPLC) methods. The samples from the experiment were centrifuged (10,000 rpm, 10 minutes) and diluted in demineralized water and 5-20 µl samples were injected into high performance liquid chromatography equipped with Shimadzu spectrophotometric detector (model SPD Pump Lc6A and system controller SCL-6B). The Peak area and retention time were calculated with Shimadzu chromatopack C-R6A data processor (Shimadzu Scientific Instrument Inc., Japan)

At the end of the experiment, the reactor was emptied to quantify the amount of biomass (in terms of volatile suspended solids) entrapped into each support and the granules were quantified gravimetrically by weighing the oven-dried samples at 105 °C for 24 h. The Oven-dried solid samples were scrapped out from the supports and ignited at 550 °C for 2 h to estimate the volatile solid (VSS) content. After the 150 days experiment, the surface of the anaerobic sludge is shown in Fig 6.2
6.2.4 Chemicals

Crystal violet [N-[4-[bis[4-(di-methylamino)phenyl]methylene]], C_{25}H_{30}ClN_{3}, molecular weight of 407.99 g mol\(^{-1}\), was used as received from (G.R. Product of CDH, India). All other chemicals used were analytical grade and supplied by CDH India.

6.3 Results and discussion

The main results of the anaerobic UASFB bioreactor are presented and summarized in Table 1. During the start up period, the solid washout was quite high and which is further reflected by an increase in suspended solid concentration. Afterwards, these values were decreased gradually and reached to a COD removal of 70 % and this is probably due to the filtration effect of the top packed-bed portion. SEM images of the sludge withdrawn at UASFB reactor are shown in Fig 6.2 and the various bacterial morphologies were observed because of mixed culture used in the reactor.

At the start up period, the sludge concentration was 16 g L\(^{-1}\). The Specific biomass activity was calculated using OLR technique at the end of the experiment and the total quantity of VSS was measured inside the reactor. The average specific activity of total biomass in the reactor was 25 g L\(^{-1}\). During the start up period gas production rate was quite low and increased to 2 L d\(^{-1}\) after 50 days and at the end of experiment it goes to 5 L d\(^{-1}\).
Use of internal packing as an alternative for retaining biomass in the UASB reactor is a suitable solution for the above mentioned problem. The packing medium in the UASB reactor is intended to increase solids retention by dampening short circuiting, improving gas/liquid/solid separation, and providing surface for biomass attachment (Rajinikanth et al. 2008). The packing medium had a triple role in the retention of the biomass inside the reactor

1. entrapment of biomass within the support and filtration of the granular biomass
2. Preventing it from going out of the reactor.
3. It also increases the reactive surface area between biomass and organic load.

After 50 days the reactor reached the steady-state conditions, after which the Organic loading rate was maintained with an average COD removal efficiency of 70%. Dye reduction occurred in the presence of oxygen when co-substrate sodium acetate was added (Donlon et al. 1997). The co-substrate sodium acetate was completely degraded in the anaerobic reactor and converted into methane and carbon dioxide. Under complete anaerobic condition almost all of the COD was recovered as methane and carbon dioxide.

Crystal Violet dye was degraded in the reactor and its biodegradation products N, N-dimethylaminophenol and Michler’s Ketone were observed as shown in Fig 6.3. Subsequently, N, N-dimethylaminophenol was also degraded to aniline in the reactor. Michler’s ketone was not degraded throughout the experiment. The other compound, N, N-dimethylaminophenol, was degraded up to 80% which was probably due to autoxidation of this compound prior to measurements or to partial anaerobic degradation of this compound. The results of the biodegradation experiment clearly show that aniline was also degraded to biogas which is a valuable product.

From the UV-Visible spectra (Fig 6.4) it is evident that 100 ppm CV (Blue line spectra) was totally decolorized (Dark yellow spectra) in UASB part of the reactor and get new pick [Lamda max-232, Abs-1.966] which is similar to aniline and after AF part of the reactor absorbance of aniline is decreased [Abs-0.584] at same lamda max. Aniline and Michler’s Ketone were also determined through HPLC analysis (which is not shown). The methanogenic activity remained high and even
increased at the end of the experiment when dye concentrations of 100 mg CV L\(^{-1}\) were applied.

![Chemical structures of Crystal violet, Michler's Ketone, and N, N-dimethylaminophenol](image)

**Fig. 6.3.** Proposed biodegradation pathways of crystal violet in UASFB reactor

Methane yield was close to the theoretical yield at all times and the biogas was found to have 69–83% CH\(_4\) and remaining being CO\(_2\). A linear relationship was found between the methane production rate and the total COD removal rate applied (Fig 6.5). At the end of the experiment, the gas production rate was quite high near about 5 Ld\(^{-1}\) for CV degradation.
Feeding was started at a soluble COD feed concentration of 400 mg L\(^{-1}\) at HRT of 24 hr (OLR=0.4 kg COD/m\(^3\) d). From the very beginning COD removal efficiencies were in the range of 50-60% and within 30 days of startup it increased to 60-70 %. After 50 days, when reactor reached steady state, the COD removal was observed around 90% (Fig 6.6). After the end of the experiment, the VSS was 25 gm L\(^{-1}\) and alkanity was maintained near about 1000 mg L\(^{-1}\) as CaCO\(_3\) through out the experiment. These results clearly show that the anaerobic UASFB bioreactor performed well, as it achieved complete decolorization of CV with increasing loads of dyes up to 100 mgL\(^{-1}\).

The importance of co-metabolism is not limited to the accumulation of biochemical products. Co-metabolism has also been used for the detection and demonstration of specific enzyme action. The accumulation of end products allowed for the isolation and identification of metabolites and the determination of specific bonds of substituted catechols which were cleaved by the co-metabolic process. A
A universally accepted definition of co-metabolism has proven elusive, but there is a general compromise that it describes the metabolism of a non-growth substrate in which no apparent benefit is accrued by the metabolizing organism (Wackett, 1996). It is further considered to be incomplete metabolism, and the intermediate(s) accumulated by one organism is assumed, but hardly ever demonstrated, to cross-feed other microorganisms. In this paper, various products were identified N,N-dimethylaminophenol, (Michler’s Ketone) N,N-bis (dimethylamino) benzophenone, aniline and biogases respectively.

![Graph](image1)

**Fig. 6.5** UASFB performance: methane gas production and % removal of CV, COD

![Graph](image2)

**Fig. 6.6** UASFB performance: Difference of COD in influent and effluent and % COD removal
6.5 Conclusions

This study reveals that the hybrid UASFB reactor can be efficiently used for the treatment of synthetic wastewater containing crystal violet (100 mg L\(^{-1}\)) and sodium acetate (1 gL\(^{-1}\)) as co-substrate at room temperature. Almost complete discoloration of the influent and 98% removal of the triphenylmethane dye (crystal violet) were observed. Michler’s Ketone, generated after CV anaerobic breakdown, and was found to remain in the effluent of the reactors because of its non biodegradable nature. The other compound generated (N,N-dimethylaminophenol) was not directly observed, although aniline was detected as a probable product of its oxidation. Subsequently, aniline is further degraded to biogas which is a valuable derivative. The gas production rate was quite high near about 5 Ld\(^{-1}\) for CV degradation. The results showed that a UASFB reactor exhibits a highly promising bioreactor for the application in the treatment of dye containing wastewater and bioremediation to remove recalcitrant triphenylmethane dye (Crystal Violet) pollutants from contaminated environment.
Table 6.1 Operational parameters and results of the hybrid UASFB reactor (average value)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>unit</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>7th</th>
<th>8th</th>
<th>9th</th>
<th>10th</th>
<th>11th</th>
<th>12th</th>
<th>13th</th>
<th>14th</th>
<th>15th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>110</td>
<td>120</td>
<td>130</td>
<td>140</td>
<td>150</td>
</tr>
<tr>
<td>*Sodium Acetate gm COD L(^{-1})</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CV mg L(^{-1})</td>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>HRT hr</td>
<td></td>
<td>24</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>22.5</td>
<td>24</td>
<td>25</td>
<td>23</td>
<td>24</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>25</td>
<td>24</td>
</tr>
</tbody>
</table>

**Efficiencies**

<table>
<thead>
<tr>
<th>*Sodium</th>
<th>Acetate</th>
<th>% Removal</th>
<th>80</th>
<th>92</th>
<th>98</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>% Removal</td>
<td>45</td>
<td>47</td>
<td>53</td>
<td>55</td>
<td>57</td>
<td>60</td>
<td>68</td>
<td>74</td>
<td>76</td>
<td>88</td>
<td>91.8</td>
<td>93</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>(at 588 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline formation</td>
<td>mg L(^{-1})</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>11</td>
<td>24</td>
<td>33</td>
<td>37</td>
<td>40</td>
<td>44</td>
<td>50</td>
<td>53</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>(at 230 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline</td>
<td>% Removal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>32</td>
<td>40</td>
<td>48</td>
<td>52</td>
<td>61</td>
<td>68</td>
<td>72</td>
<td>76</td>
<td>82</td>
</tr>
</tbody>
</table>

* Anhydrous Sodium acetate used as a co substrate 1gm=780mg O\(_2\) L\(^{-1}\)