CHAPTER 7

BIOENERGETICS AND MECHANISM OF ACID BLUE 113 DEGRADATION BY STAPHYLOCOCCUS LENTUS

7.1 INTRODUCTION

In leather manufacturing, azo dyes are commonly used since they are versatile in nature (Stolz et al 2001). During manufacturing and use of azo dyes, it has been estimated that 10 % (wt) of the dyes used is released in the environment (Vaidya et al 1982). Azo dyes contribute to 60-70 % of the world market and manufactured in large quantities. Azo dye containing effluent has been a desirable target for environmental distribution studies over the past 20 years (Vajnhandl et al 2007). The process of leather manufacturing involves a number of unit operations, including dyeing, that utilizes large quantities of water. It has been estimated that nearly 40–45 L of water per kilogram of raw-hides is used by tanneries for processing finished leather (Sarkar et al 1997). While anaerobic decolorization of azo dyes is well documented, not much information is available on the aerobic degradation (Michaels et al 1986).

Biodegradation of sulfonated aromatic compounds has been studied for many years (Feigel et al 1988, Goszczynski et al 1994 and Blumel et al 1998). Bacteria capable of degrading aromatic sulfonates have been isolated from industrial sewage treatment plants (Zimmermann et al 1982). The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of (usually colorless)

amines. These reductive processes have been associated with some aerobic bacteria, which can grow with (rather simple) azo compounds. These specifically adapted microorganisms synthesize true azoreductases, which cleave the azo group in the resence of molecular oxygen (Vaidya et al 1982). In the natural environment, azo dyes are degraded by a variety of microorganisms including aerobic and anaerobic bacteria. S. aureus was able to grow and reduce azo dyes, indicating that azoreductase was functionally expressed in the bacterium (Suzuki et al 2001). A vast literature (Chapter 2) on biological degradation of various dyes employing different kinds of microorganism is available and one gets confused with the utility of the data. It is because the studies are not comprehensive; in most of the reported studies, either the chemical characterization of the metabolites of the dye nor toxicity assessments are done to prove that the products of degradation do not contain a toxic metabolite. Only a few of the studies on microbial azo dye reduction included a clear demonstration of the total or partial, biodegradation of the metabolites (Pinherio et al 2004). In recent reviews (Pinherio et al 2004 and Anjali et al 2007), the authors concluded that the treatment of azo-dye containing waste waters presented a technical challenge and insisted on the need to assess the extent of mineralization of aromatic amines, as many amines can undergo auto-oxidation, leading to the formation of soluble recalcitrant polymers, which are toxic. Therefore, there is a requirement of microbial consortia that harbor genes for rapid degradation of mixtures of aromatic amines.

The advent of bench-scale calorimeters over the last 20 years has brought major improvements in its sensitivity, of which the isothermal mode is most suited for biological studies (von Stockar et al 1989). Heat generation is a universal feature of whole cell-catalyzed biological processes (Battley et al 1987 and Kemp et al 2004). It is particularly valuable as a control

parameter because it can provide real time insights to rapid metabolic changes (Duboc et al 1998 and Marison et al 1998). Respirometry is one of the analytical tools generally employed for assessing the physiological behavior of organism cultivated under aerobic conditions. Usually both respirometric and calorimetric data provide similar information (Voisard et al 2002). Several research studies are reported on monitoring the metabolic activity of cell culture and enzyme secretion employing the biocalorimeter in different kinds of bioprocess systems, and wastewater treatment systems (Senthilkumar et al 2007, Senthilkumar et al 2008 a and Senthilkumar et al 2008 b). About 40 to 50 % of the energy stored in a carbon source is converted to biological energy (ATP) during aerobic metabolism, and the rest is released in the form of heat, CO_2, H_2O. For actively growing cells heat evolution is directly related to their growth (Shuler and Kargi 2002).

Many biocalorimetric studies on different kinds of bioprocess systems have been published (Surianarayanan et al 2011), yet no known attempt has been done on monitoring the azo dye degradation process through metabolic heat measurements. In this study, calorimetric experiments were performed in 'real time' version of a calorimeter known 'RTCal' and metabolic heat flow rate measurements from calorimeter have been used to analyze the azo dye degradation process and study the physiological behavior of the organism. Oxygen uptake studies were performed in varying oxygen flow conditions and heat yield coefficient values determined for dye degradation process. Finally, the metabolic degradation pathway is worked out with the support of analytical instrumentation techniques such as FT-IR and GC-MS. The results could be useful during industrial bioreactor scale-up projections of a dye degradation process.

7.2 MATERIALS AND METHODS

7.2.1 Materials

The Azo dye Acid blue 113 used here has the molecular formula $C_{32}H_{21}N_5O_6Na_2S_2$ (mol.wt 68.64). It was a commercial sample obtained from one of the tanning industries located at 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.

7.2.2 Bacterial Strain Isolation and Culture Conditions

The organism used was S. lentus, which is a halotolerant bacterium. Isolation details and culturing conditions were described in previous Chapters (3&4).

7.2.3 Media for Dye Degradation

The contents of the mineral salt medium used in the degradation study are given in Table 4.1. The pH of the medium was adjusted to 7.0. The medium, without glucose, was sterilized at 121°C for 20 min. Glucose was sterilized separately and added to the medium under aseptic condition. 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. A calibration graph (Appendix 3) was plotted for different concentration of dye and the same was used to determine the % dye degradation. Dye degradation was monitored spectrophotometrically by withdrawing samples at different times. The samples were centrifuged at 10,000×g (Sigma, 3-18 k model) at 4°C for 15 min to remove the biomass (or) any other sediment. The supernatant was used for determining the degradation efficiency.

7.3 EXPERIMENTAL SETUP

7.3.1 Gas Chromatography-Mass spectrometry (GC-MS) Analysis

In this study, GC-MS is used to characterize the degradation products of the dye acid blue 113. Samples withdrawn from shake flask and biocalorimetric experiments were centrifuged. The supernatant was extracted thrice with equal volume of ethyl acetate. The extract was dried over Na_2SO_4 and concentrated in a rotary evaporator. It was then subjected to GC-MS analysis in a Perkin Elmer Autosystem XL GC with Turbomass MS spectrometer after dissolving it in 1 mL of ethyl acetate. Identification of metabolites was done by matching the fragmentation pattern with the NIST chemistry web book (NIST Chemistry Web Book).

7.3.2 High Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis was performed (Shimadzu Model CTO -10 AVP) to monitor the progress of degradation compounds. The bacterial culture medium along with the degradation products was centrifuged and filtered through 0.2 µm filters and the supernatant extracted thrice with equal volume of ethyl acetate. The extract was dried over Na_2SO_4, concentrated in a rotary evaporator and equal amount of HPLC grade methanol was added to the sample. About 25 µL of this filtrate was subjected to HPLC analysis using Gemini C-6 phenyl mobile phase column with a solvent system consisting of methanol and water.

7.3.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FT-IR analysis of the degraded samples was carried out using ABB MB3000 Spectrometer. The culture medium containing the degradation products was centrifuged and 5 µL of supernatant was sandwiched between two plates of high purity potassium bromide (KBr) salt and the spectrum recorded.

7.3.4 Glucose Analysis

Glucose analysis was performed by the Di-nitro-salicylic acid method (Miller 1959).

7.3.5 Chemical Oxygen Demand

Chemical Oxygen Demand (COD) analysis was performed by the closed reflux method and the measurement made calorimetrically, as per APHA guidelines (APHA 1998).

7.3.6 Dye Decolourization Measurement

The residual colours in the control sample and treated samples were analyzed by measuring the absorbance at 546 nm wavelength (absorbance maxima of acid blue 113) using a UV visible spectrophotometer (Shimadzu, Kyoto, Japan UV- 210 PC). The absorbance values were correlated to the calibration graph. The percentage of dye degradation was then calculated as follows:

$$\%\,\text{Degradation} = \frac{c_i - c_f}{c_i} \tag{7.1}$$

where C_i is initial concentration and C_f is final concentration.

7.3.7 Azoreductase Assay

Azoreductase activity was assayed by the Zimmermann method (Zimmermann et al 1982) using Acid blue 113 as the dye substrate. The assay mixture contained 0.8 mL of 100 mM phosphate buffer (pH 7.0) with 0.2mM of the dye Acid blue 113, 0.1 mL of 1mM NADH and 0.1 mL of enzyme in 1mL of reaction mixture. The reaction mixture without NADH was

preincubated for 4 min and the reaction started by the addition of NADH. Dye decolorization was followed by monitoring the decrease in colour intensity at 565 nm at room temperature. One unit (U) of Azoreductase activity was defined as the amount of enzyme required to reduce 1 μM of dye/min.mL under the assay conditions.

7.3.8 Cytotoxicity Testing

The cytotoxicity testing of the metabolites collected at 72 h was carried out according to the previously reported method (Adedayo et al 2004). 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 was 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 haemocytometer. At least 250 cells were counted for each measurement.

7.3.9 Heat Yield Calculation

Though power-time profiling depicts the metabolic shifts during a growth process, quantitative information on relative consumption of substrates $Y_{Q/S}$ (kJ/g of glucose consumed), energy changes associated with biomass growth $Y_{Q/X}$ (kJ/g cell dry weight formed) and oxycalorific coefficient $Y_{Q/O}$ (kJ/mol of oxygen consumed) are evaluated by calculating cumulative heat production values by integrating the power-time curve (Surianarayanan et al 2010).

7.4 RESULTS AND DISCUSSION

Prior to calorimetric experiments physical parameters such as temperature, pH, innoculum size and glucose concentration were optimized for the growth of S. lentus at shake flask experiments. The optimized parameters are summarized in Table 7.1.

Table 7.1 Optimized parameters for the growth of S. lentus at shake flask

S.No	Optimized parameters	Conditions
1.	pH	7
2.	Temperature	37^{o}C
3.	Inoculum	4%
4.	Glucose	5 g/L
5.	Acid Blue 113 (Dye)	100 mg/L

7.4.1 Optimization of Process Variables in Shake Flask for Acid Blue 113 Degradation by S. lentus

The optimization of growth parameters for S. lentus performed in shake flasks and biocalorimetry were reported in Chapter-4. Similar optimization experiments were performed by incorporating the dye under investigation at different concentrations in order to ascertain any changes in optimization of process variables. Figure 7.1 reveals that the best medium for dye degradation is MSM. Henceforth dye degradation experiments were conducted in MSM media.

The effect of salinity for acid blue 113 degradation by S. lentus was studied by varying the NaCl levels between 0.1- 0.5 % (w/v). The results are presented in Figure 7.2. It was found that at 0.4% (w/v) NaCl concentration maximum dye degradation of 90% occurs. Further dye degradation experiments were conducted with 4% NaCl concentration.

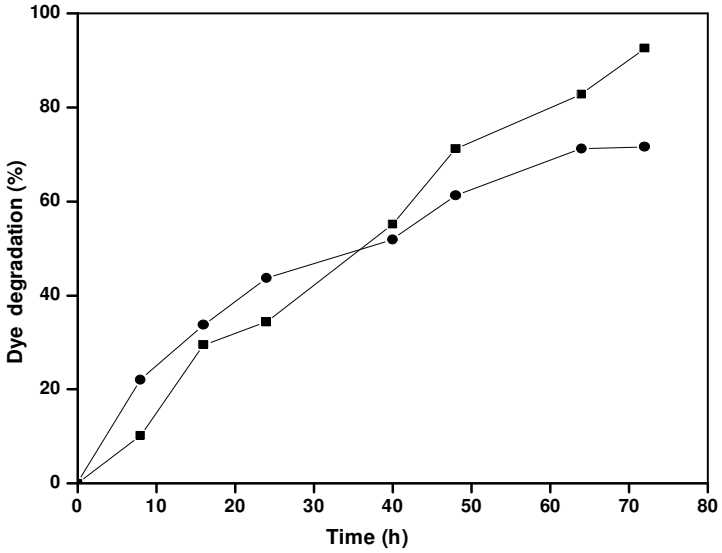


Figure 7.1 Effect of Medium for Acid blue 113 degradation: (MSM (92%) (■), BSM (75%) (●)).

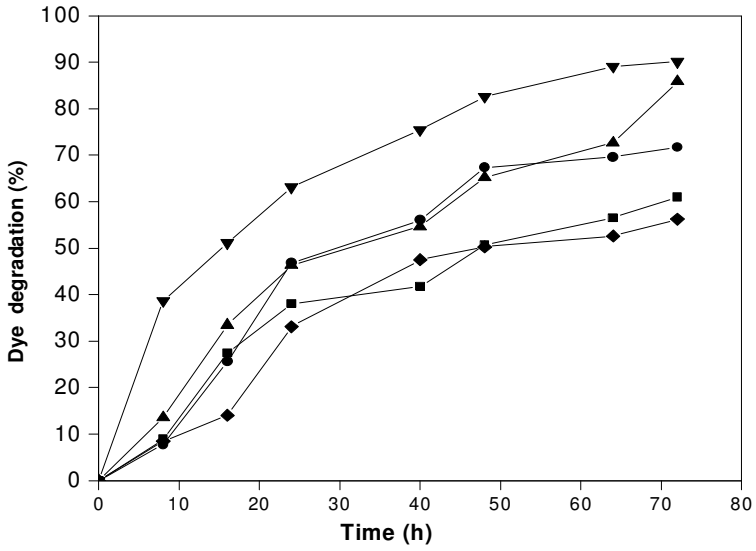


Figure 7.2 Effect of Salinity on 100ppm Acid blue 113 degradation by S. lentus in MSM at pH 7, 37°C, 4% inoculum and 100ppm dye concentration

(NaCl Concentration in % (w/v), (Dye degradation (%)): ■ – 0.1 (60%), ● – 0.2 (71%), ▲ – 0.3 (85%), ▼ – 0.4 (90%) and ♦ - 0.5 (56%)).

Figure 7.3 illustrates the effect of temperature on acid blue 113 degradation. Although degradation occurs at all the studied temperature ranges (27-50^{o}C), maximum degradation was found to occur at 37^{o}C. Therefore, for further dye degradation experiments the temperature was fixed at 37^{o}C.

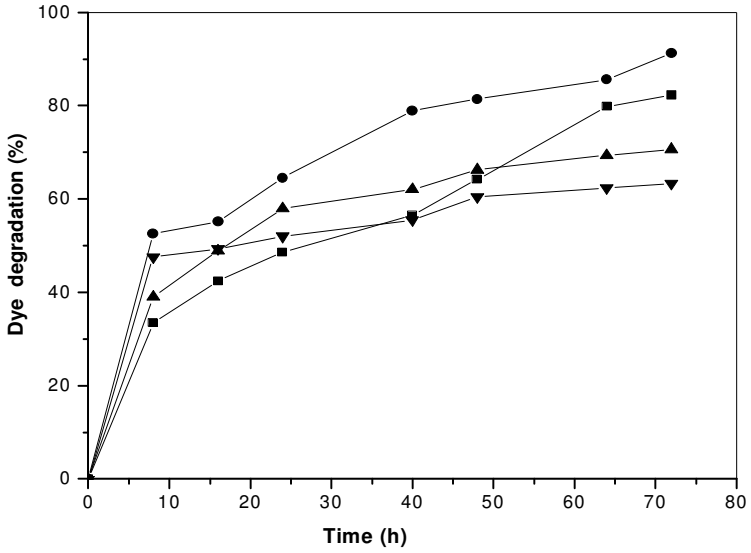


Figure 7.3 Effect of Temperature on acid blue 113 degradation by S. lentus in MSM at pH 7.0, 4% inoculum, dye 100ppm and 0.4% (w/v) NaCl.
(Temperature, °C (Dye degradation (%)): ■ – 27 (82%), ● – 37 (91%), ▲ – 45 (70%) and ▼ – 50 (63%)).

In Figure 7.4, the effect of inoculum size on the dye degradation is presented. It was found that a maximum (90%) dye degradation occurs at 4% inoculum level.

Figure 7.5 represents the comparative dye degradation profiles by S. lentus. This comparative plot depicted that maximum degradation of 92% was observed on a neutral pH of 7.

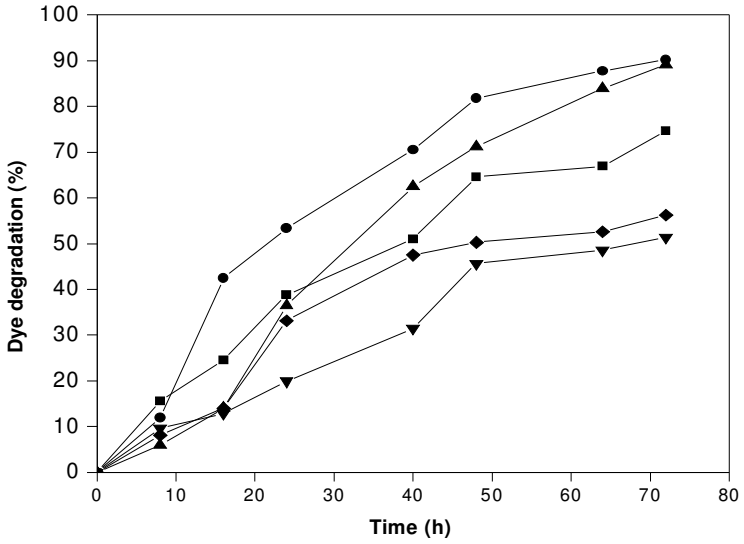


Figure 7.4 **Effect of inoculum concentration on 100ppm acid blue 113 degradation by S. lentus in MSM at pH 7.0, 37°C, 0.4% (w/v) NaCl and dye 100ppm.**
(Inoculum concentration in % (v/v), (Dye degradation(%)): ■ – 2 (74%), ● – 4 (90%), ▲ – 6 (89%), ▼ – 8 (51%) and ♦ - 10 (56%))

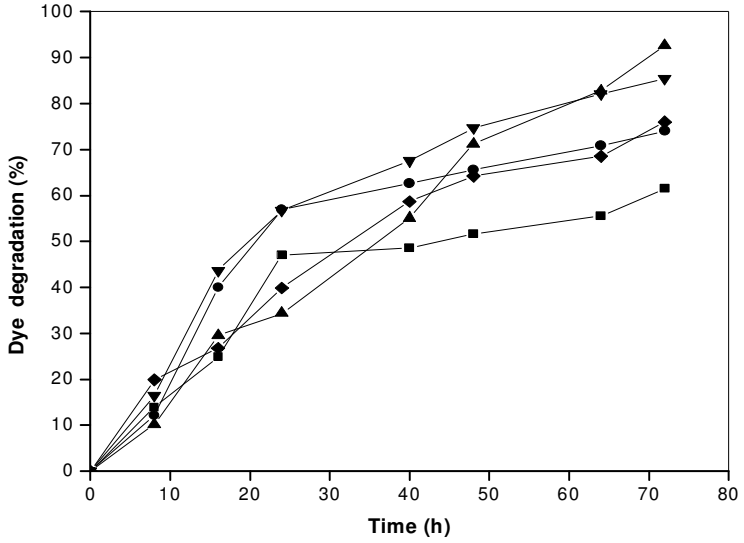


Figure 7.5 **Effect of pH on acid blue 113 degradation by S. lentus in MSM at 37°C, 4% inoculum, dye 100ppm and 0.4% (w/v) NaCl.**
(pH, (Dye degradation(%)): ■ – 6 (61%), ● – 6.5 (74%), ▲ – 7 (92%), ▼ – 7.5 (85%) and ♦ - 8 (76%)).

In order to select a suitable carbon source that maximum degrades acid blue 113, several carbon sources were screened. The resulted are presented in Figure 7.6. It was found that glucose is the best carbon source for maximum degradation.

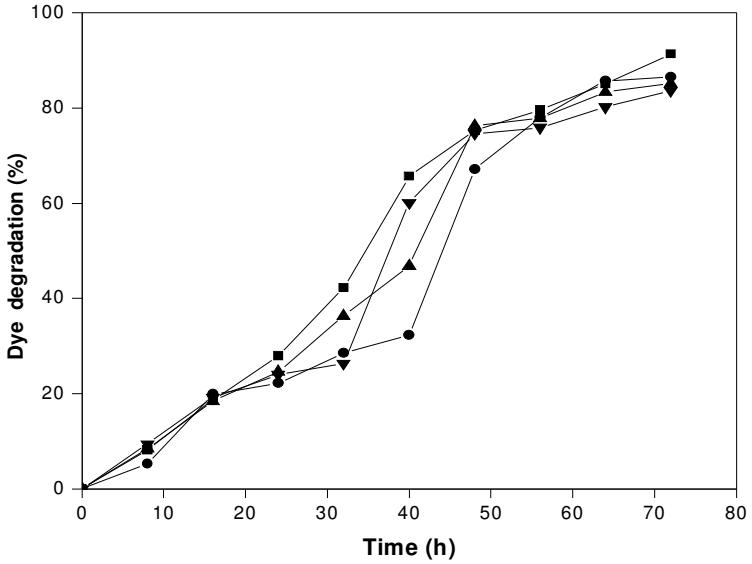


Figure 7.6 Effect of carbon source on Acid blue 113 degradation by S. lentus in MSM media 5 g/L of different carbon sources at pH 7, 37°C, 4% inoculum concentration and 0.4% NaCl. (Carbon Source (Dye degradation (%)): (■ – Glucose (91%), ● – Fructose (86%), ▲ – Sucrose (84%) and ▼ – Lactose (82%))

It was found that incorporating dye in the growth process did not alter the optimized conditions, excepting the notable difference in dye degradation efficiency. With the optimized growth parameters, dye degradation experiments were carried out by varying the initial dye concentration from 25 to 500 ppm levels in order to verify the efficiency of the organism under study. The shake flask experimental results are presented in Figure 7.7.

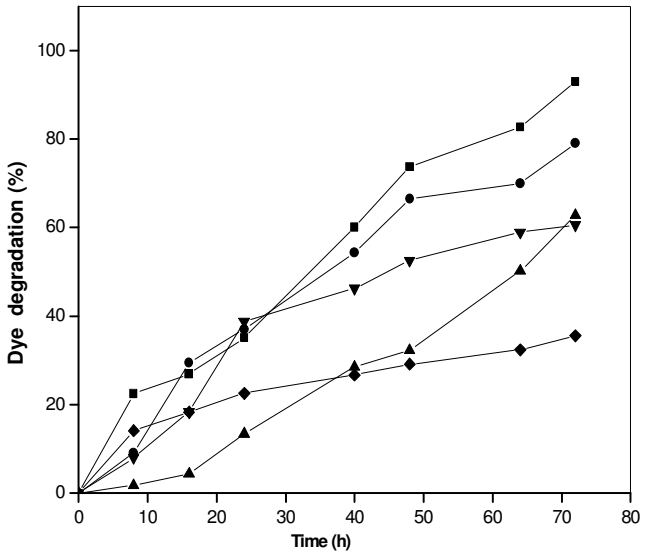


Figure 7.7 Effect of initial dye concentration on acid blue 113 degradation by S. lentus in shake flask.
(Dye concentration in ppm (Dye degradation (%)): ■ – 25 (92%), ● – 50 (79%), ▲ – 75 (62%), ▼ – 100 (60%) and ♦ - 500 (35%)).

Figure 7.7 reveals that the dye degradation efficiency depended on the initial dye concentration, a finding reported by various researchers earlier with different types of dye and bacteria (Vaidya et al 1982). In our study, beyond 100 ppm levels, the degradation efficiency decreased sharply and the results were not of commercial utility. This was one of the bottlenecks in biological degradation of colour effluents, especially with effluents containing a complex dye molecule. Upto 60% degradation could be achieved with an initial dye concentration of 100 ppm of acid blue 113. This finding from our study and other previously reported (Padmavathy et al 2003, Valli Nachiyar et al 2003, Supaka et al 2004, Kodam et al 2005, Niebisch et al 2010 and Cooney et al 1968) studies send a message to the industries generating colour effluents that their processes have to be fine tuned so that their effluents do not show dye concentrations greater than 100 ppm. However, it has been reported that in the tanning industry dye effluents, in India, the concentration levels were within this limit. (Vajnhandl et al 2007).

The shake flask optimized conditions (Table7.1) were employed in further biocalorimetric experiments for determining the influence of aeration and agitation rates on dye degradation.

7.4.2 Effect of Aeration on Heat Release Rates and Dye Degradation

The comparative plots of biocalorimetric experiments for acid blue 113 degradation at varying aeration rates 0.33, 0.66, 1.0 vvm are presented in Figures 7.8-7.10 and percent dye degraded alone are presented in Figure 7.11. The figures showed that forced aeration certainly increased the dye degradation efficiency from 60% levels in shaker flask to 85 % at 0.66vvm oxygen flow (Figure 7.9). The COD values were found to reduce from 3200 ppm to 450 ppm as a result of dye degradation. Increase in aeration rates up to 0.66 vvm resulted in the increase in dye degradation. Further increase in aeration to 1vvm resulted in decrease of maximum degradation efficiency to 70 %. The peak heat release rates, OUR and enzyme release rates were found to correspond to the degradation efficiency. As a result of growth, dye degradation and substrate consumption, the power time curve exhibited three distinct phases. In the endogenous phase the degradation rates were low compared to the early and exponential phases.

Interestingly, one can see a clear shift in the peak heat release rates (time shift) with change in aeration rates; at 0.33 vvm, the peak heat release occurred between 20- 37 h, and at 0.66 vvm it occurred between 11 and 24 h and at 1 vvm it was beyond 38 h. This observation was an indication of the organism's efficiency to adapt to various aeration rates; although the organism was able to initiate its growth and cell multiplication by consumption of glucose and dye, right from the beginning, at both 0.33 and 0.66 vvm, at higher O_2 flow rates, it could not effectively carry out the metabolism. This resulted in poor dye degradation efficiency and reflected in other parameters such as OUR, enzyme activity and COD. So aeration rates were found to be vital not only for growth but for efficient dye degradation also.

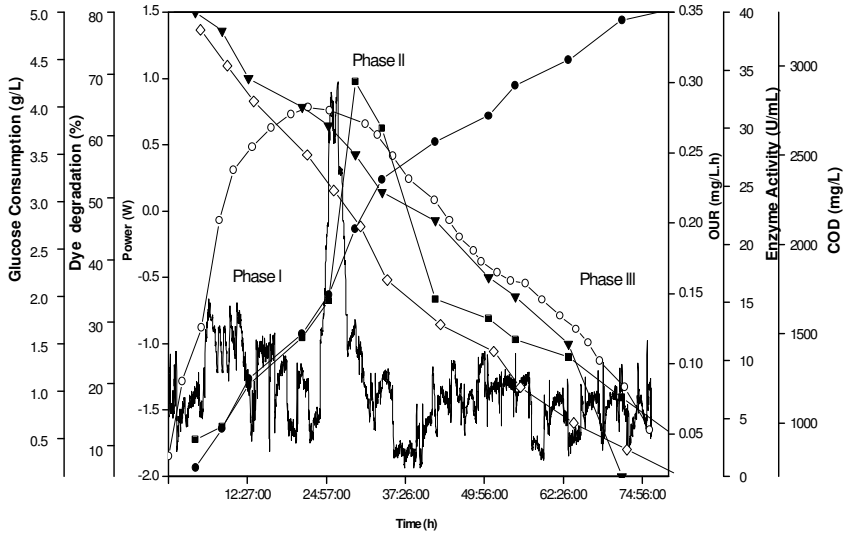


Figure 7.8 Comparative profiles of heat production during acid blue 113 degradation by S. lentus at 0.33vvm oxygen flow rate. (Heat production rate(-), oxygen uptake rate (■),dye degradation(●),Enzyme Activity (○), substrate consumption(▼) and COD (◊)).

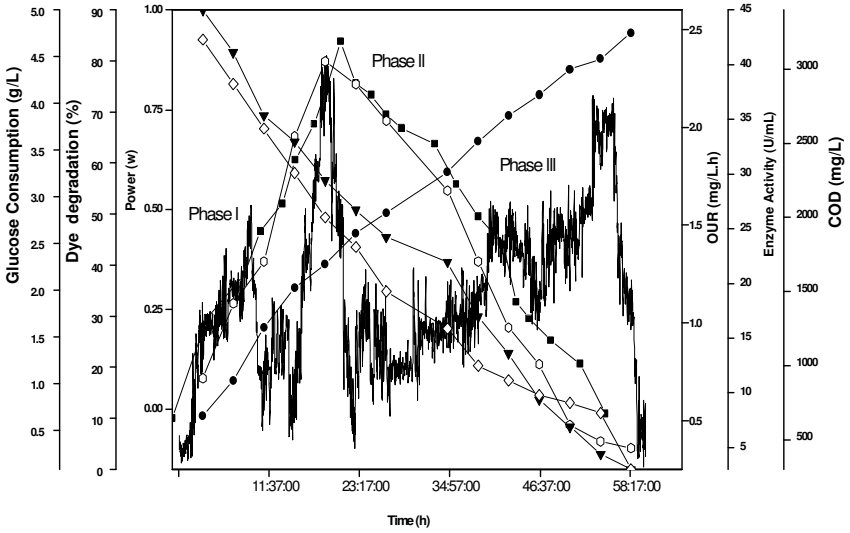


Figure 7.9 Comparative profiles of heat production during acid blue 113 degradation by S. lentus at 0.66vvm oxygen flow rate. (Heat production rate(-), oxygen uptake rate (■),(dye degradation (●), Enzyme Activity (○) , substrate consumption(▼) and COD (◊)).

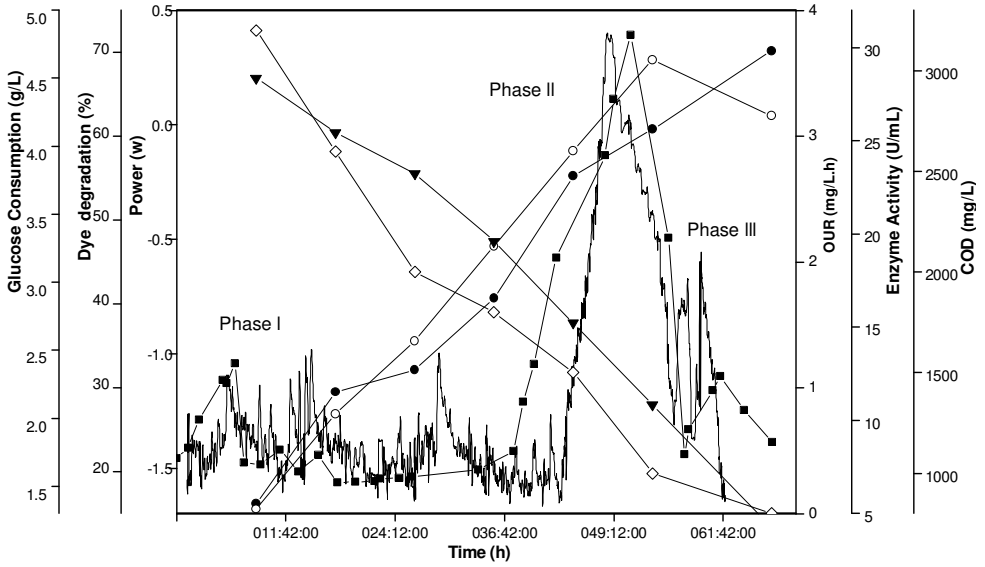


Figure 7.10 Comparative profiles of heat production during acid blue 113 degradation by S. lentus at 1vvm oxygen flow rate. (Heat production rate(-), oxygen uptake rate (■), (dye degradation (●), Enzyme Activity (○) , substrate consumption(▼) and COD (◊))

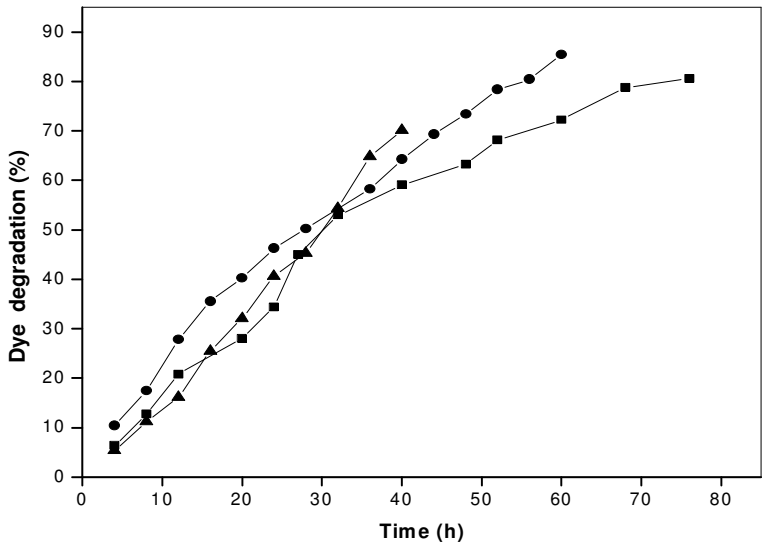


Figure 7.11 Influence of aeration rates on Acid blue 113 degradation efficiency by S. lentus.

Aeration rates (vvm) (Dye degradation (%)): ■ – 0.33 (80%), ● – 0.63 (85%) and ▲ – 1 (70%)

7.4.3 Effect of Agitation on Heat Release Rates and Dye Degradation

The effect of agitation on the acid blue 113 degradation can be seen from the comparative heat profile plots presented in Figures 7.12 and 7.13 and the degradation efficiency alone in Figure 7.14. These plots indicated that increase in agitation rate from 50 to 100 rpm increased oxygen uptake rates leading to increase in dye degradation efficiency. Further increase to 150 rpm resulted both in decrease of OUR and dye degradation efficiency, therefore the agitation did not have influence either the advancement (or) postponement of the simultaneous growth and dye degradation process, unlike the effects of aeration. Our studies led conclude that very high agitation was unnecessary and not favourable to achieve maximum dye degradation efficiency (Figure 7.13). The sample collected towards the end of the 50 h at 100 rpm and 0.66 vvm aeration was almost colourless.

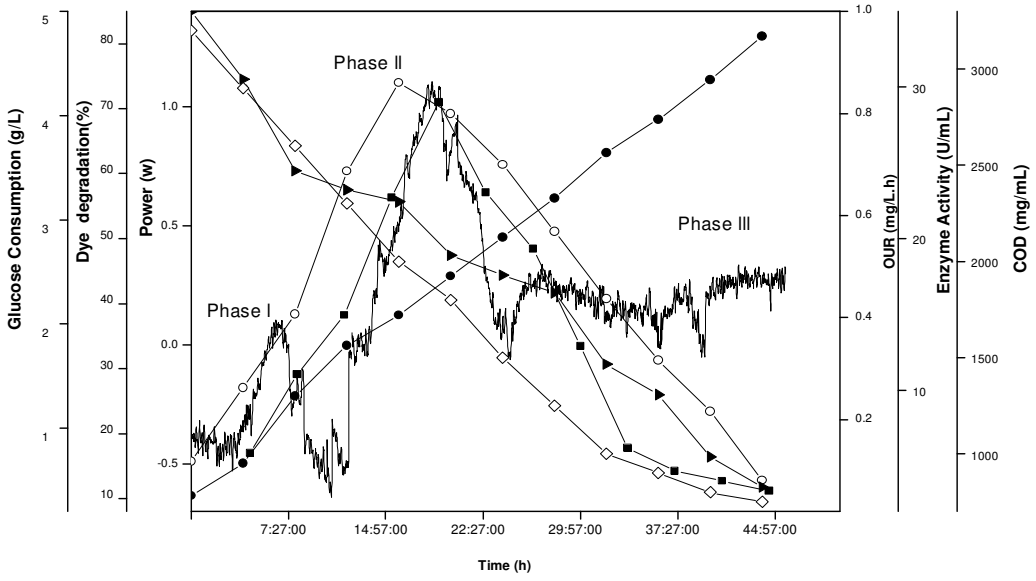


Figure 7.12 Comparative profiles of heat production during acid blue 113 degradation by S. lentus at 50 rpm.

(Heat production rate(-), oxygen uptake rate (■), offline process variables (dye degradation (●), Enzyme Activity (○), substrate consumption(▼) and COD (◊))

A cross comparison of results also indicated that optimized aeration and agitation rates were vital in bioprocess. A comparative biocalorimetric study along with other offline process variables such as substrate consumption and dye degradation helped in an insight into the bioprocess.

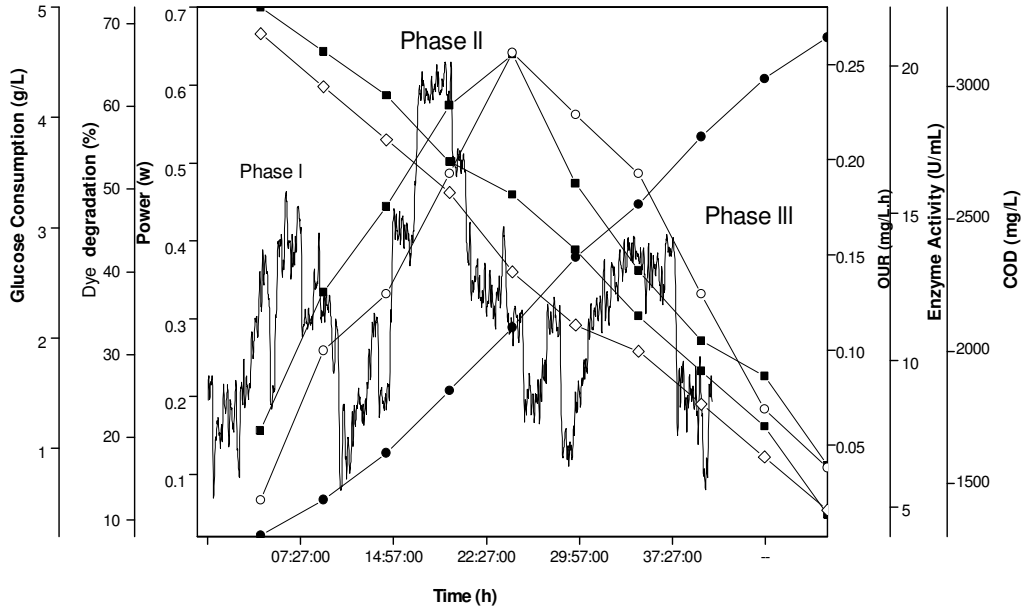


Figure 7.13 Comparative profiles of heat production during acid blue 113 degradation by S. lentus at 150 rpm.

(heat production rate(-), oxygen uptake rate (■), offline process variables (dye degradation (●), Enzyme Activity (○), substrate consumption(▼) and COD (◊))

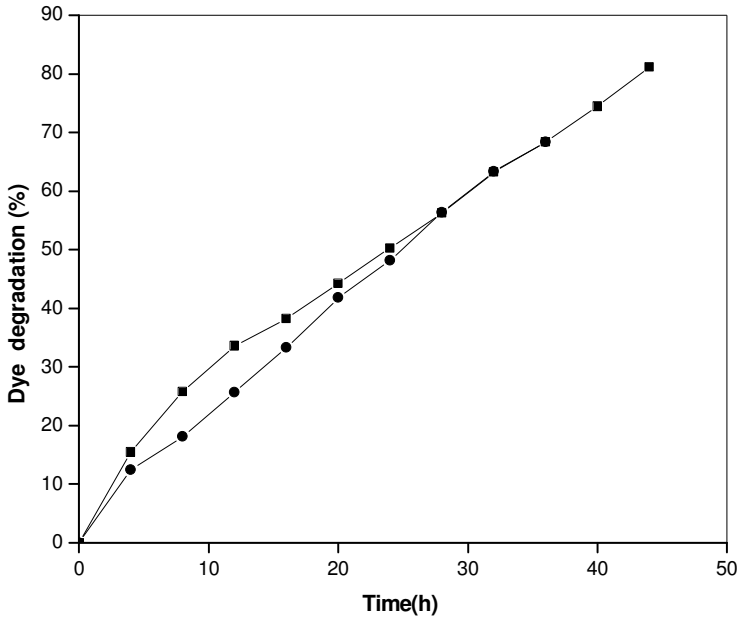


Figure 7.14 Influence of agitation rates on Acid blue 113 degradation efficiency by S. lentus.

(Agitation rates (rpm), (Dye degradation (%)): ■ – 50 (81%), and ● – 150 (68%))

7.4.4 Bioenergetics Due to Dye Degradation

In Figure 7.9, the comparative profiles of power-time, OUR, substrate consumption, enzyme activity, % dye degraded and COD removal for acid blue 113 degradation by of S. lentus performed at optimized conditions. A close examination of the power-time profile in Figure 7.9 reveals the three distinct regions, lag, exponential and the decaying phase, of the bioprocess. During the lag phase (0-14.40 h), the bacteria initially utilized the limiting substrate glucose for its growth and later shifted to the dye. During the exponential phase (14.44 -22.30 h), although simultaneous utilization of glucose and dye degradation were noticed, the slope of glucose and % dye degradation lines do indicated that the dye degradation was predominant. As the bioprocess progressed, glucose consumption rate was slow in comparison to our previously reported growth studies (Chapter 4), and

even after 40 hours, only 60 % glucose was consumed. Again after the 45th hour, the bacteria reversed the trend to consume glucose faster. The exponential phase was thus marked by simultaneous utilization of glucose and dye degradation. It was noticed that the azo reductase enzyme release enhanced the dye degradation resulting in peak heat release. After 50% dye degradation, marked by heat release and OUR, the enzyme release also decreased. Heat release and OUR pattern followed each other as reported (Senthilkumar et al 2008 b).

As can be seen from the comparative graph, the power time curve follows OUR pattern. There are three shifts in the heat curve due to (a) utilization of glucose as primary carbon source initially, (b) simultaneous utilization of glucose and dye, finally (c) dye degradation. Glucose consumption decreased as enzyme activity increased up to 40 U/mL; simultaneously dye degradation increased to 85 % and COD decreased. The results suggested that power-time profile could be used as an indirect parameter to measure COD removal and the application of calorimetry for monitoring the dye degradation process was feasible. Quantitative information on relative consumption of substrates, energy changes associated with biomass growth and oxycalorific values were evaluated using cumulative heat production values by integrating the power – time curve. The results are discussed below.

7.4.5 Biomass Yield

Heat yield coefficient due to biomass, $Y_{Q/X}$ (kJ heat evolved per g cell dry weight formed) was determined from the plot between total heat evolved by the culture (kJ/L and the biomass concentration (g/L). For dye degradation process under varying aeration and agitation the values ranged from 10-13 kJ/g (Table 7.2). Our results corroborated the earlier findings (Vonstockar et al 1989).

7.4.6 Oxycalorific Coefficient

Oxycalorific coefficient was important to asses both the metabolic efficiency and the aerobic nature of the organism under study. The theoretical value for oxycalorific heat yield was 460 kJ/mol of oxygen consumed irrespective of the nature of the microorganism, in substrate or product (Cooney et al 1968). Several researchers have (Volesky et al 1982), claimed $Y_{Q/O} >$ 400 kJ/mol to be the behind the pure aerobic nature of the process. However in our studies we obtained values from 368 to 651.2 kJ/mol (Table – 7.2) for different aeration and agitation rates. The deviation from the theoretical values was reported to be due to the degree of aerobicity, partly anaerobic nature of the organism well as to the inability to precisely measure the low OUR occurring at low values of substrate reductions. In OUR studies under optimized conditions, the $Y_{Q/O}$ value was 460 kJ/mol.

7.4.7 Heat Yield Due to Substrate Consumption

Determining the heat yield due to substrate consumption (kJ heat evolved per g of glucose consumed) could be helpful in understanding metabolism of S. lentus. Quite interestingly, in our experiments the organism was consuming glucose and dye for the entire bioprocess duration. Assuming the growth to be mainly due to glucose , a simple comparison of the heat yields due to substrate and biomass indicated that catabolic activity contributed maximum heat evolution ($Y_{Q/S} > Y_{Q/X}$). Generally for an aerobic process, when glucose acted as the substrate this value would be around 20 kJ/g. The value obtained now was higher in all cases and particularly at optimized conditions 25.4 kJ/g. The reason for such a high value and low biomass values for 0.33 vvm and 1 vvm could be the shift of limiting carbon source from glucose to the dye (Table 7.2). The detailed bioenergetics studies presented here might help resolve the issues in design and scale up of a suitable bioreactor for commercial dye degradation process.

Table 7.2 Influence of aeration and agitation rates on bioenergetics during acid blue 113 degradation by S. lentus in BioRTCal

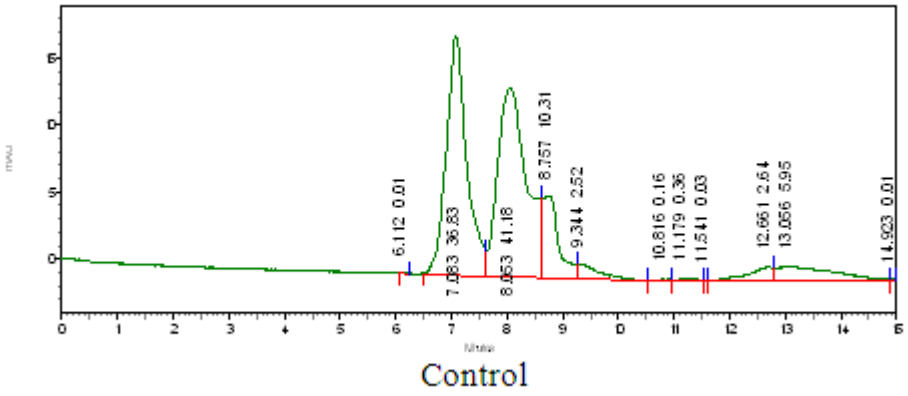
Experiments	$Y_{Q/X}$ (kJ/g)	$Y_{Q/S}$ (kJ/g)	$Y_{Q/O}$ (kJ/mol)	$Y_{Q/cod}$ (kJ/mg)
0.33 vvm / 100 rpm	11.03	17	508.6	7.97
0.66 vvm/100 rpm	13.27	25.4	460	12.2
1 vvm/100rpm	10.16	14	651.2	10.3
50 rpm	12.33	20	426	11.09
150 rpm	9.12	13.2	368	5.43

7.4.8 Analysis of Decolorized Product of Acid Blue 113

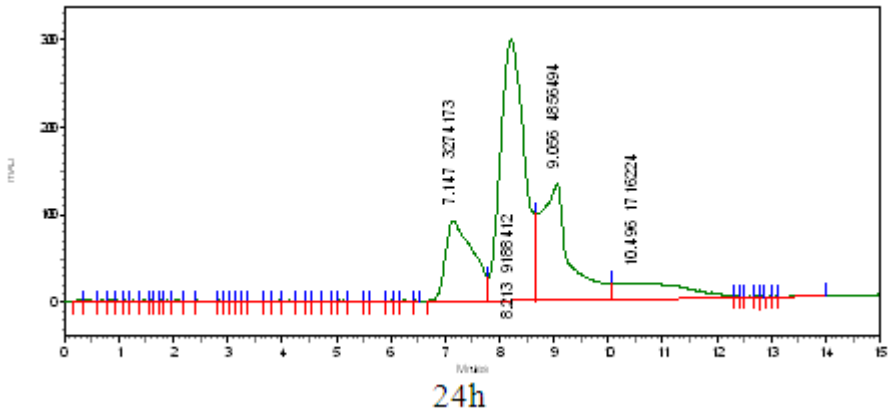
So far there are no reported studies on the elucidation of degradation pathways of acid blue 113 although carried out in other azo dyes such as RHE 7B (kalyani et al 2008), Red BL1 (Satish et al 2007) RO (Sarayu et al 2010), RY107, RB5, RR198 and DB71 (Elisangela et al 2009), reactive blue 172 (Dhanve et al 2008) and acid red GR (Xu et al 2007).

7.4.9 HPLC Analysis

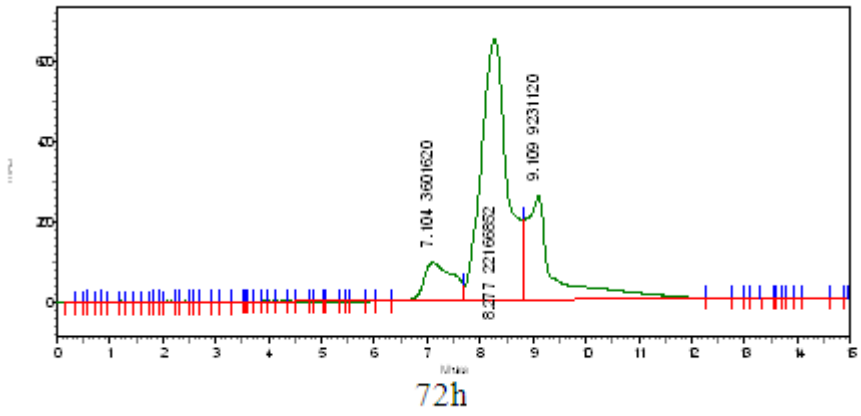
The HPLC Chromatogram of the degraded samples collected from BioRTCal at the 24 h and 72 h along with the pure dye is in Figure 7.15. The peak at 7.03 RT in Figure 7.15 (a) was due to the dye molecule (Xueheng et al 2007). As the degradation process went on, the intensity of the peak at 7.03 RT decreased. HPLC studies confirmed the efficiency of S. lentus to degrade the complex molecule of Acid blue 113.



(a)



(b)



(c)

Figure 7.15 HPLC Chromatogram showing the progress of dye degradation (a) pure dye (b) 24th h (c) 72nd h

7.4.10 FT-IR Analysis

The FT-IR spectrum of the dye acid blue 113 is shown in Figure 7.16. The samples withdrawn from shaker and BioRTCal at 72 h of inoculation during the degradation of acid blue 113 showed an identical FT-IR (Figure 7.16 (b)) spectrum. Comparison of FTIR spectra of dye (Figure 7.16 (a)) with extracted samples at 72nd h, showed the appearance of new peaks at 1328.6, 1641.1, and 2394.2, expressed breakdown of the parent dye by S. lentus. The bands characteristic of azo bond (Dhanve et al 2008) were absent in the degraded 72 h sample indicating azo reduction mechanism (breaking of N=N) occurrence in the bioprocess (Sarayu et al 2010). In comparison with the pure dye FT-IR specra, the absorption intensities in the region 3300-3500 1/cm is high and broadened in the treated

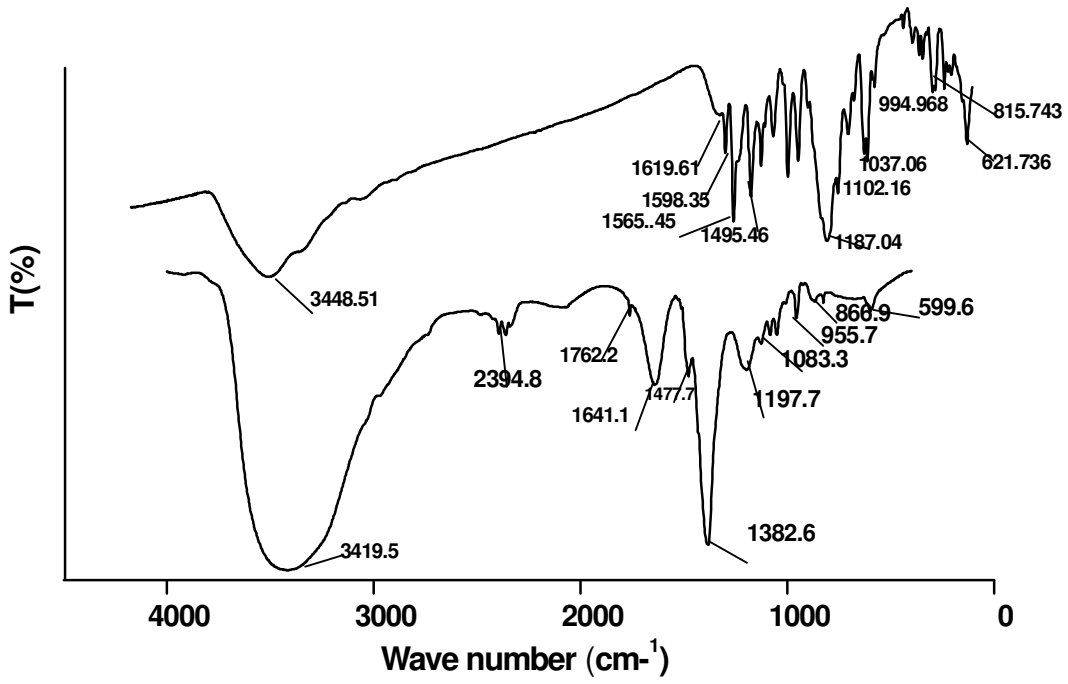


Figure 7.16 FT- IR Spectrum of (a) pure dye, (b) degradated sample collected at 72 h sample

sample, and this indicated the increase in the number of OH and NH groups as a result of biodegradation. Broadening of IR spectra is found to be due to destruction of the conjugated, aromatic structure of the dye upon microbial treatment (Pourbabee et al 2006). A peak appearing at 1641.751/cm showed the presence of compounds containing C (NH)=O. Also peaks at 1382.6 1/cm represented N-H bending vibrations. Thus the FTIR spectrum of the sample after decolourization showed characteristic change in peak positions as compared to pure dye sample.

7.4.11 GC-MS Analysis

7.4.11.1 Decolorized Product from Shake Flask Experiments

The products identified from the GC-MS trace spectra (Appendix 4) are listed in Table 7.3. The degradation pathway shown in Figure 7.17 was worked out based on the product profile listed in Table 7.3. 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 aeruginosa (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 monooxygenases 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.

Table 7.3 Major compounds identified from GC - MS studies during acid blue 113 degradation by S. lentus in shake flask

Retention time (min) (72 h sample)	m/z	Prominent compound formed
8.22	128	Naphthalene
9.35	226	Hexadecane
11.25	198	Tetradecane
12.19	268	Nonadecane
12.62	240	Heptadecane
18.32	254	Octadecane
19.52	166	Pthalic acid
21.30	278	Dibutyl Pthalate
32.18- 33.92	282	Palmitic acid vinyl ester
33.51- 33.92	93	Benzenamine

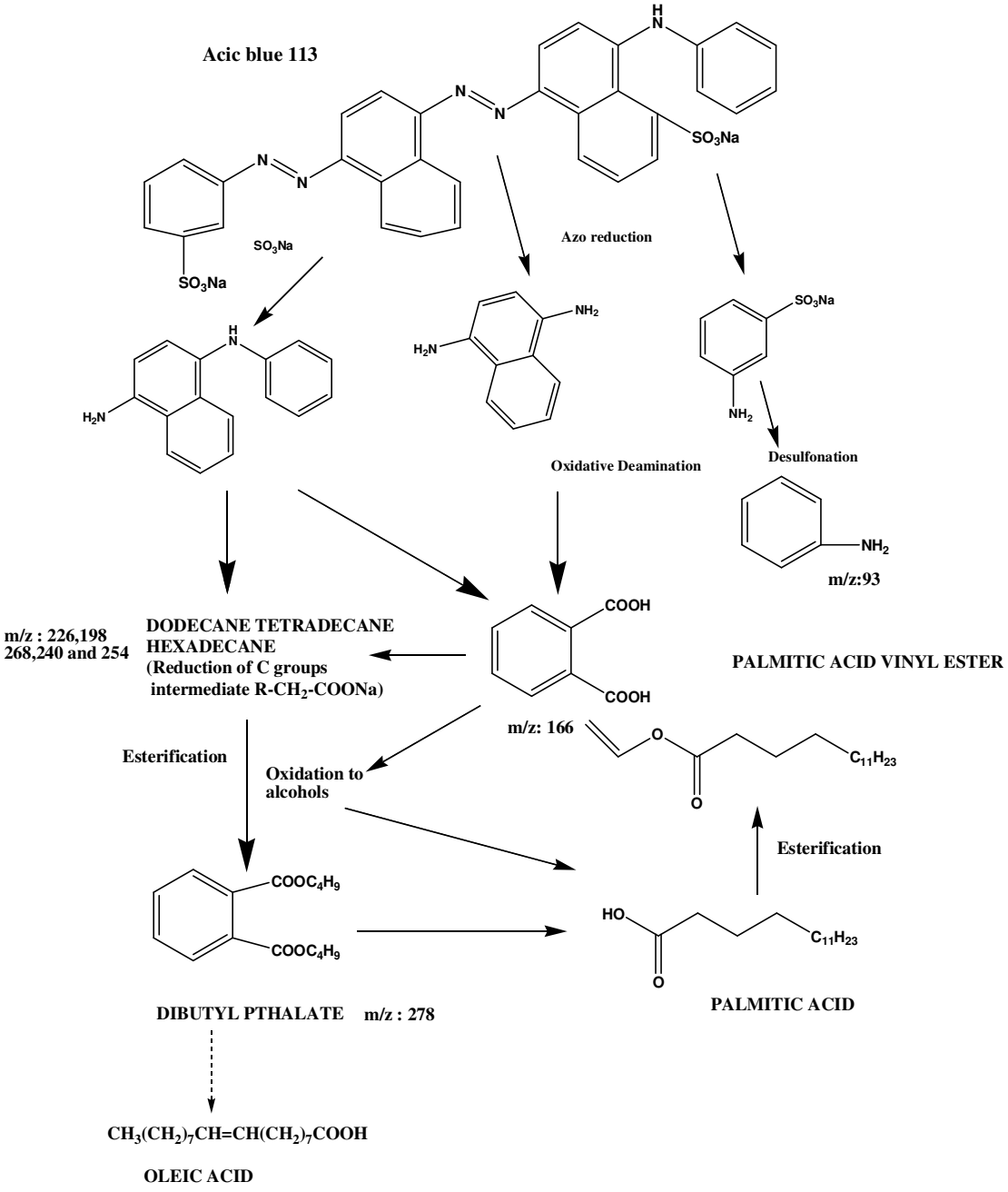


Figure 7.17 Degradation pathways of acid blue 113 by S. lentus in shake flask

7.4.12 Decolorized Product from BioRTCal Experiments

The predominant degradation products identified from the GC-MS trace spectra (Appendix 5) from the sample collected at 72 h of the inoculation are listed in Table 7.4. The degradation pathway (Figure 7.18) was worked out based on the product profile listed in Table 7.4. The dye degradation did undergo azoreduction to yield fragmented aromatic products consisting of three, two and one aromatic rings (Neill et al 2006), Intermediates such as pthalic acid were obtained followed by corresponding esters such as dibutyl phthalate. This could possibly due to formation of long chain hydrocarbons followed by oxidation to alcohols. Aniline and pthalic acid undergo oxidative deamination to resorcinol using molecular oxygen and oxygenases that undergo further extra- ring oxidation to yield products such as 1,3,5-benzenetriol.

The nitrogen moiety gets sequestered as benzeneamine via desulphonation (progesterone, oleic acid) The presence of diazo derivative of progesterone in the GC-MS analysis can be explained by the above mechanism . The degradation of primary alcohol is carried out by the alcohol-aldehyde dehydrogenase pathway to obtain the corresponding fatty acid which explains the biosynthesis of oleic acid (Eduardo Diaz 2004). These enzymes usually have a bound NAD (nicotine amine adenine dinucleotide), and are induced by the presence of hydrocarbons (Robert et al 2003). Examination of the degradation pathway undergone in shake flask and BioRTCal, revealed that only partial oxidation of the dye molecule was possible in shake flask experiments, although % decolourization was satisfactory. In BioRTCal experiments the oxidative reactions continued even after the primary azo reduction reactions leading to the biosynthesis of diazo derivatives of progesterone and less toxic products. A vast literature on

biological degradation of various dyes employing different kinds of microorganism is available and one gets confused with the utility of the data. It is because the studies are not comprehensive; in most of the reported studies, either the chemical characterization of the metabolites of the dye nor toxicity assessments are done to prove that the products of degradation do not contain a toxic metabolite. From this perspective the present study is comprehensive to prove that the product of degradation is less toxic in nature.

Table 7.4 Major compounds identified from GC - MS studies during acid blue 113 degradation by S. lentus in BioRTCal

Time(h)	Retention Time (min)	m/z	Prominent Compound Formed
24	16.13	166	Pthalic Acid
24	8.06	198	Tetradecane
24	17.91	278	Dibutyl Pthalate
36	30.20	282	Oleic acid
72	6.40	110	Resorcinol
72	12.39	126	1,3,5, Benzenetriol
72	33.26	340	Diazoprogesterone
72	34.4	144	2-naphthalenone

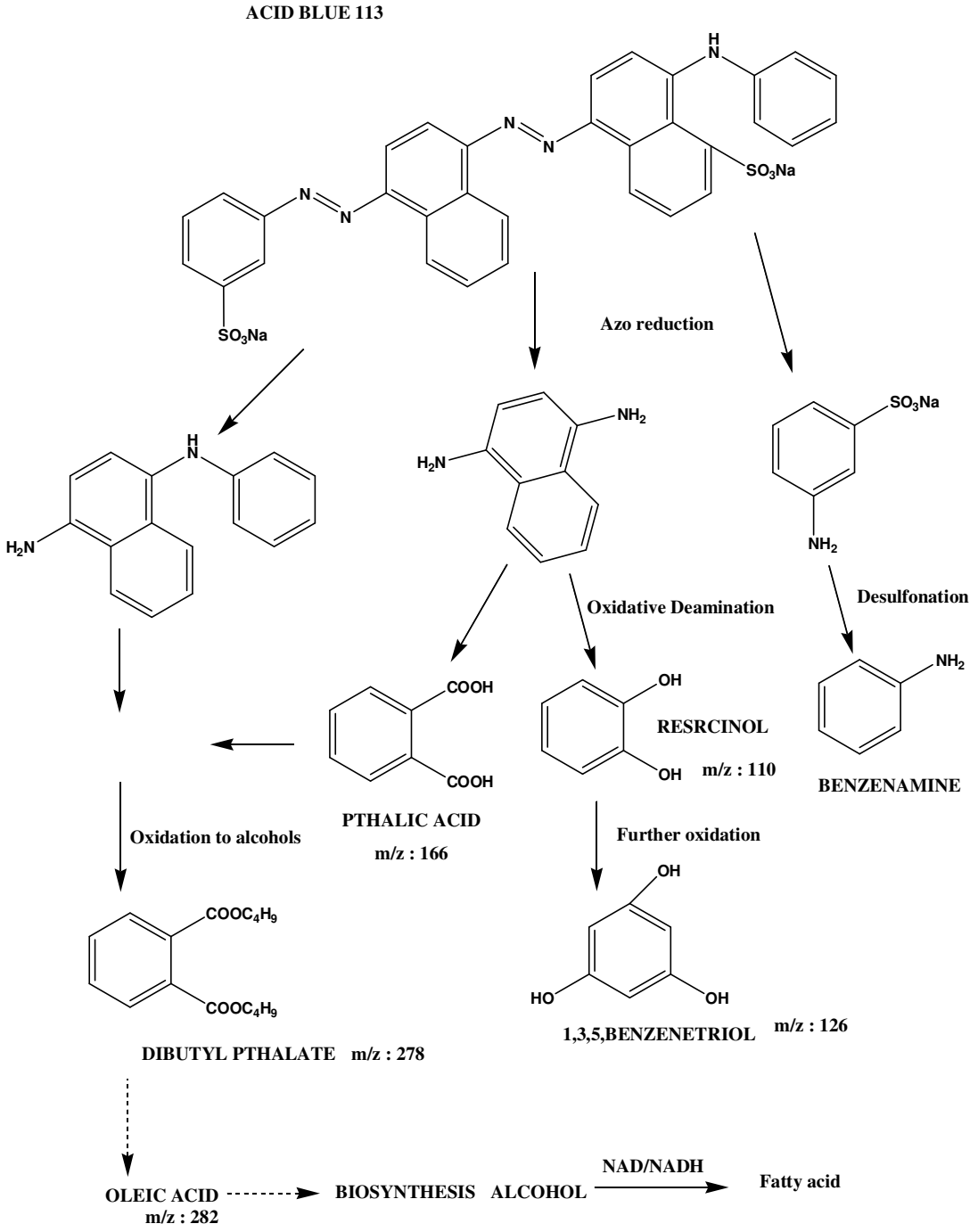


Figure 7.18 Degradation pathways of acid blue 113 by S. lentus in BioRTCal

7.4.13 Heat of Reaction for the Degradative Pathway of Acid Blue 113

The bio-degradation of the acid blue 113 dye was carried out in the bio-reaction calorimeter using the bacterial strain S. lentus. Here glucose was used as the carbon source. Using the CHN analysis the empirical formula of S. lentus was determined. The molecular structures of compounds in the degradation pathway (Figure 7.19) were drawn in CHEMDRAW software version 7.3 and incorporated into CHETAH software (Shanley et al 1995) using smiley input. The experimental heat of reaction values obtained from BioRTCal (7586.3kJ/mol) was due to simultaneous growth of the organism and dye degradation. The heat of reaction values (7545.3 kJ/mol) determined from the growth experiments performed at identical conditions were subtracted from the dye degradation experiment to obtain the heat of reaction for degradation alone (7258.23kJ/mol) The values were found to be close and thus further validated the mechanism of acid blue 113 degradation.

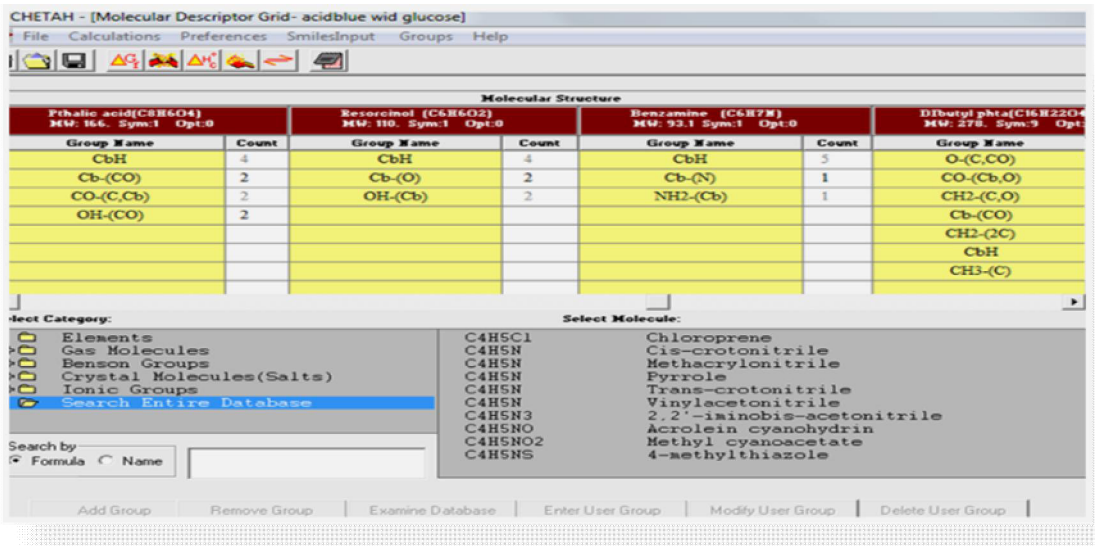


Figure 7.19 CHETAH for biodegradation of acid blue 113

7.4.14 Cytotoxicity Assessment

Figure 7.20 the percent viability of Vero cells to the toxins are shown. The IC50 values for the samples were found out 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 (Adedayo et al 2004), it was thought appropriate to consider the IC90 values to assess the toxic nature. The IC 90 values (78 mg/mL) shows 91 % 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 less due to dilution of large quantities of water. Oxidation of aromatic amines in the aerobic stage was found to be responsible for less toxicity of the extracts (Elisangela et al 2009).

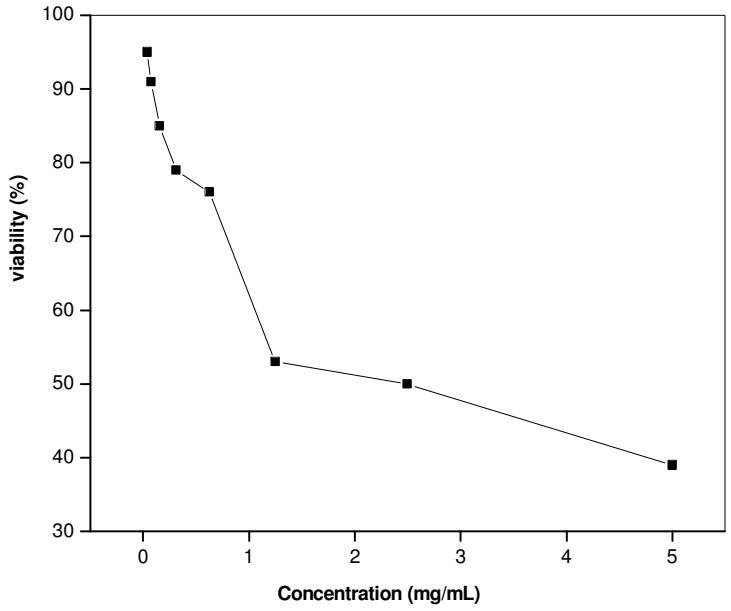


Figure: 7.20 MTT assay showing the cytotoxicity of Vero cells

7.5 CONCLUSION

- Dye degradation of Acid blue 113 by the bacteria S. lentus was successfully performed in a BioRTCal for the first time.
- Power-time and OUR curve exhibited similar trends suggesting that profiles could be used for monitoring the biological degradation of color effluents.
- The shifts observed in power- time profile indicated three distinct phases of the bioprocess, and suggested simultaneous utilization of glucose (primary) and dye (secondary carbon source). Secretion of azoreductase enzyme enhanced the degradation process.
- Optimization of aeration and agitation rates were found to be vital for efficient dye degradation. The degradative pathway of acid blue 113 by S. lentus was worked by performing HPLC, FT-IR, and GC–MS analysis.
- Interestingly, the predominant products identified were less toxic and the final end product was found to be Diazoprogesterone.
- The biochemical energetics and the detailed mechanistic pathway presented will be useful for designing a suitable bioreactor for degradation of dye effluent.
- Moreover, this study proves the feasibility of application of calorimetry as an in-line analytical tool for monitoring dye degradation process. Biocalorimetric data for dye degradation have been reported for the first time here.