CHAPTER 2

LITERATURE

The main focus in the thesis is to investigate the biocalorimetric and respirometric responses during the acid blue 113 degradation by an identified halotolerant bacterial strain. The investigation requires that the current status of knowledge on three major areas viz. aerobic degradation of azo dyes, biocalorimetric studies and application of ionic liquids in biological processes are reviewed before scope and objectives are deduced for the present work. The contents of literature survey include.

2.1 THE CONTENTS OF LITERATURE SURVEY

- Halotolerant bacteria
- Aerobic degradation of Azo dyes
- The concept of Biocalorimetry
- Application of Ionic Liquids as nutrients in biological processes
- The Leather Industry and its effluents
- Summary

2.2 HALOTOLERANT BACTERIA

Some of the halotolerant species employed were Vibrio. Sp., Pseudomonas sp., Staphylococci and E.coli (Kubo et al 2001). The saline

wastewater from various industries (wine making and agri-food industry, pickled plum production plant and tartaric industry) has been also treated successfully (Vlyssides et al 2005) through biological treatment. However, there is no reported information on the use of halotolerant bacteria for the treatment of saline waste stream form leather industry. Some of the above reported halophiles strains are high saline tolerant (about 25 % NaCl by wt) and more virulent in nature. Furthermore, azo dye degrading microbial communities are sensitive to high concentrations of salts that are used in the dye process (Carliell et al 1994 and Manu et al 2003). This can limit growth and activity of the degrader bacteria such that the process treatment times become impractical. As far as tannery wastewater is concerned, the salinity ranges from 0.1 % - 0.5 % by NaCl (w/v). Halotolerant strains are sufficient to treat the organics, and xenobiotics present in the wastewater. Such work has not been attempted yet.

2.3 AEROBIC DEGRADATION OF AZO DYES

2.3.1 Degradation of Azo Dyes

Treatment of dye-contaminated wastewater discharged from the leather, textile and other dye-stuff industries is necessary to prevent contamination of soil and surface and ground water. Currently, there are several physicochemical and biological methods for the removal of dyes from effluents (Alinsafi et al 2007, Arslan-Alaton 2007, Behnajady et al 2004, Brosillon et al 2008, El-Gohary et al 2009, Golab et al 2005, Hao et al 2007, Lux et al 2009, Moustafa et al 2005, Pearce et al 2003, Saxe et al 2006 and Wang et al 2004). Among these, biotechnological approaches are receiving increased attention worldwide as Eco-friendly methods that are becoming increasingly efficient and cost-effective for the remediation of dye-contaminated wastewater (Dubrow et al 1996 and Khalid et al 2009). Many biotreatment systems rely on the use of sludge as an inoculum to initiate the

dye degradation process (Bromley - challenor et al 2000, Ekici et al 2001, Pazdzior et al 2009 and Worch et al 2002). While generally effective, it is nonetheless important to assure complete mineralization and detoxification for use as a reliable treatment method. Azo dyes and their degradation intermediates vary in their recalcitrance to biodegradation due to their complex structures and xenobiotic nature and in some cases are both mutagenic and carcinogenic (Catwright et al 1983, Chung and cerniglia 1992, Makinen et al 1993, Miller et al 1983, Ozturk et al 2001, Pinheiro et al 2004, Saupe et al 1999 and Weisburger et al 2002). Furthermore, azo dye degrading microbial communities are sensitive to high concentrations of salts that are used in the dye process (Carliell et al 1994 and Manu et al 2003). This can limit growth and activity of the degradation bacteria such that the process treatment times become impractical.

With the discovery and isolation of very efficient, halotolerant azodye degrading bacteria, biotreatment systems with specific microbial strains has now become an effective strategy to improve wastewater treatment systems and to enhance the bioremediation of azo dyes (Boon et al 2000, McClure et al 1991, Rittman et al 1994, van Limbergen et al 1998 and Yu et al 2001). Although activated sludge is used in the process of treating the contaminants, the microbial species that are contained in this material are uncharacterized and the system is a “black box” (Dabert et al 2002). This may lead to inconsistent results, such that in some cases, up to 90% of the dyes in an effluent can remain untreated after an activated sludge process (Lucas et al 2006). On the other hand, activated sludge can provide a useful starting medium from which individual strains or consortia can be isolated and cultured for use as inoculants (Khalid et al 2009, Chunli et al 2008, Elisangela et al 2009, Francison et al 2009, Khalid et al 2008a and Khalid et al 2008b). The microbial species and consortia can then be studied to determine the environmental factors that affect their growth and the rate of degradation. Often the effectiveness of individual isolates can be enhanced by co-culture

with other highly efficient dye-decolorizing strains (Chen et al 2006, Qu et al 2006 and Qu et al 2005). Here, it is speculated that the combined enzyme systems of the mixed bacterial culture are more effective than the enzymes from the individual isolates, each of which may have different substrate kinetics and efficiency at different dye concentrations. Cooperation within microbial communities also can occur through exchange of growth cofactors and removal of toxic metabolites. Before individual isolates can be recommended, comprehensive research is required to understand the role of individual microorganisms and their interactions with other microflora (Pinheiro et al 2004, Dabert et al 2002 and Wagner et al 2002).

Several studies have demonstrated partial or complete degradation of dyes by pure and mixed cultures of bacteria (Table 2.1). In many biotreatment systems, mixed bacterial cultures have proved to be superior to single pure cultures. It has been reported that a higher degree of azo dye biodegradation might be achieved by mixed bacterial cultures due to complementary catabolic pathways within the microbial community that may not be accomplished by individual pure strains (Khehra et al 2005a, Khehra et al 2005b, Moosvi et al 2007, Chang et al 2004, He et al 2004 and Nigam et al 1996). Recently, several researchers have identified single bacterial strains that have very high efficacy for removal of azo dyes (Khalid et al 2009, Khalid et al 2008a, Khalid et al 2008b, Xu et al 2005, Pearce et al 2006 and Hong et al 2007). In contrast to mixed cultures, the use of a pure culture has several advantages. These include predictable performance and detailed knowledge on the degradation pathways with improved assurance that catabolism of the dyes will lead to nontoxic end products under a given set of environmental conditions. Another advantage is that the bacterial strains and their activity can be monitored using culture-based or molecular methods to quantify population density of the bacteria over time. Knowledge of the population density can be extrapolated to quantitative analysis of the kinetics of azo-dye decoloration and mineralization.

Table 2.1 Bacterial species capable of degrading azo dyes

Bacterial species	Dyes	Comments	References
Acinetobacter calcoaceticus NCIM 2890	Direct brown MR (DBMR)	Decolorization of DBMR was 91.3% in static anoxic condition, whereas agitated cultures showed less decolorization (59.3%) after 48 h	Ghodake et al 2009
Acinotobacter sp., Citrobacter freundii, Klebsiella oxytoca	Acid Red 88, Reactive Black 5, Direct Red 81, Disperse Orange 3	The mixed culture of bacteria removed 88–100% dyes (100 mg L^{-1}) in 10 h	Khalid et al 2009
Aeromonas caviae, Proteusmirabilis, Rhodococcus sp.	Acid Orange 7	More than 90% decolorization of the dye was achieved in 16 h	Joshi et al 2008
Aeromonas, Pseudomonas,Bacillus, Shewanella and Massillia spp.	Reactive Black 5, Direct Red 81, Acid Red 88 Disperse Orange 3	Treatment times required by the most efficient strain, AS96 (Shewanella putrefaciens), were as short as 4 h for complete decolorization of 100 mg L^{-1} of AR-88 and DR-81 dyes under static conditions, and 6 and 8 h, respectively, for complete decolorization of RB-5 and DO-3	Khalid et al 2008a and Khalid et al 2008b
Bacillus cereus DC11	Acid Blue 25, Malachite Green, Basic Blue	High decolorization efficiency (95-98%) achieved within 6 h for 100 mM Acid Blue 25 (anthraquinone dye), 4 h for 55 mM Malachite Green (triphenylmethane dye), and 2 h for 750 mM Basic Blue X-GRRL under anaerobic conditions	Deng et al 2008
Bacillus fusiformis	Disperse Blue 79, Acid Orange 10	The dyes were completely mineralized within 48 h	Kolekar et al 2008
Bacillus sp.	Congored	The complete decolorization was achieved in 24–27 h for a concentration of 100–300 mg dye L^{-1}	Gopinath et al 2009
Bacillus subtilis HM	Fast Red	Under the near-optimal conditions, 99% of the decolorization was achieved in 6 h	Mabrouk et al 2008
Bacillus thurengiensis	Acid Red 119	The dye was decolorized up to 70% in 24 h	Dave et al 2009
Bacillus velezensis AB	Direct Red 28	The dye (25 mg L^{-1}) was completely decolorized within 10 h.	Bafana et al 2008a
Citrobacter sp. CK3	Reactive Red 180	About 95% dye (200 mg L^{-1}) was removed in 36 h	Wang et al 2009
Enterococcus Gallinarum	Direct Black 38	The bacterium removed 53–63% of the dye in 24 h in minimal medium while 71–85% of decolourization was observed in Luria broth medium.	Bafana et al 2008b
Escherichia coli NO3	Reactive Red 22	After acclimation, time for 50% color removal lowered from 5.7 to 4.3 h	Chang et al 2000
Escherichia coli, Pseudomonas sp.	Congo Red, Direct Black 38	The complete decolorization was achieved at the end of 9 days of incubation in case of E. coli while Pseudomonas sp. decolorized in 5 days	Isik et al 2003
Eschericia coli YB	Acid Red 27	The dye was decolorized up to 75% in 2 h	Liu et al 2009
Halomonas sp.	Reactive Brilliant Red X, Acid Black 10B, Acid Scarlet GR, Acid Red B, Acid Red G, Reactive Brilliant Red K	The decolorization of the dyes was up to 90%in 24 h	Guo et al 2008a, Guo et al 2008b and Guo et al 2008c
Kerstersia sp. VKY1	Amaranth, Fast R, Ponceau S, Congo R, Orange II, Acid O 12, Acid R 151	The first four dyes decolorized by the bacterium by 100% while the remaining three decolorized by 84, 73 and 44%, respectively, in 24 h	Vijayakumar et al 2007

Table 2.1 (Continued)

Bacterial species	Dyes	Comments	References
Klabisiella sp. VN-31	Reactive Yellow107, Reactive Red 198,Reactive Black 5,Direct Blue 71	Monoazo dyes RY107 and RR 198 were decolorized in 72 and 96 h; the diazo dyes (RB5 and triazodye DB71) decolorized in 120 and 168 h	Chunli et al 2008
Lactobacillus casei TISTR 1500	Methyl Orange	The complete decolorization of the dye was achieved in 2.5 h	Seesuriyachan et al 2007
Paenibacillus polymyxa, Micrococcus Luteus	Reactive Violet 5R	The bacterial consortium showed complete decolorization in 36 h	Moosvi et al 2007
Proteus vulgaris, Micrococcus Glutamicus	Scarlet R	Bacterial consortium decolorized 90% dye in 3 h	Saratale et al 2009
Pseudomonas Luteola	Reactive azo dyes, Direct azo dyes and leather dyes	The 59–99% color removal after 2–6 days static incubation, at dye concentration of 100 mg L^{-1}, monoazo dyes showing fastest rate of decoloration	Hu 2001
Pseudomonas aeruginosa, P. oleovarons, P. putida	Methyl Orange, Y87, B86, R91, B19, R90, B69, B31, B36, Y15, R34, B15, Y79, and B54	P. aeruginosa showed decolorization efficiency over 98% after 48 h while 76% decolorization was achieved by P.oleovarons after 54 h. P. putida showed lower efficiency	Silveira et al 2009
Pseudomonas Desmolyticum	Direct Blue 6, Green HE4B, Red HE7B	The dye GHE4B was completely decolorized in 12 h while DB 6 and RHE7B were decolorized in 16h	Kalme et al 2009
Pseudomonas luteola, Eschericia coli	Reactive Red 22	The E. coli improved the ability of Pseudomonas sp. to decolorize the dye by producing decolorization – stimulating extracellular metabolites	Chen et al 2006
Pseudomonas putida mt-2	Acid Violet 7	Complete biodegradation of azo dye up to 200 mg L^{-1} was achieved in 49 h under shaking while the biodegradation time was reduced to 37 h under static conditions	Ben-Mansour et al 2009
Pseudomonas sp. SUK1	Reactive Red 2	The strain was capable of degrading dye in a wide range of concentration (up to 5 g L^{-1}) and almost 80% dye was removed in 114 h	Kalyani et al 2009
Rhodopseudomonas Palustris	Reactive Black 5	The dye up to 700 mg L^{-1} concentration was complete decolorized in 40 h	Wang et al 2008
Shewanella decolorationis S12	Fast Acid Red GR	After 4 h incubation, more than 90% of the color was removed under anaerobic conditions while 12.8 and 33.7% decolorizing rates were observed under aerobic and microaerophilic conditions	Xu et al 2007
Shewanella decolorationis sp. nov. S 12^T	Fast Acid Red GR, Reactive Brilliant Blue	The 90% decolorization of the dyes was achieved within 12 h	Xu et al 2005
Shewanella J18 143	Remazol Black B, Acid Orange 7	Anaerobic cultures of Shewanella strain J18 143 rapidly removed color from the azo dye Remazol Black B in the growth medium to produce an absorbance at 597 nm of less than 1 in under 40 min	Pearce et al 2006
Sphingomonas herbicidovorans	Anthraquinone dyes	The bacterium was capable of decolorizing bromoamine acid dye (1,000 mg L^{-1}) more than 98% within 24 h	Fan et al 2009

Similarly, the ability of bacteria to remove the color of azo dyes from solid agar medium suggested the accumulation of redox active enzymes or biochemical substances that were released into the medium during growth of the bacterial cells (Khalid et al 2008a). These studies imply that reducing equivalents are transferred from an intracellular electron transport chain to the mediators, which consequently reduces the extracellular dye non-enzymatically shown in Figure 2.1. Another possibility is that the bacteria establish a link between their intracellular electron transport systems and the extracellular dye via electron transferring proteins in the outer shell.

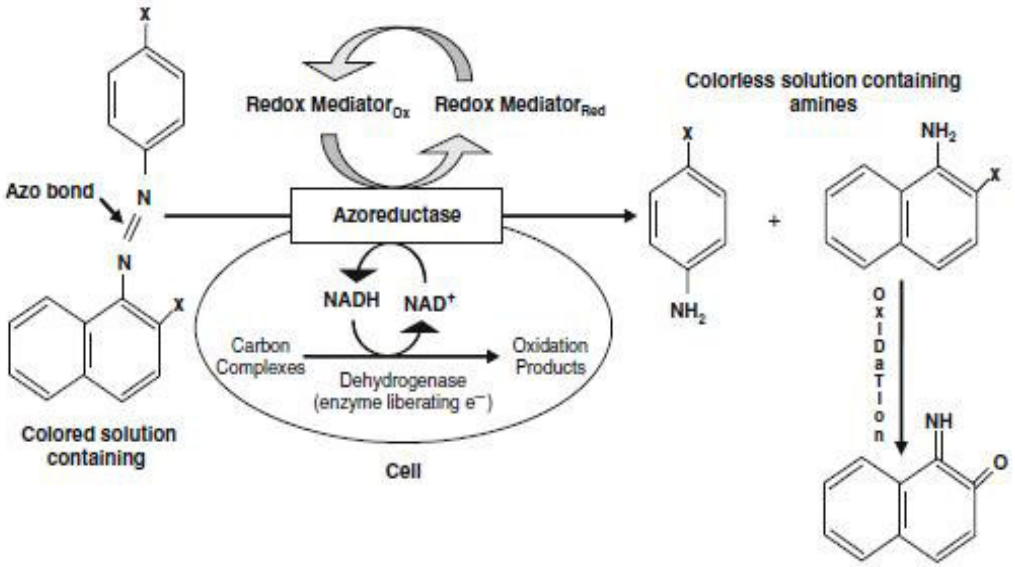

Figure 2.1 Possible mechanisms for the removal of Azo dyes by bacteria (Pearce et al 2006)

Since the azo dye does not yield carbon or energy for growth during the first stage of enzymatic attack, various organic compounds (cosubstrates) are required for the dye decolorization step, in which the dyes act as acceptors of electrons that are supplied through the reducing equivalents that are generated by the electron transport chain (Brige et al

2008). Azo dye decolorization by mixed as well as pure cultures, generally, requires organic sources such as glucose, starch, acetate, ethanol, peptone yeast extract, or a combination of complex organic sources and carbohydrates (Khalid et al 2008a, Chen et al 2006, Khehra et al 2005a, Khehra et al 2005b, Moosvi et al 2007, Xu et al 2007, Xu et al 2005, Chen et al 2003 and Brige et al 2008). As a result of the anaerobic reduction step, a variety of colorless aromatic compounds are synthesized. Depending on their chemical properties, these metabolites will accumulate under the anaerobic conditions, in which case further degradation can be achieved at accelerated rates under aerobic conditions (Khalid et al 2009 and Pearce et al 2006). Previously, (Kudlich et al 1999) reported that such types of compounds undergo rapid oxidation reactions, forming a range of polycyclic intermediates. In this manner, a sequential anaerobic–aerobic system is preferred for complete degradation of dyes (Khalid et al 2009, Khalid et al 2008a and Xu et al 2007). The cleavage of azo linkages is not specific under anaerobic conditions (Bromley - challenor et al 2000, Rafii et al 1993, Rafii et al 1995, Rafii et al 1990, Rafii et al 1992 and Stolz 2001); however, the electron withdrawing nature of the azo linkages impedes the susceptibility of dye molecules to oxidative reaction (Fewson 1988) and, thus, azo dyes show resistance to aerobic biodegradation (Ganesh et al 1994, Pagga et al 1986, Pagga et al 1994 and Shaul et al 1991). Nonetheless, some bacteria with azo dyes-reducing enzymes, both specific and nonspecific, were capable of degrading azo dyes under aerobic conditions (Khalid et al 2008a, Stolz 2001, Ghosh et al 1993, Ghosh et al 1992, Suzuki et al 2001, Zimmermann et al 1984 and Zimmermann et al 1982). A comprehensive study with halotolerant bacteria thus has not yet been attempted and this forms one of the major objectives of this thesis.

2.3.2 Substrate Specificity of Azoreductase for Different Types of Azo Dyes

Azo dyes are a diverse class of chemicals in which various moieties confer a wide range of colors. The number and position of sulfonate and other substituent groups on the azo dye are particular features that affect the rate of decolorization. Hitz et al (1978) illustrated that acid dyes exhibit low color removal due to the number of sulfonate groups present in the dye, while the direct dyes exhibit high levels of color removal, being independent of the sulfonate groups. As illustrated in studies with Lactobacillus casei TISTR 1500, methyl red with a mono-azo bond and lacking a sulfonate group is relatively easily degraded, while acid red 151 and congo red with two azo bonds are difficult to cleave (Seesuriyachan et al 2007). Similarly, the decolorization rates observed in case of acid red and acid orange 8 were lower than those of other dyes containing sulfonate groups (Seesuriyachan et al 2007). The resistance to degradation shown by the latter dyes could be attributed to their complicated chemical structures consisting of polyaromatic and sulfonate groups. This can be attributed to steric interference and increased difficulty for azoreductases to form enzyme substrate complexes with acid red 151 and acid orange 8. Likewise, dyes with methyl, methoxy, sulfo, or nitro groups in their structures and substituent groups in the molecule also affect azoreductase activity (Pearce et al 2003, Blumel et al 2002 and Chen et al 2004).

Nigam et al (1996) suggested that azo compounds with a hydroxyl group or with an amino group are more likely to be degraded at faster rates than those with a methyl, methoxy, sulfo, or nitro groups. Zimmermann et al 1982 suggested some general structural features of dye substrates for reduction by azoreductases. They viewed that a hydroxy group in the ortho position of the naphthol ring is a prerequisite for the azoreductase reaction, and charged groups in the proximity of the azo group could cause hindrance

in the reaction. Similarly, a second polar substituent on the dye molecule inhibits the reaction by lowering its affinity to the enzyme, while the electron withdrawing substituents on the phenyl ring increases the rate of the reaction. The dye reduction rates are also influenced by changes in electron density in the region of the azo group. The substitution of electron withdrawing groups in the para position of the phenyl ring, relative to the azo bond, causes an increase in the reduction rate (Walker et al 1971). Hydrogen bonding, in addition to the electron density in the region of the azo bond, has a significant effect on the rate of reduction (Beydilli et al 2000). It was also shown that sulfonated dyes were reduced faster than carboxylated dyes due to the higher electronegativity of the sulfo group, which renders the azo group more accessible to electrons (Kulla 1981). Likewise, Martins et al (1999) reported that dyes with low polarity and having an electron-donating methyl substituent group in the ring are quite recalcitrant. Thus, it can be concluded that the decolorization of azo dyes is highly dependent on the specificity of azoreductase for different types of azo dyes that affect formation of substrate–enzyme complexes and the ability of the dye to accept an electron and cleave the azo group from the parent molecule.

Enrichment culture is the most common method for isolating azo dye degrading bacteria, using specific dyes individually or in mixtures, where the dyes are provided as the sole source of C or N (Khalid et al 2008a, Silveira et al 2009, Fan et al 2009, Adedayo et al 2004 and Coughlin et al 1999). Such bacteria cleave azo (–N=N–) bonds reductively and utilize aromatic amines as the source of C and N for their growth and they are specific towards their substrate. On the other hand, other bacterial strains cannot utilize dye as the growth substrate (Stolz 2001), but can be isolated using other organic compounds that are added as a co-substrate along with the dye to support their growth. The latter method has led to the isolation of many efficient dye-degrading strains (Khehra et al 2005a, Khehra et al 2005b, Kolekar et al 2008, Wang et al 2009, Kalyani et al 2009 and Kalyani et al

2008), but has the disadvantage that the co-substrate must be added to the wastewater. Depending on the co-substrate, this can increase the cost of the treatment process. Moreover, addition of co-substrates to mixed microbial communities containing undefined mixtures of bacterial species from the environment can lead to competition between the inoculants and other bacteria that degrade the cosubstrate. Following isolation of strains, screening under controlled conditions by conducting repeated trials is critical to identify the most effective dye-decolorizing bacterial strains. Similarly, bacterial strains capable of effectively converting/degrading highly toxic intermediates/byproducts of dyes can be screened. Ideally a strain or consortium that is able to decolorize azo dyes under anaerobic conditions would also be efficient for further degrading the dye intermediates (aromatic compounds) under aerobic conditions.

Finally, strains showing good results under controlled conditions should be tested further for their performance to degrade dyes and their byproducts in a bioaugmented system by co-culturing with bacterial communities from an activated sludge system to determine if the strains are competitive and are able to enhance the dye degradation rates over that which is achieved by a nonaugmented sludge community (Khalid et al 2009).

2.3.3 Bioaugmentation with Azo Dye Degrading Bacteria

Although conventional activated sludge systems are commonly used to treat azo dye containing wastewater (Ekici et al 2001, Boon et al 2000, Guo et al 2008a, Guo et al 2008b, Guo et al 2008c, Fu L et al 2001 and Kardi et al 2005) , these treatment systems are inconsistent for removal of recalcitrant azo dyes and are subject to failure due to poor environmental conditions (van Limbergen et al 1998, Chen et al 2006, Qu et al 2006 and Castillo et al 1999). The bioaugmentation of treatment systems commonly involves the use of mixed cultures of microorganisms, and similarly can result

in varying treatment efficacy depending on the abilities of the individual strains to compete with indigenous populations that are often well acclimated to the existing environmental conditions (Banat et al 1996). More recently, individual strains of bacteria have been reported to have exceptional traits and can greatly accelerate dye decolorization rates (Table 2.2). In addition to azo dye degraders, degradation rates sometimes can also be improved by augmentation with a bacterial sp. with nonessential functions to influence treatment performance (Chen et al 2006 and Chen et al 2003). For example, Escherichia coli DH5α increases the decolorization efficiency of P. luteola even though DH5α is not an active decolorizer of azo dyes among the microbial community. In this case, extracellular metabolites expressed by DH5α stimulated decolorization activity of P. luteola. In recent work, genetically engineered microorganisms (GEM) have also received attention for biodegradation studies and been widely applied in bioaugmentation systems (Jin et al 2009)

Table 2.2 Rate of azo dye decolorization by different microbial strains

Dye	Strain	Dye concentration	Decolorization rate (mg dye h^{-1})	References
Acid Red 119	Bacillu thuringiensis	300 mg L	218	Dave et al 2009
Acid Red 88, Direct Red 81, Reactive Black 5, Disperse Orange 3	Shewanella putrefaciens AS96	100 mg L	22.1–25.0	Khalid et al 2008b
Direct Black 38	Enterococcus Gallinarum	491 mg L	12.8	Bafana et al 2008a
Direct Fast Scarlet 4BS	Bacterial and fungal Consortium	1,000 mg L	81.2	He et al 2004
Direct Red 28 _1	Bacillus velezensis	25 mg L	2.5	Bafana et al 2008b
Direct Red 81	Bacterial consortium	100 mg L	2.5	Junnarkar et al 2006
Methyl Orange, Methyl Red	Lactobacillus casei TISTR 1500	0.23 mmol L	6.1–31.0	Seesuriyachan et al 2007
Reactive Red 22	Escherichia coli NO3	200 mg L	17.0	Chang et al 2000
Scarlet R	Consortium GR comprised of Proteus vulgaris and Micrococcus glutamicus	50 mg L	16.7	Saratale et al 2009

2.3.4 Aerobic Fate of Aromatic Amines

Aerobic biodegradation of aromatic amines formed during anaerobic stage seems more promising compared to anaerobic biodegradation of aromatic amines. Since aromatic amines can be easily biodegraded aerobically through hydroxylation and ring opening of the aromatic ring, it is suggested to combine the anaerobic cleavage of the azo dyes with the aerobic biodegradation of the accumulated amines (Dave et al 2009 and Bafana et al 2008a). However, it was reported that some aromatic amines are readily autoxidized in the presence of oxygen (Chung et al 1992 and Wang et al 2009). That is why researches focus on the determination of specialized cultures, which can mineralize aromatic amines under anaerobic conditions. In aerobic degradation of aromatic compounds by microorganisms, catechol, protocatecuate, and gentisate play a key role, since they are ring cleavage substrates in which an exceptionally large number of peripheral pathways converge. These central intermediates are then cleaved by dioxygenases such as catechol 1,2 dioxygenase, catechol 2,3 dioxygenase, protocatecuate 3,4 dioxygesae, protocatecuate 4,5 dioxygenase, and gentisate 1,2 dioxygenase (Bafana et al 2008b, Chang et al 2000 and Isik et al 2003). Previous studies suggested that enzymes responsible for the aromatic amine removal become more active when the color removal rates are high, which resulted in more aromatic amine production within the sequential batch reactor. Beside this, adverse effect of anaerobic conditions on aerobic enzymes was also reported, and results indicated that their activities increased in aerobic stage and decreased in anaerobic stages due to the absence of dissolved oxygen (Chung et al 1992 and Liu et al 2009). Guo et al (2008a) found that the activity of catechol 1,2-dioxygenase is dependent on the dissolved oxygen. concentration and is influenced by the oxygen concentration. It was also found that the activity of catechol 1,2-dioxygenase is likely to be low in systems with more limited oxygen concentrations.

Azo dye-containing wastewater seems to be one of the most polluted wastewaters, which require efficient decolorization and subsequent aromatic amine metabolism. On the basis of the available literature, it can be concluded that anaerobic–aerobic SBR operations are quite convenient for the complete biodegradation of both azo dyes and their breakdown products. Nevertheless, like the other methods used for biological treatment, SBRs treating colored wastewaters have some limitations. Presence of forceful alternative electron acceptors such as nitrate and oxygen, availability of an electron donor, microorganisms, and cycle times of anaerobic and aerobic reaction phases can be evaluated as quite significant. Though treatment of azo dye-containing wastewaters needs combined anaerobic–aerobic phases, microorganisms are subjected to continually alternating anaerobic and aerobic conditions. Thus, it is presumable that anaerobic enzymes involved in the azo dye reduction may be adversely affected by aerobic conditions, as well as aerobic enzymes involved in the aromatic amine mineralization may be adversely affected by anaerobic conditions. Since little is known about the regulations of the enzymes involved in complete biodegradation of colored wastewaters, there is a need to initiate advanced investigation to improve color removal and aromatic amine mineralization.

2.4 THE CONCEPT OF BIOCALORIMETRY

Thermodynamics of life states that all biochemical reactions of living systems involve alteration in heat and it is proved that their stored internal energy is dissipated in the form of heat with surroundings to sustain their metabolism (von Stockar et al 2006). Therefore, heat measurement gives an overall estimation of biological activity of any living system. Of all living microbes are considered as powerful sources for heat generation in contrast with other higher level organisms and hence calorimetry finds significant application on fingerprinting their metabolic activities (von Stockar et al

1989b). This principle finds application in areas usually referred to as "Biochemical or Biological" calorimetry. In general, calorimetry, being nonspecific, non-invasive and insensitive to the electrochemical and optical properties of the investigated system, can serve as an analytical tool to measure exothermic heat in the bioprocess (Winkelmann et al 2004). Control of bioprocesses requires reliable and robust sensors capable of providing real-time information on the main variables of the processes. Nowadays, sensors to monitor pH, oxygen or carbon dioxide and ammonia are plentiful in use for bioprocess applications (Harms et al 2002). In-line measurements of biomass can be done using sophisticated biosensors (Novemeber et al 2004). But, the evaluation of metabolic activity of organisms is now dealt off-line after analyzing the kinetic data obtained from the experimental results. Biocalorimetry has proved to be an efficient tool (Wadso et al 2002) for the in-line monitoring of bioprocesses, i.e. studying the growth and metabolic activity of cell cultures (or) for the detection of biological key components (Schneiders et al 1995). Redl and Tiefenbrunner (Redl et al 1980) reported that it was possible to employ the measured heat flux signal to characterize a bioprocess system based on cellular activity response with a specific substrate, where a quick rise in heat production indicated a well-adapted system, and a slow rise indicated a poorly adapted one. Moreover, many metabolic events occurring in a biological system, such as shifts from one substrate to another (diauxic growth) or change in the cell metabolism, e.g. from oxidative to fermentative (von Stockar et al 1989b), can be identified by measuring the heat evolution patterns. The advantage of calorimetry is that it measures the total energy flow; under certain conditions this can also be measured by respirometry. In contrast to calorimetry, however respirometry is restricted to aerobic conditions. On combining calorimetry with other in-line bioprobes, it is feasible to achieve significant goals in bioprocess engineering Gustaffson et al 1994). Duboc et al (1999) provided an extensive introduction to quantitative calorimetry. The application of calorimetric

techniques for bioprocesses monitoring is related to the sensitivity of the instrument, to the net enthalpy change of the bioreaction under study and to its rate.

Two main types of calorimeters have been intensively developed during the past few decades: Microcalorimeters (Wadso et al 1986 and Wadso et al 1997) and bench scale calorimeters (Marison et al 1998a). Microcalorimeters were developed first and attained sufficient sensitivity for monitoring biological processes. In the late 1960's, following the pioneering work of Calvet in France and Benzinger in the USA, the development of modern isothermal microcalorimeters began to accelerate (Calvet et al 1963). Gustaffson et al (1991) employed microcalorimetry to study the aerobic growth of the yeast S.cervisiae with glucose as the only carbon and energy source and reported that a continuously changing proportion of the respiratory catabolism in relation to fermentative catabolism and explained the mixed respiratory-fermentative metabolism. The use of microcalorimeters including flow-through measurements, which achieve the necessary sensitivity to measure a low heat flow signal, was hindered by the technical difficulties in fulfilling the biological environmental needs (oxygen and substrates supply, a controlled pH, mixing, etc.) in the small volume of the micro calorimeter measuring cell (typically around 1 ml) and sample transfer time (Kemp et al 1991, van Kleeff et al 1998**,** von Stockar et al 1991). These technical limitations led to development of bench-scale biocalorimeters. Bench-scale biocalorimeters were broadly classified in three major categories viz., dynamic calorimetry, continuous calorimetry and heat flux calorimetry based on heat measurement principle. A more detailed explanation of principle of operation of these three modes of bench-scale calorimetry is available in a review by von Stockar (1989). Heat flux calorimetry has a significant role in bioprocess application since its inception into market by 1980. The design of a bench-scale heat flux calorimeter was first developed by Ciba-Geigy AG,

Basel, Switzerland and later commercialized as a 'Reaction Calorimeter RC1' by Mettler Instruments AG, Greifensse, Switzerland (Grob et al 1987). Reaction calorimeters have proved suitable for cultivation of different cell lines mimics the real time process conditions of a bioreactor. They have become powerful tools for quantitative thermodynamic studies and reliable monitoring and control of many bioprocess systems (Battley 1998). Bench scale heat flux biocalorimeters are high performing (bio) reactors, whose potential as tools for quantitative monitoring and control of (bio) processes is now well established (Zentgraf et al 1991). The operating principle of a bench scale heat-flux biocalorimeter could be briefly explained as follows:

In the isothermal mode, the reactant temperature (T_r) was maintained constant by controlling the jacket temperature (T_j) by circulating low-viscosity silicone oil through the reactor jacket at a higher rate (2 L/s). The jacket temperature was carefully controlled by blending oils from a 'hot' and a 'cold' circuit, via an electronically controlled metering valve. Thus, an exothermic or endothermic process would decrease or increase T_j leading to a temperature gradient across the reactor wall which was directly proportional to the thermal flux liberated by the process (q) according to the following equation (2.1)

$$q = UA(T_r - T_j) \tag{2.1}$$

The above equation facilitates real-time measurement of heat flow rate resultant due to metabolic process in a biocalorimeter. However, the low sensitivity of the measured heat rate signal has hindered their application to weakly exothermic bioprocess systems.

The advent of bench-scale calorimeters over the last 20 years has brought major improvements in its sensitivity. Currently, calorimetric systems can be operated in isothermal, isoperibolic or adiabatic modes, of

which the isothermal mode is most suited for biological studies (Von stockar and Marison 1989a). It has been pointed out that heat-flux measurements can be expected to be a particularly appropriate way for monitoring bioprocesses at large scale. Biocalorimeters have become powerful tools for quantitative thermodynamic studies and for reliable monitoring and control of many bioprocesses (Voisard et al 2002). Thermodynamic studies indicate that there is a direct correlation between heat production rate and oxygen requirement for aerobic growth. Biocalorimetry has been applied to investigate metabolic activity of heterotrophic and autotrophic nitrifying bacterial populations in sludge samples from wastewater treatment plants (Ligthart and Daverio 2003). Though there are several reported studies on the calorimetric response of different isolated strains, that there is no comprehensive study incorporating the calorimetric responses for bacterial activity during azo dye degradation, and the metabolism of Ionic liquids. The objective of the present study is to fill that gap.

2.4.1 Baseline Heat Flow Rate Estimation Method

Estimation of baseline heat flow (accounting all heat gain and loss effects) at defined operating conditions of a bioprocess system seems to be less complicated and has been widely adopted in almost all heat-flux based biocalorimetric measurements.

A basic description of calorimetry is presented here to facilitate the understanding of the measurement principle and modifications made to suit our measurement. Heat flows are defined here as positive if heat is released inside the bioreactor. For an aerobic process, the heat balance around the reactor can be written as:

$$q_{ac} = q_r - q_j + q_s - q_g - q_e - q_f - q_a - q_{CO_2} \qquad (2.2)$$

In the above equation (2.2), q_{ac} is the heat accumulation in the bulk, q_r the heat flow of the running process, q_j the heat flow through the reactor wall to the jacket oil, q_s the stirring power, q_g the heat flow induced by aeration, q_e the heat flow to the environment through the non-jacketed part of the reactor, q_f the heat flow of the feed, q_a the heat flow of the acid or base addition and q_{CO_2} the heat flow of the CO_2 vaporization.

For bioprocesses with a good bioreactor temperature controller, the heat accumulation term can usually be neglected. The jacket heat flow is the most important to monitor since it contains all other heat flows, especially the heat flow generated by the (bio) processes. In the isothermal mode, it will be varied by the temperature controller to keep the bioreactor temperature constant. In the case of RC1 it can be written:

$$q_j = UA.(T_r - T_j) = UA.\Delta T \qquad (2.3)$$

When all other heat flows are constant, the heat transfer coefficient can be determined with a known or measured calibration heat flow (q_e). In case of the Bio-RC1, calibration was carried out by means of an electrical heater which releases a measured quantity of heat in the reaction (20 W).

The heat flow due to stirring is important compared with the process heat flow and is usually not directly measured. In our experiment a torque meter was used to measure it and the heat flow can be calculated as:

$$q_s = 2\pi.\frac{S}{60}.Mt \qquad (2.4)$$

The heat flow induced by aeration is a complex function depending on parameters described as

$$q_g = f\left(T_{gi}; T_{go}; RH_i; RH_o; \dot{m}_{gi} \right) \tag{2.5}$$

The heat losses to the environment can be calculated with a single relation, considering one lumped heat transfer coefficient for the upper part of the bioreactor. It has to be used only for high ambient temperature variations.

$$q_e = K_e.\left(T_r - T_e\right) \tag{2.6}$$

Any matter added to the bioreactor will also induce a heat flow. The heat flow for substrate feed is given below:

$$q_f = m_f.C_{p_f}.\left(T_r - T_f\right) \tag{2.7}$$

During fermentations, carbon dioxide is produced and the associated heat term is given as follows:

$$q_{CO_2} = n_{CO_2}.\Delta H_r \tag{2.8}$$

The heat flow of an on-going (bio) process, q_r, is the result of the energy dissipated by all biochemical reactions. It cannot be directly measured, but can be determined from the following relation.

$$q_r = q_j - q_s - q_c + q_g + q_e + q_f + q_a + q_{CO} \tag{2.9}$$

When there is no bio chemical activity in the bio reactor, the sum of all the heat flows on the right side of equation (2.9), should always be zero, provided they are properly measured and calibrated. In practice, equation can be reduced by lumping all the constant heat flows in a "base-line" term, q_{bl}, which has to be determined before and after any experiment. If culture conditions are not varied, q_S, q_g, q_C and q_f are potentially constant. Finally,

for monitoring purposes, q_a and q_{CO_2} can be lumped in q_r, since they relate to metabolic activity of cells. They have to be separated only for the thermodynamic evaluation of q_r. Equation (2.9) can be reduced to equation (2.10), which is used with the basic (Bio-RC1) setup:

$$q_r = q_j - q_{bl} \tag{2.10}$$

When using the basic BioRC1 setup, the process heat flow was obtained from equation (2.10) and the following procedure is applied. The bioreactor was maintained under culture conditions and stabilized to determine the initial base-line (ΔT_{bl}). A UA calibration was made immediately before inoculation (Figure 2.2).

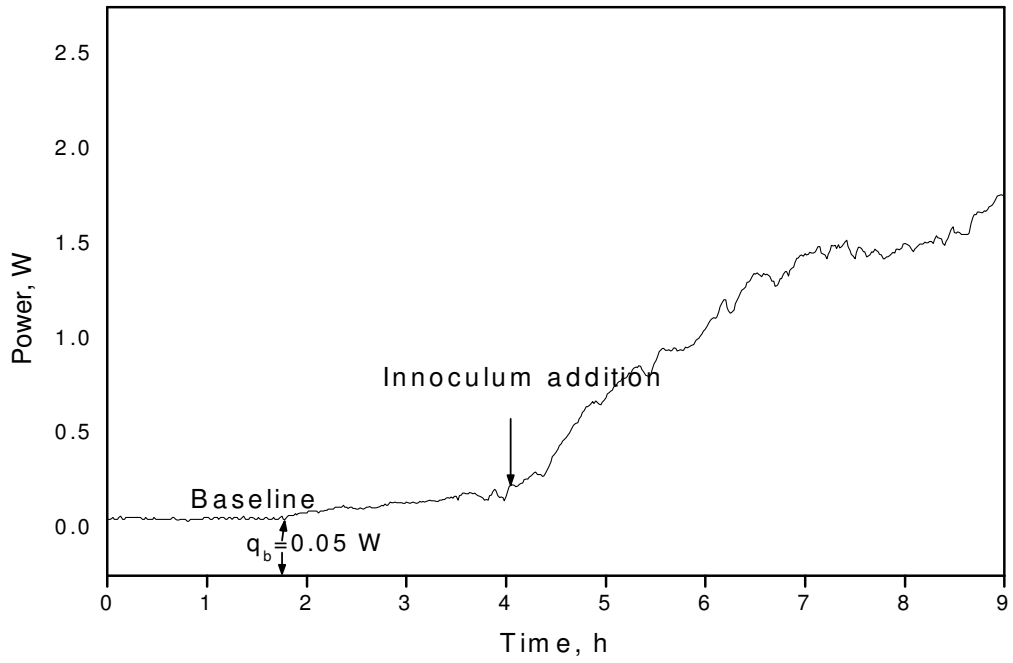

Figure 2.2 Baseline heat evaluation

UA calibration was performed during experiment at regular time intervals by means of in situ calibration heater (20 W) and accordingly the measured heat signal (q_r) was corrected. When the culture conditions were modified (aeration rate, temperature, stirring speed etc.), the baseline stabilization and UA calibrations were repeated for each set of culture conditions. Heat effects due to inlet aeration, stirring speed and other heat losses were eliminated by pre-thermostatting and insulating the respective streams. The exhaust gas stream was allowed to another membrane filter (0.2 μm) to remove the bacterial species trapped in the gas stream and sterile condition was maintained inside the lab.

After the experiment, cells had to be inactivated and the RC1 stabilized to determine the final base-line. A second UA calibration was then made for the off-line evaluation of the process heat flow. Several situations could occur,

1. UA did not vary and the final base-line heat flow (q_{bl}) was equal to the initial one. In this case, q_r was not corrected off-line.
2. UA varied but the final q_{bl} was equal to the initial one. In this case, UA was re-calculated linearly with time or volume (if available) to correct q_r off-line.
3. UA did not vary but the final q_{bl} was different from the initial one. In this case, q_{bl} was re-calculated linearly with time or ambient temperature (if available) to correct q_r off-line.
4. UA varied and the final q_{bl} was different from the initial one. In this case, both UA and q_{bl} were re-calculated as described under points 2 and 3.

However, the heat flow evaluation was carried out using a user-friendly evaluation software (supplied with the equipment) known as RTCal. Heat loss terms and calibration for obtaining heat flux data were done in an automatic mode of the instrument.

2.4.2 Calorimetry for Studying Microbial Growth Process

The literature on the dynamics and energetics of cell metabolism can be subdivided in three main areas viz. employment of calorimetry for studying the growth process, development of correlations between the dynamics of cell metabolism to the heat dissipation, application of calorimetry to study the dynamics and energetics of species in different areas viz. fermentation, wastewater treatment, photoautotrophic reactions and mammalian cells.

The first review in the use of calorimetry in biotechnology dates back to 1989 by von Stockar and Marison. The author has very clearly shown that microbial heat generation depends in very specific ways of growth, biomass yield, maintenance metabolism, nature of substrate and energetic efficiency of growth, oxygen uptake and product formation. It is possible to resort to heat measurements to gain information on any of the above factors. In the review it has been demonstrated that thermograms can be used to determine the monitoring of general microbial activity and for observing and pinpointing all kinds of metabolic events such as product formation, oxidation, medium limitations, inhibition and diauxics. Calorimetry is convenient and a rapid determination tool for microbial growth kinetics. Using a heat flux calorimeter the authors showed that the heat dissipation measurements on industrial scale would hold some potential for process control.

The first review on the thermodynamic considerations for constructing the energy balances in cellular growth was done by von Stockar et al in 1993 wherein they considered the species as a chemical compound in a given thermodynamic state as defined by the state of aggregation. In this treatment the authors assumed that physical transition from one species to another and transformations between different species, catalyzed by living cells were described by one (or) several overall processes with fixed stoicheometry. The resulting enthalpy balance was generally applicable to closed adiabatic and closed isothermal system, open systems at steady state and also to open system in transient stages. A correct evaluation of baseline evolution is necessary to obtain instantaneous heat signals. It's possible to determine the enthalpy growth efficiency in a very simple way by calorimetry (von Stockar et al 1993). The free energy efficiency can be computed based on the knowledge of growth stoicheometry, which remained constant at 60 % for K.fragilis under aerobic, facultative and anaerobic conditions.

Application of calorimetry to biotechnology processes appears to have begun at the time it was applied to chemical reaction systems during the mid 1980's. A number of authors reported the possibility of employing isothermal and heat flow calorimetry for investigating the biological process development work, thermodynamic data generation for understanding the energetics and dynamics of cell biology under aerobic (Gnaiger 1989, Zentgraf 1991, Marison and von Stockar 1985 and Gustaffson 1991), anaerobic and fermentative processes (Wadso 1985 and Sandler and Orbey 1991). The above contribution of the researchers led to the development of the calorimetry suitably modified for studying the biological reactions (Wadso 1997).

Sandler and Orbey 1991 provided a rigorous thermodynamic analysis of microbial growth process that could be used for the study of fermentation, wastewater treatment and similar processes. The authors showed methods to get accurate energy balances with the use of combination of energy balance and stoicheometric equations. Gustaffson (1991) employed microcalorimetry to study the aerobic growth of the yeast S.cervisiae with glucose as the only carbon and energy source and reported that a continuously changing proportion of the respiratory catabolism in relation to fermentative catabolism could explain the mixed respiratory-fermentative metabolism. (Marison et al 1998a) modified Mettler-Toledeo reaction calorimeter by changing both hardware and software to increase the resolution to 2 to 15 mW / L for studying aerated and non-aerated processes.

Since 1997 a rigorous research programme was undertaken by von Stockar et al (von Stockar 1997 – 2000), reporting the thermodynamic analysis of microbial growth (von Stockar et al 1999). They concluded that the amount of Gibbs energy dissipated per C-mole of new biomass grown, ΔG_0^X, was the key parameter for understanding the thermodynamics of microbial growth. Further they stated that on one hand it was linked to the rate of metabolism and therefore regarded as a driving force for growth and on the other hand the Gibbs energy balance ($\Delta_R G_0^X$) determined the biomass yield ($Y_{x/s}$). ie. higher the value of $\Delta_R G_0^X$, lower would be the biomass yield. von Stockar et al reported the first calorimetric investigation of an extreme case of entropy-driven (endothermic) microbial growth. (Voisard et al 2002) explored the development of a large scale biocalorimeter to monitor and control bioprocess and concluded that it was indeed possible to apply real-time quantitative calorimetry at pilot to production scale for easy online monitoring and control. In order to achieve this it was necessary to correctly measure four heat-flows viz. the jacket flow, the calibration heat flow, and stirring power and heat losses to the environment.

Heat is a global measurement of metabolic activity which when correlated with other process variables may give important insights in to the metabolic state of the cells. The metabolism of the microbial system P.denitrificans (DSMS strain 65) was investigated employing calorimetry to determine the growth rate, the occurrence of limiting conditions and the formation of metabolites (Winkleman et al 2004). In a recent review (von Stockar et al 2006) of the thermodynamics of microbial growth and metabolism, it was stated that wide variations of biomass yield reported for different microbial growth system could be explained on thermodynamic reasoning. These variations appeared to be the result of an evolutionary adaptation of the amount of Gibb's energy dissipation towards a reasonable compromise between growth efficiency and growth rate. More work is needed in the thermodynamics of processes the living cells. Most important, the database concerning the Gibb's energy of the chemicals of life and the biochemical reactions, and knowledge on intracellular chemicals affecting the forces during these reactions must be proved. This includes more accurate and more detailed data on the metabolites, whose concentrations have a decisive impact on the thermodynamic calculations.

2.4.3 Predicting Important Heat Yields

An obvious use of the microbial heat evolution data would be the indirect determination of biomass concentration.

$$Y'_{Q/X} = \frac{\Delta H^*_S - Y'_{P/S}\Delta H^*_X}{Y'_{X/S}} - \Delta H^*_X \qquad (2.11)$$

In equation (2.11), the modified heats of combustion could be estimated.

$$Y'_{Q/X} = Q_0\left[\frac{\gamma_S - Y'_{P/S}}{Y'_{X/S}} - \gamma_X\right] \tag{2.12}$$

where Q_0 is about 115 kJ C-mol (degree of reduction).

The amount of heat released per C-mol of biomass formed is thus expected to increase for microbial growth on more reduced substrates. Furthermore, more heat will be dissipated per unit biomass in less efficient growth processes, i.e., with decreasing $Y'_{X/S}$. The appearance of a byproduct will probably lower $Y'_{X/S}$, thereby tending to increase the heat dissipation.

The heat dissipated per mole of oxygen consumed, designated by $Y'_{Q/O}$, can be predicted.

$$Y'_{Q/O} = \frac{\Delta H^*_S - Y'_{X/S}\Delta H^*_P}{1/4\left(\gamma_S - Y'_{X/S}\gamma_X - Y'_{P/S}\gamma_P\right)} \tag{2.13}$$

$$Y'_{Q/O} = 4Q_0 = 460\,\text{kJ mol}^{-1} \tag{2.14}$$

The amount of heat released per mole of oxygen consumed should be nearly the same for all strongly aerobic growth processes regardless of microbial strain, nature of substrate or product and has been discussed by many researchers (Birou et al 1987 a, Roels 1983, Erickson et al 1978 and Erickson et al 1978 a).

The rate of heat to CO_2 evolved could be useful because both evolution rates can be measured on-line in industrial bioreactors. This ratio is related to $Y'_{X/S}$ and $Y'_{P/S}$ as follows:

$$Y'_{Q/C} = Q_0\,\frac{\gamma_S - Y'_{X/S}\gamma_X - Y'_{P/S}\gamma_P}{1 - Y'_{X/S} - Y'_{P/S}} \tag{2.15}$$

2.4.4 Nature of Substrate and Growth Efficiency

The nature of the carbon and energy substrate has a profound effect on the heat released by a microbial culture. The amount of heat generated per unit biomass formed $\left(Y'_{Q/X}\right)$ depends on the enthalpy content of the substrate as shown by equation (2.12):

$$Y'_{Q/X} = Q_0\left[\frac{\gamma_S - Y'_{P/S}\gamma_P}{Y'_{X/S}} - \gamma_X\right] \tag{2.16}$$

A study of microbial heat generation using a range of substrates with widely different degrees of reduction has been published by Birou et al (1987). Their experiments were conducted under strongly aerobic conditions in order to suppress the formulation of products as much as possible. The results of these experiments (Table 2.3) reflect the influence of γ_S on the heat yield and may be compared with the influence predicted by equation (2.16).

Table 2.3 Heat yields as a function of reductance degree of substrates γ_S

Organism	Substrate	γ_X	$Y'_{X/S}$ (g/g)	$Y_{Q/X}$ (g/g)	$Y_{Q/O}$ (kJ/mol)
C .lipolytica	Citrate	3	0.290	14.99	423
C. lipolytica	Succinate	3.5	0.344	16.76	492
K. fragilis	Glucose	4	0.520	12.51	447
K. fragilis	Glucose (1)	4	0.465	12.51	456
K. fragilis	Glucose (2)	4	0.388	15.16	-
C. pseudotropicalis	Glucose (3)	4	0.465	13.50	408
C. utilis	Glucose	4	0.481	11.66	421
E. coli	Glucose	4	0.416	8.70	-
Ent. cloacae	Glucose	4	0.345	9.35	-
K. fragilis	Galctose	4	0.494	13.63	418
K. fragilis	Lactose		0.485	14.58	495
C. utilis	Acetate	4	0.406	17.78	385
C.utilis	Glycerol	4.67	0.562	10.79	474
C.boidinii	Ethanol	6	0.790	16.7	-
C.utilis	Ethanol	6	0.557	19.91	421
M.methylotrophus	Methanol	6	0.406	23.7	437

The heat yield $Y_{Q/X}$ ranges from about 10 to 15 kJ/g cell dry weight when biomass is grown on substrates with an average degree of reduction, such as hexoses (γ_S=4). As predicted by equation (2.13) much higher values of 16-26 kJ/g for $Y_{Q/X}$ are obtained with more reduced substrates such as ethanol, methanol, and hexadecane.

2.4.5 Monitoring and Control of Bioprocesses

Application of calorimetry to bioprocess monitoring began at the time it was applied to chemical reaction systems during the mid 1980's. A number of authors reported the possibility of employing isothermal and heat flux calorimetry for investigating aerobic (Marison et al 1985), fermentative processes (Wadso et al 1985 and Sandler et al 1991) and anaerobic systems (Liu et al 2001). Since heat is a global parameter for all living species to far analysis of their metabolic activity, employment of calorimetry for in-line monitoring and control of bioprocesses is gaining importance since the last decade.

Recently Jungo et al (2007a) employed bench scale calorimetric investigations on fed-batch fermentation of Pichia pastoris for recombinant protein production. Through the heat flow profile responses, it was observed that in a mixed substrate feed P.pastoris fed-batch fermentation, sorbitol was found to be a suitable co-substrate compared with glycerol. The interesting finding of their research work is prediction of the start of induction phase at low specific growth rate based on heat rate measurements. High specific growth rate rendered high heat generation, caused limitation on oxygen and cooling water supply. On extension of this work, some more calorimetric trials were performed by Jungo et al (2007a) to study mixed feed strategy of methanol and glycerol on fed batch fermentations of P.pastoris for avidin

production (Jungo et al 2007b) and alcoholic oxidase expression (Jungo et al 2007c). Change in composition of methanol in feed and shift in substrate uptake was well depicted by corresponding change in heat flow profile. These findings proved the application of calorimetry for effectively monitoring fed batch fermentation systems. Most of their calorimetric results were focused on discussing the pattern of heat flow fluctuations for change in biomass growth, substrate limitation, diauxic growth and type of substrate (e.g., readily metabolized, complex long chain). Senthil kumar et al (2011) employed calorimetry to correlate enzymatic activity with heat flow profile as it was an inherent parameter of metabolism. Calorimetric trials were performed on BioRC1 to investigate proteolytic activity of P.aeruginosa cultivated in a peptone-enriched medium. The authors reported that respirogram closely followed power–time curve in all the phases of growth and a linear correlation between respirometric and calorimetric data was achieved. Low oxy-calorific coefficient (355 kJ mol^{-1}) value and low peptone-heat yield (6 kJ g^{-1}) showed the existence of fermentation coupled metabolism of P. aeruginosa. A biomass yield of 13.4g /gmol of oxygen consumption showed that dissolved oxygen as an inevitable substrate for optimum biomass growth and protease secretion. Biochemical reactions involving protease production account for higher heat generation compared to cell culture growth and break down of substrates. This study revealed that both growth and non-growth related reactions involved in this cell culture metabolism could be monitored efficiently by calorimeter and the heat yield values can be used for better design of fermentors and their scale up. Liu et al (2001) proved the existence of endothermic microbial growth by cultivation of acetotrophic methanogen, Methanosarcina barkeri in BioRC1. This was the first reported study in ‘anaerobic process monitoring’ area employing heat flux calorimeter (Liu et al 2001). Heat evolution curve was observed to be endothermic and indicated the different phases of growth during cultivation of

M.barkeri. Moreover, the heat profile provided real-time information on growth limitation effect well in advance compared to other process parameters. This study proved the capability of heat flux calorimetry to monitor even a slow growth process with low heat evolution rate. The success witnessed above ensures the application of heat-flux calorimetry as a potential tool for monitoring commercially significant anaerobic bioprocesses (e.g., biofuel production) in near future.

Control of biotechnological process is rather important in industrial perspective in order to achieve high product yield. Fed-batch mode is the commonly adopted bioprocess strategy, in which the limiting substrate is fed in to process system based on a measured key process parameter. Hence, there is a need of reliable measurement to directly measure (or) estimate the key process parameter. As seen from the previous sections, heat evolution rate is observed to be the robust signal providing real-time information on cell growth, cell physiology, process limitations and product yield. Control based on calorimetric signal started a decade before, where high-sensitive BioRC1 is employed for monitoring fed-batch growth of S. cerevisae (Von stockar et al 1997b). Here, heat rate is used as an indicative parameter to track the metabolic state of S. cerevisae and the feeding rate of substrate is controlled by means of Respiratory Quotient (RQ), derived from heat rate values. Recently, Senthilkumar et al (2011). Employed directly the heat rate measurements for estimating cell concentration and specific growth rate (Senthilkumar et al 2011). A feed-back control strategy was designed based solely on heat rate measurements and employed for real-time feed control. The results suggested that heat rate signal was more robust than other measured signals and have wide scope for application in different industrially relevant bioprocess systems.

2.5 APPLICATION OF IONIC LIQUIDS AS NUTRIENTS IN BIOLOGICAL PROCESSES

Ionic liquids are discussed as alternative to organic solvents, in view of sustainable and ultimately green processes, (Martyn et al 2000 and Roosen et al 2008). Ionic liquid has been increasingly found useful in biotechnological process as a catalyst, a medium, (solutes and solvents) and in biotransformations (Gangu et al 2009, Shan et al 2008 and Dreyer et al 2008). Besides hydrophobicity and nucleophilicity the spatial configuration of ionic liquids was considered a key factor affecting the behavior of the enzyme in ionic liquids (Shan et al 2008). The ionic liquid, Ammoeng110, containing cations with oligoethyleneglycol units was found to be highly effective for the formation of aqueous two-phase systems (ATPS) and could be used for the biocompatible purification of active enzymes (Dreyer et al 2008). Focusing on the toxic effect of the anion, ionic liquids were designed by combining the benign cholinium cation, [NMe3 (CH2CH2OH)]+, with a range of linear alkanoate anions ($[CnH_2n+1CO_2]$-, n = 1-9), as well as two structural isomers (n = 3 or 4). The toxicity of these ionic liquids was evaluated using filamentous fungi as model eukaryotic organisms. Surprisingly, most of the tested species showed active growth in media containing extremely high ionic liquid concentrations, up to molar ranges in some cases (Petkovic et al 2010).

In yet another study, the metabolic foot printing revealed that fungal cultures responded to specific liquid salts by changing their cell biochemistry resulting in a different pattern of secondary metabolites. Further, ionic liquids were molecularly flexible through different cations and anions combinations and enabled fine – tuning of the physical properties, especially the substrate product partitioning and biocompatibility (Gangu et al 2009). It was shown that normal physiological properties of bacterium (S. Pneumonia)

were observed if trimethylamino group of choline was present in the medium. When these trimethylamino groups were replaced with ethanolamine, they showed numerous abnormalities, were found resistant to bacteriophage and could not undergo genetic transformation (Tomasz et al 1968). These abnormalities were further confirmed by studies in which S. Pneumonia was grown in choline free medium (Damjanovic et al 2007 and Kharat et al 2008). The metabolic activity of imidazolium based ionic liquids in lactic acid producing bacteria revealed L. delbruekii subsp. lactis NRIC 1683 could grow in these ionic liquids and with the increase in the alkyl chain length of imidazolium, microbial activity became low (Matsumoto et al 2004).

Two important aspects are to be considered to enhance the green aspects of any biological process. The first is the nutrient salt, used to increase the populations of bacteria. This should not be toxic to the environment. Secondly, the biomass produced should not pollute the environment. Keeping these ideas in mind and after careful investigations, choline cation based ionic liquids were chosen in this study especially because cholines were easily biodegradable and the products of biomass were not toxic to the environment. There are reports on choline based ionic liquids in a variety of applications. The enhanced solubility and stability of proteins and DNA in novel biocompatible ionic liquids based on the dihydrogen phosphate anion and the choline cation (Vijayaraghavan et al 2010a) was recently reported. Bio-compatibility of some of the choline salts with and without the use of substrate, such as collagen, has also been investigated (Vijayaraghavan et al 2010). As a result, families of novel biocompatible salts have been grown and are beginning to find broader range of application. There have been independent studies on either choline (Meck et al 2003) or lactate (Gladden et al 2004) metabolisms on different microorganisms. So far no reports have been made on the combined use of choline and lactate as ionic liquids. Here

choline based ionic liquids (suitably combining the anions such as tartrate, saccharinate, citrate, lactate etc. and make them biocompatible) were used to ensure normal physiological properties of bacterium (S. lentus) and metabolic activities monitored.

2.6 THE LEATHER INDUSTRY AND ITS EFFLUENTS

The overall environmental impact of leather industry is significant. Leather making is almost wholly a wet process that generates large amounts of wastewater, as shown in Table 2.4. The characteristics of the different streams of wastewater generated during the process are given in Table 2.8. In addition, significant volumes of solid wastes are produced and include trimmings, degraded hides and hair from the beam house processes. The solid wastes can represent up to 70 % of the wet weight of the original hides (Joseph and Nagendran 2004). Large quantities of sludge are also generated. Decaying organic material produces strong odor. Hydrogen sulphide is released during dehairing and ammonia is released in deliming.

In the context of water scarcity and intrusion of salt in freshwater in Tamilnadu (the hub of leather industry in South India), the management of the tannery saline wastewater, high levels of organic load, suspended solids (sand, lime, hair, flesh, dung, etc.) and color, is of great concern for the tannery management. Because of its high complexicity, tannery color effluents should be segregated treated whenever it is possible. Since biodegradation of the color effluents is the cheapest method, there is an interest in treating it through biological methods, using halotolerant micro-organisms, to reduce the amount of organic matter in the effluent leading to complete mineralization of the complex dye molecule. Although there is huge amount of literature on the treatment azo dyes containing wastewater yet the utility of their data for commercial scale-up was limited.

Table 2.4 Characteristics of tannery wastewater. Note: volume of wastewater applicable is for hides (cow and buffalo) and goatskins. Source: Central leather research institute, Chennai, India.

Parameter	Soaking	Beam house operation	Pickling and chrome tanning	Wet finish – rechroming, dyeing and fat liquoring	Composites (including washings)
Volume of the effluent (m^3/t of hides or skins)	6-9	6-10	1.5-3	3-5	30-40
pH	7.5-8	8-12	2.2-4	3.5-4.5	7-9
BOD5 (mg/L)	1100-2500	2000-8000	400-800	1000-2000	1000-3000
COD (mg/L)	3000-6000	3000-15000	1000-3000	2000-7000	2000-8000
Sulphide (mg S/L)	-	50-200	-	-	30-150
Total solids (mg/L)	35000-55000	6000-20000	30000-60000	4000-10000	15000-25000
Dissolved solids (mg/L)	32000-48000	5000-15000	29000-58000	3400-9000	13000-20000
Suspended solids (mg/L)	3000-7000	3000-15000	1000-2000	600-1000	2000-5000
Chlorides (mg Cl/L)	15000-30000	3000-6000	15000-25000	500-1000	600-9500
Total chromium (mg Cr/L)	-	-	1500-3000	30-60	80-200

2.7 SUMMARY

- Biological treatment of the Acid Blue 113, an azo dye widely used in Leather and Textile Industry has not been dealt with in detail.
- Calorimetric studies relating the heat production to metabolic activity of S. lentus have not been dealt with.
- Data on energetics, dynamics and heat yields of the halotolerant bacteria are not available in literature.

- Calorimetric monitoring of the enzymatic activity of halotolerant bacteria has not been studied.
- Use of Choline salts based ionic liquids as a carbon source for halotolerant bacteria are not available in literature
- The metabolic pathway for the consumption of choline salts by the bacteria are not dealt with so far
- The chemical pathway of degradation of acid blue 113 is not available in literature

Thus the review revealed that there was no comprehensive study on the calorimetric monitoring on biological treatment of acid blue 113, an azo dye widely employed in leather and textile industry by the identified halotolerant bacteria. The present thesis study will address the above gaps.