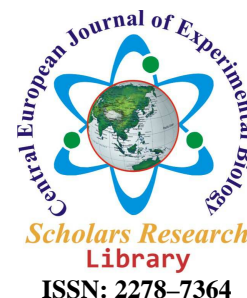




Scholars Research Library

Central European Journal of Experimental  
Biology, 2014, 3 (2):19-25  
(<http://scholarsresearchlibrary.com/archive.html>)



## Separation, purification and characterization of dye degrading enzyme azoreductase from bacterial isolates

P. Saranraj\*, D. Stella and P. Sivasakthivelan

Department of Microbiology, Annamalai University, Annamalai Nagar, Chidambaram.  
Tamil Nadu, India

---

### ABSTRACT

Azoreductases are the enzymes which catalyze the reductive cleavage of azo bonds to produce colorless aromatic amine products. Azoreductases are observed in many organisms, including the rat liver enzyme, rabbit liver aldehyde oxidase and intestinal microbiota. Several studies have been investigated bacterial Cytoplasmic azoreductases, and suggested that they can be applied for the purpose of environmental biotechnology. In the present study, the enzyme Azoreductase was assayed for extracellular activity. As there was no significant activity observed, intracellular release of the enzyme was performed. After each step of purification the activity was assayed and it was found that the specific activity of the enzyme increased after each step of purification. Azoreductase activity in the crude cell extract was found to be 0.0015 U/mg. After ammonium sulfate precipitation, the activity increased to 91.66 U/mg. The optimum pH found for the activity of azoreductase enzyme was pH 7 with an activity of 0.0010 U/mg. The optimum temperature was found to be 40°C with an activity of 0.00098 U/mg.

**Key words:** Azoreductases, Bacteria, Optimization, Purification and SDS-PAGE.

---

### INTRODUCTION

Bioremediation is the microbial clean up approach, microbes can acclimatize themselves to toxic wastes and new resistant strains develop naturally, which can transform various toxic chemicals to less harmful forms. A major mechanism behind biodegradation of different recalcitrant compounds in microbial system is because of the biotransformation enzymes [1, 2]. Bacterial degradation of azo dyes is often mediated by azoreductases, are the enzymes which catalyze the reductive cleavage of azo bonds ( $-N-N-$ ) to produce colorless aromatic amine products [3] which are more efficient under static and anoxic conditions. Bacterial degradation of azo dyes is often initiated by cleavage of azo bonds by azoreductases which are followed by the aerobic degradation of the Microbial degradation of azo dyes is commonly initiated by the reduction of the azo bonds by a group of NADH or NADPH dependant azoreductases with many requiring flavin as a cofactor. Azo dyes are mainly metabolized by bacteria to colorless aromatic amines, some of which are carcinogenic, by azoreductases that catalyze a NAD(P)H-dependent reduction. The resulting amines are further degraded aerobically by bacteria.

Azoreductase is the key enzyme responsible for the reductive azo dye degradation in bacterial species. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH, thus impeding electron transfer from NADH to the azo bonds [4]. The advantage of the anaerobic reduction of azo dyes is that oxygen depletion is easily accomplished in microaerophilic cultures thus enabling anaerobic,

facultative anaerobic and microaerophilic bacteria to reduce azo dyes. The reaction takes place at neutral pH values and is extremely unspecific [5]. However, the precise mechanism of anaerobic azoreduction is still not totally understood. It was recently suggested that microbial anaerobic azoreduction was linked to the electron transport chain, and that Dissimilatory azoreduction was a form of microbial anaerobic respiration [6]. In addition, different models for the nonspecific reduction of azo dyes by bacteria, which do not require transport of the azo dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested [7].

## MATERIALS AND METHODS

### 2.1. Microorganisms used

Six different bacterial isolates *viz.*, *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes* sp. and *Nocardioopsis alba*. The bacterial isolates were isolated and identified from the textile dye effluent.

### 2.2. Purification and characterization of azoreductase enzyme

Cells from the mid log phase culture were harvested by centrifugation at 10000 rpm for 10 minutes at 4°C. Pellets were disrupted by sonication at 40% power for 6 minutes. The cell lysate was subjected to fractionated ammonium sulfate precipitation at 40% saturation to remove impurities, followed by 70% saturation in a second step to precipitate the azoreductase. After 24 hrs, the precipitated protein is centrifuged for 10 minutes at 10000 rpm at 4°C and the pellet was dissolved in equal volume of 50mM potassium phosphate buffer (pH 7.2). Ammonium sulfate precipitated sample was then desalted by dialysis against phosphate buffer (50 mM, pH 7) overnight under room temperature. 2mL of the resulting solution was fractionated by anion exchange chromatography using DEAE sephadex column installed in an Amersham Pharmacia Biotech AKTA purifier; pump P- 900, monitor pH/C- 900, monitor UV-900, auto sampler Frac-950. Elution buffer (sodium phosphate buffer containing 1M NaCl was set to a gradient of 100% for 150 minutes. Proteins were eluted at a flow rate of 1.5 mL/minute. The fractionated sample was concentrated using protein purification column. Active fractions were collected and stored as the purified enzyme preparation.

### 2.3. Assay of Azoreductase activity

Assays were carried out in cuvettes with a total volume of 1mL using Ultrospec 2100 UV-VIS Spectrophotometer (Amersham Biosciences). The reaction mixture consists of 400µl of potassium phosphate buffer with 200 µl of sample and 200 µl of reactive dyes (500 mg/l). The reaction was started by addition of 200 µl of NADH (7mg/mL) and was monitored photometrically at 502 nm. The linear decrease of absorption was used to calculate the azoreductase activity [8]. One unit of azoreductase can be defined as the amount of enzyme required to decolorize 1 µ mol of acid red per minute.

### 2.4. pH and Temperature optima for the stability of the Azoreductase

The effect of pH on azoreductase activity was determined by incubating the reaction mixture at pH values ranging from 5 to 9. The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 30°C to 70°C in 50mM potassium phosphate buffer (pH 7). The relative activity of azoreductase at each temperature was determined.

### 2.5. Protein estimation

The protein concentration of Azoreductase was estimated by following the method of Lowry *et al.* [9].

### 2.6. Molecular weight estimation by SDS - PAGE

The molecular weight of the Azoreductase was estimated by Sodium Dodecyl Sulphate – Polyacrylamide Agarose Gel Electrophoresis (SDS – PAGE) Technique.

## RESULTS

### 3.1. Total activity and specific activity of crude azoreductase obtained from the bacterial isolates and consortium

The total activity and specific activity of crude azoreductase obtained from the bacterial isolates and consortium was assessed and the results were furnished in Table – 1. The total activity of crude azoreductase was maximum in the

azoreductase obtained from bacterial consortium (8.320 U) followed by *Bacillus odyssey* (7.859 U), *Bacillus thuringiensis* (7.120 U), *Bacillus subtilis* (5.550 U), *Bacillus cereus* (3.602 U) and *Alcaligenes* sp. (1.273 U). Least azoreductase activity was noticed in the azoreductase produced by *Nocardiopsis alba* (0.988 U). The best specific activity of crude azoreductase was recorded in the bacterial consortium.

### 3.2. Total activity and specific activity of partially purified azoreductase obtained from the bacterial isolates and consortium

The total activity and specific activity of partially purified azoreductase obtained from the bacterial isolates and consortium was determined and the results were given in Table – 2. The total activity of partially purified azoreductase was high in the azoreductase obtained from bacterial consortium (5.275 U) followed by *Bacillus odyssey* (4.936 U), *Bacillus thuringiensis* (4.521 U), *Bacillus subtilis* (2.115 U), *Bacillus cereus* (1.615 U) and *Alcaligenes* sp. (0.973 U). Least azoreductase activity was noticed in the azoreductase produced by *Nocardiopsis alba* (0.388 U). The best specific activity of partially purified azoreductase activity was recorded in the bacterial consortium.

Table – 1: Total activity and specific activity of crude azoreductase obtained from the bacterial isolates and consortium

S.No	Bacterial isolates	Total activity (U)	Specific activity [U (mg protein) <sup>-1</sup> ]
1.	<i>Bacillus odyssey</i>	7.859	0.043
2.	<i>Bacillus thuringiensis</i>	7.120	0.062
3.	<i>Bacillus subtilis</i>	5.550	0.110
4.	<i>Bacillus cereus</i>	3.602	0.139
5.	<i>Alcaligenes</i> sp.	1.273	0.220
6.	<i>Nocardiopsis alba</i>	0.988	0.272
7.	Bacterial consortium	8.320	0.015

### 3.3. Effect of pH stability on azoreductase activity

The effect of pH stability on azoreductase activity was evaluated and the results were presented in Table – 3. The azoreductase activity was assessed at pH – 5, pH – 6, pH – 7, pH – 8 and pH – 9. Maximum azoreductase activity was recorded by the bacterial consortium at pH – 7 (9.641 U/ml) followed by pH – 6 (7.561U/ml), pH – 5 (6.770 U/ml), pH – 8 (5.236 U/ml) and pH – 9 (4.550 U/ml). Next to bacterial consortium, maximum azoreductase activity was observed in *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes* sp. and *Nocardiopsis alba*.

### 3.4. Effect of temperature stability on azoreductase activity

The effect of temperature stability on azoreductase activity was investigated and the results were listed in Table – 4. The azoreductase activity was assessed at 30°C, 40°C, 50°C, 60°C and 70°C. Maximum azoreductase activity was recorded by the bacterial consortium at 40°C (9.431U/ml) followed by 30°C (8.896 U/ml), 50°C (8.621 U/ml), 60°C (7.772 U/ml) and 70°C (7.623 U/ml). Next to bacterial consortium, maximum azoreductase activity was observed in *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes* sp. and *Nocardiopsis alba*.

### 3.5. Total protein content of crude and partially purified azoreductase obtained from the bacterial isolates and consortium

The total protein content of crude and partially purified azoreductase obtained from the bacterial isolates and bacterial consortium was studied and the results were showed in Table – 5. Total protein content was maximum in the crude azoreductase when compared to the partially purified azoreductase. Protein content was maximum in the azoreductase produced by bacterial consortium (Crude azoreductase – 1455 µg/ml; Partially purified azoreductase – 1025 µg/ml), *Bacillus odyssey* (Crude azoreductase – 1390 µg/ml; Partially purified azoreductase – 990 µg/ml), *Bacillus thuringiensis* (Crude azoreductase – 1261 µg/ml; Partially purified azoreductase – 829 µg/ml), *Bacillus subtilis* (Crude azoreductase – 1150 µg/ml; Partially purified azoreductase – 711 µg/ml), *Bacillus cereus* (Crude azoreductase – 981 µg/ml; Partially purified azoreductase – 628 µg/ml), *Alcaligenes* sp. (Crude azoreductase – 805 µg/ml; Partially purified azoreductase – 585 µg/ml) and *Nocardiopsis alba* (Crude azoreductase – 719 µg/ml; Partially purified azoreductase – 471 µg/ml).

### 3.6. Molecular weight estimation by SDS – PAGE

The molecular weight of an enzyme azoreductase was estimated by SDS – PAGE technique and the result was given in Figure – 1.

Fig – 1: SDS - PAGE of Azoreductase

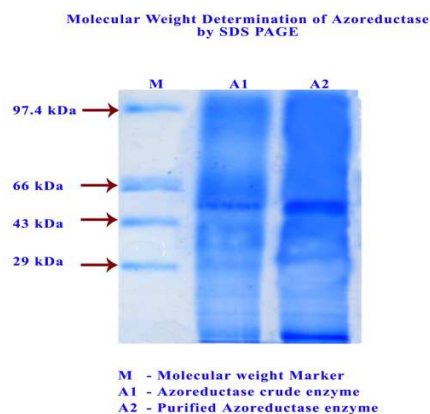


Table – 2: Total activity and specific activity of purified azoreductase obtained from the bacterial isolates and consortium

S.No	Bacterial isolates	Total activity (U)	Specific activity [U (mg protein) <sup>-1</sup> ]
1.	<i>Bacillus odyssey</i>	4.936	0.057
2.	<i>Bacillus thuringiensis</i>	4.521	0.096
3.	<i>Bacillus subtilis</i>	2.115	0.105
4.	<i>Bacillus cereus</i>	1.615	0.239
5.	<i>Alcaligenes sp.</i>	0.973	0.279
6.	<i>Nocardiopsis alba</i>	0.388	0.285
7.	Bacterial consortium	5.275	0.042

Table - 3: Effect of pH stability on azoreductase activity

S.No	Bacterial Isolates	Enzyme activity (U/ml)				
		pH 5	pH 6	pH 7	pH 8	pH 9
1.	<i>Bacillus odyssey</i>	6.770	7.561	8.320	5.236	4.550
2.	<i>Bacillus thuringiensis</i>	5.862	6.110	7.120	4.440	3.897
3.	<i>Bacillus subtilis</i>	4.997	5.497	5.550	3.666	3.101
4.	<i>Bacillus cereus</i>	2.886	3.111	3.602	2.109	1.975
5.	<i>Alcaligenes sp.</i>	0.888	0.960	1.273	0.768	0.652
6.	<i>Nocardiopsis alba</i>	0.836	0.911	0.988	0.729	0.603
7.	Bacterial consortium	7.881	8.672	9.641	6.347	5.661

Table - 4: Effect of temperature stability on azoreductase activity

S.No	Bacterial isolates	Enzyme activity (U/ml)				
		30°C	40°C	50°C	60°C	70°C
1.	<i>Bacillus odyssey</i>	7.785	8.320	7.510	6.661	6.512
2.	<i>Bacillus thuringiensis</i>	6.828	7.120	6.239	5.789	5.231
3.	<i>Bacillus subtilis</i>	5.136	5.550	4.779	4.269	4.111
4.	<i>Bacillus cereus</i>	3.436	3.602	3.085	2.888	2.441
5.	<i>Alcaligenes sp.</i>	1.007	1.273	0.890	0.710	0.661
6.	<i>Nocardiopsis alba</i>	0.823	0.988	0.776	0.698	0.546
7.	Bacterial consortium	8.896	9.431	8.621	7.772	7.623

Table – 5: Total protein content of crude and partially purified azoreductase obtained from the bacterial isolates and consortium

S. No	Bacterial isolates	Total protein content of crude azoreductase (µg/ml)	Total protein content of partially purified azoreductase (µg/ml)
1.	<i>Bacillus odyssey</i>	1390	990
2.	<i>Bacillus thuringiensis</i>	1261	829
3.	<i>Bacillus subtilis</i>	1150	711
4.	<i>Bacillus cereus</i>	981	628
5.	<i>Alcaligenes sp.</i>	805	585
6.	<i>Nocardiopsis alba</i>	719	471
7.	Bacterial consortium	1455	1025

## DISCUSSION

Azoreductases isolated from several bacteria have been shown to be inducible flavoproteins and to use both NADH and NADPH as electron donors [10]. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH; thus impeding electron transfer from NADH to the azo bonds. The advantage of the anaerobic reduction of azo dyes is that the depletion of oxygen is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerobic bacteria to reduce azo dyes. The reaction takes place at neutral pH values and is extremely unspecific [11]. However, the precise mechanism of anaerobic azo-reduction is not yet totally understood. A different model was recently suggested for the nonspecific reduction of azo dyes by bacteria, which does not require transport of the azo dyes or reduced flavins through the cell membrane [12].

Bacterial degradation of dyes is often an enzymatic reaction linked to anaerobiosis [13, 14]. Thus, the bacterial reduction of dyes under anaerobic or anoxic conditions is non-specific to the kind of dye involved [15]. The total activity and specific activity of crude azoreductase obtained from the bacterial isolates and consortium was assessed in the present study. The total activity of crude azoreductase was maximum in the azoreductase obtained from bacterial consortium (8.320 U) followed by *Bacillus odyssey* (7.859 U), *Bacillus thuringiensis* (7.120 U), *Bacillus subtilis* (5.550 U), *Bacillus cereus* (3.602 U) and *Alcaligenes sp.* (1.273 U). Least azoreductase activity was noticed in the azoreductase produced by *Nocardiopsis alba* (0.988 U). The best specific activity of crude azoreductase was recorded in the bacterial consortium. Fatemeh *et al.* [16] also reported extracellular enzymatic activity (azoreductase) for anaerobic bacteria.

The total activity and specific activity of partially purified azoreductase obtained from the bacterial isolates and consortium was determined. The total activity of partially purified azoreductase was high in the azoreductase obtained from bacterial consortium (5.275 U) followed by *Bacillus odyssey* (4.936 U), *Bacillus thuringiensis* (4.521 U), *Bacillus subtilis* (2.115 U), *Bacillus cereus* (1.615 U) and *Alcaligenes sp.* (0.973 U). Least azoreductase activity was noticed in the azoreductase produced by *Nocardiopsis alba* (0.388 U). The best specific activity of partially purified azoreductase activity was recorded in the bacterial consortium.

Fatemeh *et al.* [16] also reported extracellular enzymatic activity (azoreductase) for anaerobic bacteria. The isolated strains from the microbial consortium identified as *Pseudomonas aeruginosa* and *Bacillus circulans*. Identification of some other isolated strains (NAD1 and NAD6) is in progress. A number of *Pseudomonas sp.* and *Bacillus sp.* have been known to produce azoreductas [17].

Earlier studies provided evidence that microbial anaerobic azo-reduction was linked to the electron transport chain, and suggested that dissimilatory azo-reduction was a form of microbial anaerobic respiration [18]. In addition, different models for the non-specific reduction of azo dyes by bacteria, which did not require transport of the azo dyes or reduced flavins through the cell membrane and that described the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested. These results suggested that azo dye reduction was a strain specific mechanism that could be performed by an azoreductase enzyme or by a more complex metabolic pathway.

The effect of pH stability on azoreductase activity was evaluated. The azoreductase activity was assessed at pH – 5, pH – 6, pH – 7, pH – 8 and pH – 9. Maximum azoreductase activity was recorded by the bacterial consortium at pH – 7 (9.641 U/ml) followed by pH – 6 (7.561U/ml), pH – 5 (6.770 U/ml), pH – 8 (5.236 U/ml) and pH – 9 (4.550 U/ml). Next to bacterial consortium, maximum azoreductase activity was observed in *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes sp.* and *Nocardiopsis alba*. The effect of temperature



stability on azoreductase activity was investigated. The azoreductase activity was assessed at 30°C, 40°C, 50°C, 60°C and 70°C. Maximum azoreductase activity was recorded by the bacterial consortium at 40°C (9.431U/ml) followed by 30°C (8.896 U/ml), 50°C (8.621 U/ml), 60°C (7.772 U/ml) and 70°C (7.623 U/ml). Next to bacterial consortium, maximum azoreductase activity was observed in *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes* sp. and *Nocardiopsis alba*. Temperature optima for azoreductases from *Bacillus subtilis* reported in the literature range from 40 to 45°C [19, 20]. Similarly, in this work, the azoreductase from *Bacillus cereus* showed a temperature optimum at 40°C with a pH optimum at pH 7.

The major mechanism involved in the microbial biodegradation of synthetic azo dyes is based on their biotransformation by enzymes [21]. The initial step involved in the biodegradation of azo dyes is the reductive cleavage of azo bond (–N N–) with the azoreductase [22]. Further, a significant induction in the activity of azoreductase was observed in the cell free extracts of both the strains AK1 and AK2 during Metanil Yellow decolorization when compared to the control cells. Such inductive pattern of azoreductase was observed during decolorization of sulfonated azo dyes by *Kerstersia* sp. strain VKY1 [23] and *Galactomyces geotrichum* MTCC 1360 [24].

The total protein content of crude and partially purified azoreductase obtained from the bacterial isolates and bacterial consortium was studied. Total protein content was maximum in the crude azoreductase when compared to the partially purified azoreductase. Protein content was maximum in the azoreductase produced by bacterial consortium (Crude azoreductase – 1455 µg/ml; Partially purified azoreductase – 1025 µg/ml), *Bacillus odyssey* (Crude azoreductase – 1390 µg/ml; Partially purified azoreductase – 990 µg/ml), *Bacillus thuringiensis* (Crude azoreductase – 1261 µg/ml; Partially purified azoreductase – 829 µg/ml), *Bacillus subtilis* (Crude azoreductase – 1150 µg/ml; Partially purified azoreductase – 711 µg/ml), *Bacillus cereus* (Crude azoreductase – 981 µg/ml; Partially purified azoreductase – 628 µg/ml), *Alcaligenes* sp. (Crude azoreductase – 805 µg/ml; Partially purified azoreductase – 585 µg/ml) and *Nocardiopsis alba* (Crude azoreductase – 719 µg/ml; Partially purified azoreductase – 471 µg/ml).

According to a review by Hanson *et al.* [25], sulfatase enzyme was involved in hydrolytic desulfonation of sulfate esters (CO–S) and sulfamates (CN–S) and this enzyme was discovered in *Enterococcus faecalis*. More biological functions of this enzyme from prokaryotic sources have yet to be discovered and explored. Desulfonation was previously reported as a step in biodegradation of other sulfonated aromatic compounds, including substituted naphthalene sulfonates and benzene sulfonates [26, 27].

Dawkar *et al.* [28] showed induction in the intracellular lignin peroxidase, laccase and NADH-DCIP reductase during decolorization of Brown 3REL by *Bacillus* sp. VUS. They also showed the induction (upto 42% decolorization) of the riboflavin reductase activity and then decreased afterwards. Recently, Selvakumaran *et al.* [29] reported on the genetic diversity of aromatic ring-hydroxylating dioxygenase (ARHD) genes from *Citrobacter* spp. which code for dioxygenase enzymes. The variability of these dioxygenases would be reflected upon their functional diversity and their ability to completely degrade of benzoate, hydroxybenzoic acid and phenol.

## REFERENCES

- [1] L. Amor, C. Kennes, M. C. Veiga, *Bioresource Technology*, **2001**, 78, 181–185.
- [2] R. G. Saratale, G. D. Saratale, J. S. Chang, S. P. Govindwar, *Biodegradation*, **2010**, 93 - 60.
- [3] J. S. Chang, C. Chou, Y. Lin, J. Ho, T. L. Hu, *Water Resources*, **2001**, 35, 20 - 41.
- [4] J. Chang, C. Lin, *Biotechnology Letter*, **2001**, 23, 631.
- [5] A. Stolz, *Applied Microbiology and Biotechnology*, **2001**, 56, 69 - 80.
- [6] Y. Hong, M. Xu, J. Guo, Z. Xu, X. Chen, G. Sun, *Applied and Environmental Microbiology*, **2007**, 73, 64 - 72.
- [7] Luciana Pereira, Ana Coelho, Cristina A. Viegas, Margarida Correia dos Santos, Maria Paula Robalo, Ligia Martins, *Journal of Biotechnology*, 2009, 139, 68 - 77.
- [8] J. Maier, A. Kandelbauer, A. Erlacher, A. Cavaco – Paulo, G. M. Gubits, *Applied Environmental Microbiology*, **2004**, 70, 837 – 844.
- [9] O. H. Lowry, N. J. Rosebrough, A. Farr, R. J. Randall, *Journal of Biological Chemistry*, **1951**, 193, 265 - 275.
- [10] A. Moutaouakkil, Y. Zeroual, F. Zohra, Dazayri, M. Tarbi, K. Lee, **2003**, *Process Biochemistry*, 41, 139-146.
- [11] A. Stolz, *Applied Microbiology and Biotechnology*, **2001**, 56, 69 - 80.
- [12] M. Kudlich, A. Keck, J. Klein, A. Stolz, **1997**, *Applied Environmental Microbiology*, 63(9), 3691 - 3694.

- [13] I. M. Banat, P. Nigam, D. Singh, R. Marchant, *Bioresource Technology*, **1996**, 58, 212–227.
- [14] K.C. Chen, J. Y. Wu, D. J. Liou, S. C. J. Hwang, **2003**, *Journal of Biotechnology*, 101, 57 - 68.
- [15] C. I. Pearce, J. R. Lloyd, J. T. Guthrie, *Dyes and Pigments*, **2003**, 58, 179 - 196.
- [16] R. Fatemeh, W. Franklin, C. E. Cerniglia, *Applied Environmental Microbiology*, **1990**, 56(7), 2146 – 2151.
- [17] M. Mazumder, J. R. Logan, A. T. Mikell, S. W. Hooper, *Journal of Industrial Microbiology and Biotechnology*, **1999**, 23, 476 - 483.
- [18] Y. Hong, M. Xu, J. Guo, Z. Xu, X. Chen, G. Sun, *Applied and Environmental Microbiology*, **2007**, 73, 64 - 72.
- [19] T. L. Hu, *Water Science Technology*, 2001, 43, 261 - 269.
- [20] N. Matsudomi, K. Kobayashi, S. Akuta, *Agricultural Biology and Chemistry*, **1977**, 41(12), 2323 – 2329.
- [21] C. Raghukumar, D. Chandramohan, J. Michel and C. A. Reddy, *Biotechnology Letters*, 18, 105 - 106.
- [22] J. S. Chang, T. S., Kuo, Y. P. Chao, J. Y., Ho, P. J. Lin, *Biotechnology Letter*, **2000**, 22, 807.
- [23] M. H. Vijaykumar, A. Parag, S. S. Vaishampayan, S. S. Yogesh, T. B. Karegoudar, *Enzyme Microbial Technology*, **2007**, 40, 204 - 211.
- [24] S. Jadhav, M. U., Jadhav, A. N. Kagalkar, S. P. Govindwar, *Journal of Chemical Engineering*, **2008**, 39, 563 – 570.
- [25] S. R. Hanson, M. D. Best, C. H. Wong, *Chemistry International*, **2004**, 43, 5736 – 5763.
- [26] D. Zurrer, A. M. Cook, T. Leisinger, *Applied Environmental Microbiology*, **1987**, 53, 1459 - 1463.
- [27] W. Haug, A. Schmidt, B. Nortemann, D. C. Hempel, A. Stolz, H. J. Knackmuss, *Applied Environmental Microbiology*, **1991**, 57, 3144 - 3149.
- [28] V. V. Dawkar, U. U. Jadhav, S. U., Jadhav, S. P. Govindwar, *Journal of Applied Microbiology*, **2008**, 105, 24 - 28.
- [29] S. Selvakumaran, A. Kapley, S. M. Kashyap, H. F. Daginawala, V. C. Kalia, H. J. Purohit, *Bioresource Technology*, **2011**, 102, 4600 - 4609.