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Alkaline Protease Production by *Bacillus subtilis*

Antifungal Activity of *Bacillus subtilis* Soil Isolates

Azo Dye Biodegradation by Azoreductase from *Bacillus megaterium*

Anti-hyperlipidemic Effects of *Agaricus bisporus*

Abstracts

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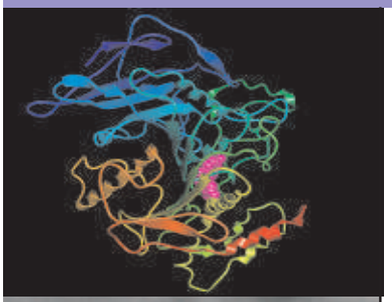

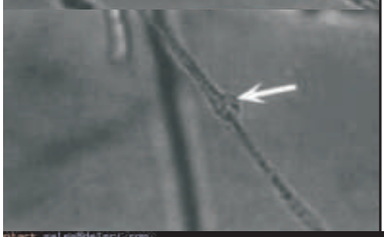

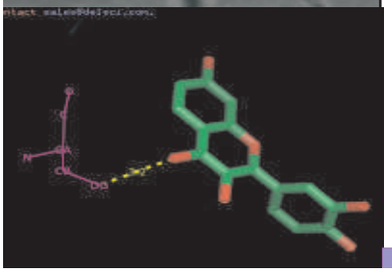

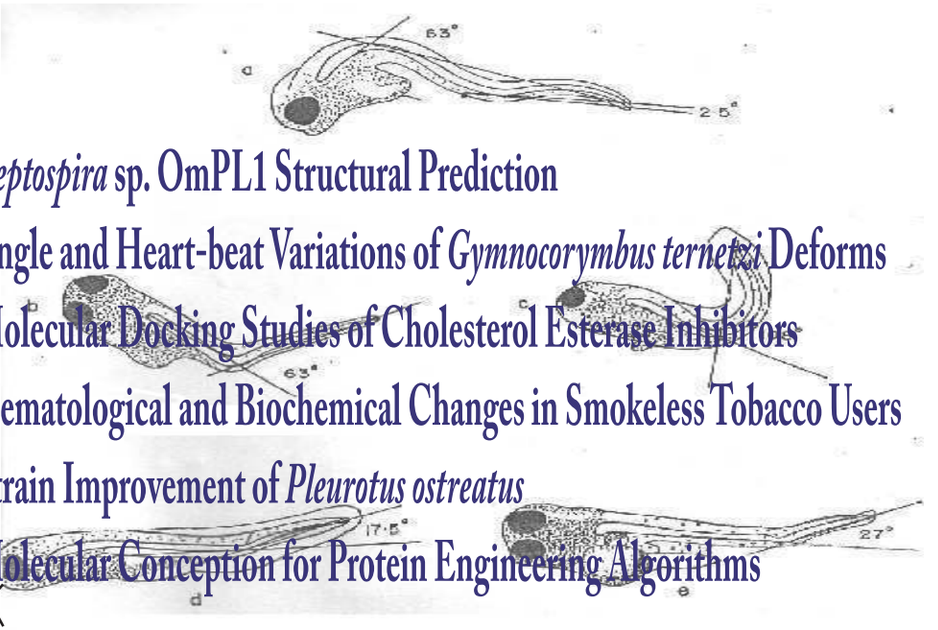

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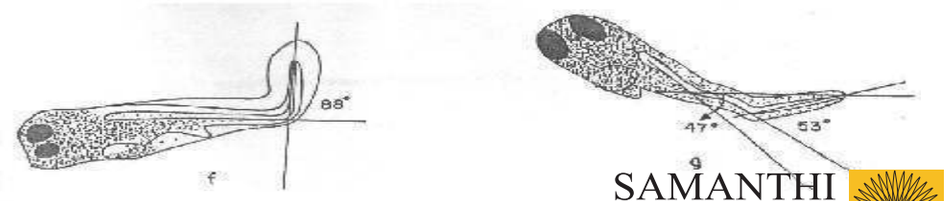

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Biodegradation of Azo Dye by Moderately Halotolerant *Bacillus megaterium* and Study of Enzyme Azoreductase Involved in Degradation

Jahir Alam Khan

Abstract

Halophiles are exteremophiles able to survive in extreme conditions of salt concentration, and are gaining interest in areas such as industrial waste treatment .A moderately halotolerant microbe (0.5-2.5 M NaCl sustaining) was isolated from water sample from Marina beach and was identified as *Bacillus megaterium*. Percentage dye degradation by the isolated *Bacillus megaterium* was found to be 64.89%. The enzyme involved in degradation Azoreductase was assayed and purified by anion exchange chromatography. Total activity of the purified enzyme was 36.03U. The enzyme gave a single band in the SDS–PAGE with a molecular weight of 65 kDa (approximately).

Key words: Azoreductase, *Bacillus megaterium*, Halotolerant, SDS-PAGE.

Introduction

Azo dyes have been used increasingly in industries because of their ease and cost effectiveness in synthesis compared to natural dyes. It has been estimated that about 10,000 different types of dyes are being used industrially worldwide. During the dyeing processes about 10–90% of the dye stuff do not bind to the fibres and therefore, released into the sewage treatment system or the environment (Zollinger 1991; Abdullah, *et al.,* 2000). This implies the wide occurrence of synthetic dyes in dye waste waters. Most of Azo-dyes are toxic, carcinogenic, and mutagenic (Pinheiro *et al.,* 2004). Azo bonds (–N≡N–) in the dyes are resistant to breakdown, with the potential for the persistence and accumulation of high-levels of dye in the environment. They are very stable in acidic and alkaline conditions and are resistant to temperature and light. However, they can be degraded by bacteria under anaerobic and aerobic conditions (Talarposhti *et al.,* 2001). There are two different methods for bacterial Azo-dye decolorization: aerobic and anaerobic mechanisms. For aerobic bacteria, they need to be acclimatized long-term under aerobic chemostatic growth in the presence of a simple azo compound. Then, the bacteria synthesize an azoreductase specifically corresponding to the azo compound added in the presence of oxygen. In contrast, decolorization under anaerobic activity is relatively nonspecific with respect to the azo compounds involved (Pearce *et al.,* 2003). Halophiles are defined as the microbes requiring NaCl for growth. Most bacteria growing in halophilic conditions are moderately halophilic growing optimally within the range of 0.5–2.5M NaCl. Halophiles are metabolically diverse and are acquainted with the saline environment which is the condition of most of the industrial effluents containing azo dyes, so these halophiles can be used directly for the treatment of the effluents at the industrial sites, which will be cost effective than the conventional methods of treatment using non-halophilic microflora, where first the effluent has to be pretreated via reverse osmosis, electro dialysis, or fresh water dilution in order to make the effluents favourable for the non halophilic microflora to grow and act upon the substrates (Margesin and Schinner, 2001; Peyton *et al.,* 2002).

Azo dye Red 2G being used in this study is mainly used as a food dye it is also used as a dye for coatings inks ,paper, crepe paper and fine tissue. The present study on the degradation of the azo dye (Red 2G) using halophiles was carried out with the following objectives: (i) To isolate a moderately halotolerant bacterial Species capable of dye degradation, (ii)To calculate the Percentage dye degradation by the screened bacterial species and (iii) To purify and characterize the Azoreductase enzyme.

Materials and methods

For the isolaton of halophilic bacteria water sample was collected in sterile plastic bottles from the Marina beach, Chennai the capital city of Tamilnadu. 1ml of the sample was serially diluted and the dilutions 10^{-1}–10^{-6} were inoculated on the nutrient agar plates (10% NaCl) by pour plate method, Colonies got after incubation were purified on quadrant streaking, purity of colony was checked by Grams staining procedure, the purified colonies were than streaked on the modified DSC plates (10–12% NaCl) [for halophiles] in order to countercheck the halophilic nature. The media used for the screening was the mineral salts basal media as the one used by Oranusi and Mbah (2005) modified in order to give carbon source, had deficiency of nitrogen source so that the dye can be utilized as a nitrogen source by microorganisms.

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All the six purified colonies named as S_1,S_2,S_3,S_4,S_5,S_6 were streaked on the screening media and were kept for incubation for one weak. A control plate was also maintained for comparison. After one weeks incubation the plates were observed for decolourization of the dye at and around the culture position. After incubation the dye decolourization was ranked on the basis of visual identification as Intense (+++), Moderate (++), Slight (+), No decolourization (-). The isolate (S4) showing intense decolourization was than identified based on the Grams staining, spore test, and various biochemical tests from the key cited by Aneja from the Bergeys manual of determinative biology.

The procedure is based on the one used by Oranusi and Ogugbue (2005). The standard inoculum of the isolated strain S4 giving intense decolourization of azo dye was prepared by inoculating it in 25 ml nutrient broth containing high salt concentration. The standard inoculum (25 ml) was than used to inoculate 225 ml mineral salts basal media in which 15 ml of the dye solution has been added after autoclaving. A control flask was also maintained. After inoculation 5 ml of the sample was taken out in sterile condition and centrifuged at 6000 rpm for 10 min. Supernatant was taken and its optical density was determined spectrophotometrically (Szimatzu), at 532 nm the absorbance maxima of azo dye Red 2G being studied for decolourization.Thereafter the percent of dye decolourization on zero day was calculated by the formula (Oranusi and Ogugbue, 2005).

$$\% \text{ Dye decolorization} = \frac{O.D_{\text{zero day}} - O.D_{\text{sample}}}{O.D_{\text{zero day}}} \times 100$$

The inoculated media was then incubated in shaking incubator at 150 rpm. Percentage dye degradation was calculated every alternate day for twenty days and the above described method and formula was used every time.

The enzyme was produced in large quantity by the inoculation in the production media containing limited nitrogen source after which azo dye was the only source of nitrogen.

Stock Dye Solution: 2 g dye Red 2G in 100ml distilled water, sterilized by membrane filter.

Components	Quantity (gl-1)
Potassium Dihydrogen Phosphate-	3.0
Disodium Hydrogen Phosphate-	6.0
Glucose-	2.0
Sodium Nitrate-	0.42
Sodium Chloride-	100
Red 2G (stock) -	50ml
pH-	7.0

10 ml of S_4 in nutrient broth was than inoculated in the production media 90 ml containing 5 ml of the stock dye solution in order to get the enzyme in large amount. The inoculated media in flasks were kept in the shaking incubator at 150 rpm for 20 days incubation. The broth was taken at the end of incubation time and was centrifuged at 6000 rpm for 15 min. The supernatant was taken and was treated as crude extract.

Assay for azoreductase activity was performed using the following materials:

1. Potassium phosphate buffer 50mM (Reagent A).
2. Azo dye solution 0.1%, (Reagent B)
3. Enzyme solution (Reagent C)
4. Reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH) 2 mM (Reagent D).

The assay procedure is based on the principle that with the addition of NADH to the reaction mixture containing substrate, buffer and enzyme solution, the substrate azo dyes azo bond is degraded and there is a decrease in the absorbance of the dye after an initial lag phase.

Unit Definition: One unit will reduce 1.0 µmole of azo dye per minute in the presence of NADH using Millimolar extinction coefficient of azo dye Red 2G (31.56) at pH 7.0 and 30°C temperature.

Calculation

$$\text{Units/ml enzyme} = \frac{(\Delta A\,532\text{nm / min Test} - \Delta A532\text{nm / min Blank})\,(3)\,(df)}{\varepsilon \times (0.1)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
ε= Millimolar extinction coefficient of azo dye Red 2G
0.1 = Volume (in milliliter) of enzyme used

The procedure adopted was based on the one done by Zimmermann *et al*. (1982).

1. Pipetted (in milliliters) the following reagents into test tubes:

	Test	Blank
Reagent A (Buffer)	2.80	2.80
Reagent B (Azo dye solution)	0.05	0.05
Reagent C (Enzyme solution)	0.1	0.1 (Distilled water)

Mixed by inversion and equilibrate at room temperature. Then add:

Reagent D (NADH)	0.05	0.05

2. Immediately mixed by inversion and recorded the decrease in absorbance at 532 nm for approximately 5 min. obtained the ΔA 532 nm/ minute for both the Test and Blank.
3. Enzyme activity was than calculated by the formula given above.

The enzyme purification was performed as follows: The crude extract was then subjected to ammonium sulphate precipitation at 80% saturation; this was carried out by adding 50.51g of ammonium sulphate to 90 ml crude extract. The precipitated enzyme was collected by centrifugation, and the pallet was dissolved in 10 ml of phosphate buffer (50 mM, pH 7.0). The solution was then desalted by dialysis against phosphate buffer (50 mM, pH 7.0) overnight.

Further purification was carried out by anion exchange chromatography, using DEAE anion exchanger, the enzyme was eluted by buffers in the range of 100–500 mM NaCl concentration. The protein concentration was estimated by Lowry's method of protein estimation for both crude extract and all the elutes of ion exchange. Molecular weight of the enzyme purified was determined by SDS–PAGE, running the enzyme along with a marker of known molecular weight.

For characterization, the purified enzyme was studied for the effect of temperature and pH on its activity. For the effect of temperature, during the azoreductase assay the different temperatures were maintained during the incubation time. Similarly for the effect of pH, the buffer used in the azoreductase assay was manipulated to get different pH values of interest.

Results

After the serial dilution of samples and further plating on the DSC-97 media six colonies of moderately halotolerant *Bacillus* species were obtained which were purified by quadrant streaking. As the colonies were able to sustain salt concentration between (0.5 M and 2.5 M) in both the nutrient agar and DSC-97 (selective media for halophiles) they were called as moderately halotolerant.

Screening of all the six colonies was done they were ranked for the decolourization on the basis of visual identification as shown in Table 1. The formation of colourless zones around the colony indicates that the isolate is able to utilize the Azo dye as a source of nitrogen.

S.No.	Purified colonies	Ranking of Decolourization
1.	S_1	+
2.	S_2	+
3.	S_3	++
4.	S_4	+++
5.	S_5	-
6.	S_6	+

Table 1- Screening for azoreductase activity.
Ranking of dye decolourizers based on visual identification. +++ :Intense decolourization, ++ :Moderate decolourization, + : Slight decolourization., – :No decolourization

It can be seen from Table 1 that the isolate S_4 showed maximum decolourization and therefore it was selected for further studying the dye decolourization, purification of azoreductases and there characterization.

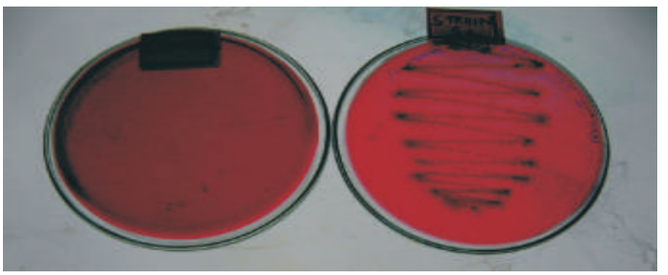

Figure : 1- Showing screening plate

The isolate S4 showing maximum decolourization was identified by staining and biochemical activities, the results of the same can be seen in Table 2.

S.No.	Characteristics	Reaction
1.	Grams staining	Gram positive
2.	Shape	Rod
3.	Spore	Present
4.	Catalase test	Positive
5.	Voges-Proskauer test	Positive
6.	Mannitol fermentation	Positive

Table 2-Grams staining & other characteristics of isolate S_4.

Percentage dye degradation by the isolate S_4 was studied in detail because it showed maximum decolourization in the screening media plates and the dye degradation percentage was calculated by the formula described earlier, it was found that the dye was found to be decolourized up to 64.89% within 20 days. Table 3 shows the result of percentage dye degradation on every alternate day. As can be seen from the Figure 2 / Table 3 there was a continuous increase in the degradation percentage of dye degradation it shows that with the increase in the number of colonies the in the culture flask the dye was readily degraded by the isolate in order to get the nitrogen source from the dye.

S.No.	Days	O.D at 532 nm for control	O.D at 532 nm for test	%Dye degradati-on for control	% Dye degradation for test
1.	0.	2.914	2.914	0.00	0.00
2.	2.	2.914	2.801	0.00	3.87
3.	4.	2.914	2.611	0.00	10.39
4.	6.	2.914	2.412	0.00	17.22
5.	8.	2.914	2.261	0.00	22.40
6.	10.	2.914	1.861	0.00	36.13
7.	12.	2.914	1.628	0.00	44.13
8.	14.	2.914	1.412	0.00	51.54
9.	16.	2.914	1.202	0.00	58.75
10	18.	2.914	1.141	0.00	60.84
11.	20.	2.914	1.023	0.00	64.89

Table 3- Percentage Dye degradation.

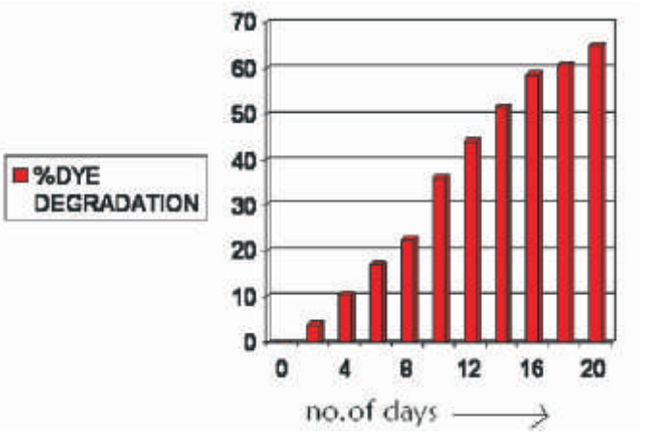

Figure: 2-Representation of %dye degradation

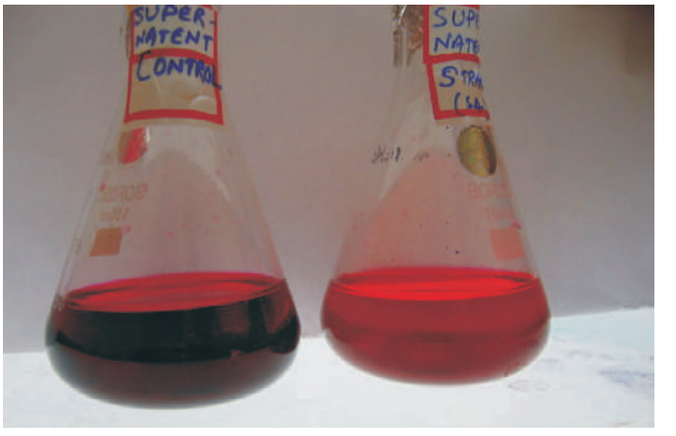

Figure: 3-Supernatants of both control ant test flask after 20 days

Histogram in figure 2 shows that there was a continuous increase in the percentage dye degradation with respect to the time elapsed.

The figure 3 shows that there was a decolourization in the flask being inoculated with the isolate S_4 thus showing that the microbe is involved in dye degradation.

The concentration of protein in crude and purified sample was estimated by the Lowry's method of protein estimation, a standard graph was plotted in order to get the concentration of the protein in the crude extract and the purified samples. The concentration of both the crude extract as well as the different elutes after ion exchange chromatography has been shown in the Table 4. It can be seen that the concentration of protein in the crude extract was 1390 µg/ml, and the maximum protein concentration of 610 µg/ml was found in the 100 mM elute.

S.No.	BSA (ml) (mM)	Distilled water (ml)	Reagent C (ml)	Fc Reagent (ml)	O.d at 660 (nm)	Concentration of protein (ìg/ml)
1.	0.0 (blank)	1.0	5	0.5	0.0	0.0
2.	0.2	0.8	5	0.5	0.111	200
3.	0.4	0.6	5	0.5	0.209	400
4.	0.6	0.4	5	0.5	0.312	600
5.	0.8	0.2	5	0.5	0.410	800
6.	1.0	0.0	5	0.5	0.506	1000
7.	Crude extract (0.2 ml)	0.8	5	0.5	0.712	1390
8.	100 elute (0.2 ml)	0.8	5	0.5	0.306	610
9.	200 elute (0.2 ml)	0.8	5	0.5	0.112	220
10	300 elute (0.2 ml)	0.8	5	0.5	0.091	180
11.	400 elute (0.2 ml)	0.8	5	0.5	0.072	140
12.	500 elute	0.8	5	0.5	0.050	100

Table 4 : Estimation of protein in crude & ion exchange elutes.

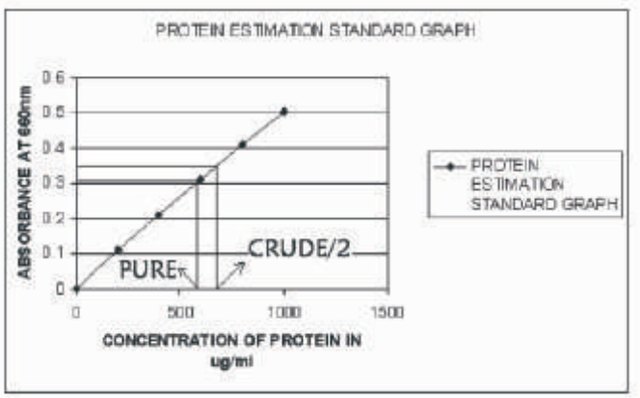

Figure : 4 -Standard graph for protein estimation

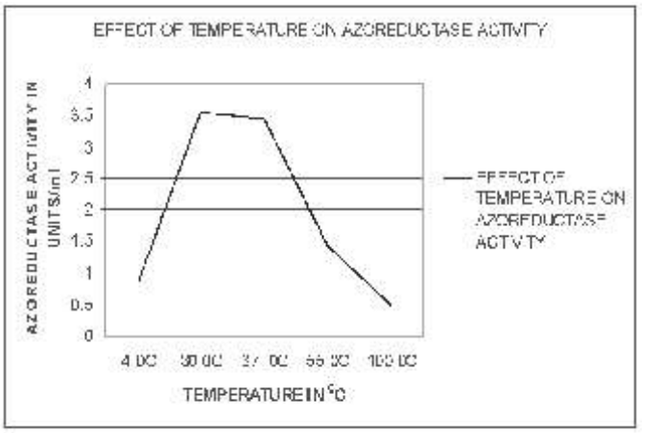

Figure: 5 - Effect of temperature on enzyme activity

Figure 4 above is the standard graph plotted by getting the absorbance of the known concentration of the protein, from this graph the concentration of the protein in the crude and the purified samples was easily determined by the help of absorbance readings of crude and purified samples.

Azoreductase assay was carried out for the crude as well as all the elutes of ion exchange chromatography. Table 5 depicts the results of the assay, it can be seen that the 100 mM elute of the ion exchange shows the maximum Azoreductase activity, thus it can be said that 100 mM elution buffer eluted out the maximum amount of enzyme in the ion exchange chromatography. This 100 mM elute was thus called as purified enzyme, and thus was characterized, for the effect of pH, and Temperature on its activity, and finally its molecular weight was determined by running it in SDS –PAGE along with a marker of known molecular weight.

As depicted by the results (Table 6) of effect of temperature on the enzyme activity it can be said that the Azoreductase enzyme purified from *Bacillus megaterium* has a temperature optimum ranging between 30^0 C and 45^0 C. This Optimum is quite good for the degradation of the azo dye containing effluents which normally have this range of temperature.

Figure 5 is the graphical representation of the effect of temperature on the activity of the Azoreductase purified from *Bacillus megaterium.*

The results shown in the Table 7 & Figure 6 clearly depicts that the Azoreductase purified from the *Bacillus megaterium* has a pH optimum ranging between pH 7-9 but the enzyme was found to be showing maximum activity at pH-7.

Molecular weight of the Azoreductase purified from *Bacillus megaterium* was determined by running the pure sample of Azoreductase in the SDS PAGE along with a marker BSA (Bovine serum albumin) of molecular weight 66k Da. As seen in the Fig. 7, after the staining and destaining procedures a band of pure protein is present nearly parallel to

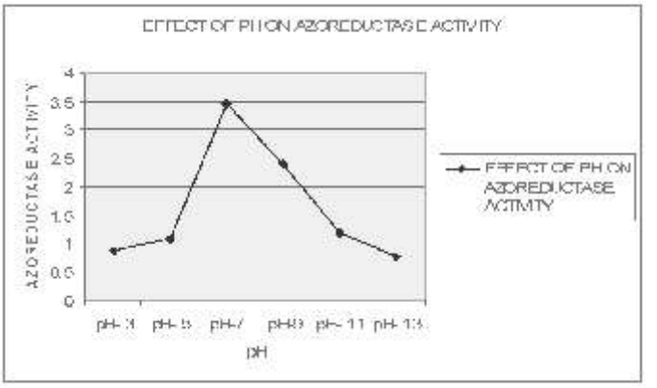

Figure : 6 - Effect of pH on Enzyme activity

S.No.	Tubes (mM)	Phosphate buffer (ml)	Azo dye solution (ml)	Enzyme solution (ml)	Equilibrate at room temperature for 5 minutes	2 mM NADH solution (ml)	δa 532(average decrease in o.d/minute)	Enzyme activity units/ml
1.	Blank	2.8	0.05	0.1 (Distilled water)		0.05	0.0	0.0
2.	Crude extract	2.8	0.05	0.1		0.05	0.750	7.129
3.	100 elute	2.8	0.05	0.1		0.05	0.379	3.602
4.	200 elute	2.8	0.05	0.1		0.05	0.150	1.425
5.	300 elute	2.8	0.05	0.1		0.05	0.140	1.330
6.	400 Elute	2.8	0.05	0.1		0.05	0.134	1.273
7.	500 Elute	2.8	0.05	0.1		0.05	0.104	0.988

Table 5 - Enzyme activity of crude and purified enzyme

S.No./Tubes (^{0}C)	Phosphate buffer ml)	Azo dye solution (ml)	Enzyme solution 100 mm(ml)	Equilibrate at room temper-ature for 5 min.(^{0}C)	2 mM NADH solution (ml)	Δa 532 (average decrease in o.d/min.)	Enzyme activity units/ml
Blank	2.8	0.05	0.1	25	0.05	0.0	0.0
4	2.8	0.05	0.1	4	0.05	0.090	0.855
30	2.8	0.05	0.1	30	0.05	0.370	3.517
37	2.8	0.05	0.1	37	0.05	0.360	3.422
55	2.8	0.05	0.1	55	0.05	0.150	1.425
100	2.8	0.05	0.1	100	0.05	0.050	0.475

Table 6 - Effect of temperature on enzyme activity of crude and pure enzyme

S. No.	Phosphate buffer (2.8ml)	Azo dye solution (ml)	Enzyme solution 100mm (ml)	Equilibrate at room temperature for five minutes	2mM NADH solution (ml)	äa 532 (average decrease in o.d/minute)	Enzyme activity units/ml
1.	pH-7 blank	0.05	0.1		0.05	0.0	0.0
2.	pH- 3	0.05	0.1		0.05	0.092	0.874
3.	pH- 5	0.05	0.1		0.05	0.114	1.083
4.	pH-7	0.05	0.1		0.05	0.365	3.469
5.	pH-9	0.05	0.1		0.05	0.254	2.414
6.	pH- 11	0.05	0.1		0.05	0.125	1.188
7.	pH- 13	0.05	0.1		0.05	0.080	0.760

Table 7- Effect of pH on enzyme activity

Figure 7: SDS–PAGE gel showing bands of marker and pure protein

the markers band and thus the molecular weight can be said to be equal 65 kDa.

Discussion

The isolate decolorized the dye substrates and Decolourizing efficiency was dependent on the growth of the isolate in the flask (Table3).There was neither growth nor decolourization in the control flasks. This showed that the decolourization was due to the metabolic activity of the organisms and not due to any abiotic factors.

Halophiles have been reported to be involved in the dye decolourization (SalahUddin *et al.,* 2007) .The moderately halotolerant *Bacillus* sp.isolated in this study decolourized the azo dye Red 2G to a extent of 64.89%.This rate of decolourization may be due to the high metabolic diversity being seen in the halophiles due to there extremophilic nature (Oren *et al.,* 1992; Ventosa *et al.,* 1998).

Decolourization of synthetic dyes is due to the cleavage of the chromophoric group which generates colourless metabolic

intermediates. This intermediate metabolites of the dye substrates are aromatic amines (Ganesh, 1992; Brown and DeVito, 1993). The results obtained in Table 3 show that the chromophoric groups of the dye were cleaved. The cleavage of the chromophoric group of dyes is a reduction process which requires redox equivalents (electron donars) that transfer electrons to the chromophoric group (electron acceptor) of dyes (Russ *et al.,* 2000).

The dye substrates were the only source of nitrogen left for the microbe after the initial utilization of the limited nitrogen source available in the media, the increase in the cell density as was visible in the broth evidenced that the isolate was utilizing the nitrogen available in the form of dye substrate. Bacterial utilization of azo dyes as a source of carbon, and energy have been reported (Yatome et al. 1993; Dykes *et al.,* 1994) as nitrogen source (Coughlin *et al.,* 1997) and as corbon,energy and nitrogen source (Oranusi and Mbah, 2005).This study is perhaps one of the few reports in the literature on the utilization of azo dye Red 2G as a source of nitrogen.

Aromatic amines generated by the reductive cleavage of the azodyes are potentially toxic, mutagenic and carcinogenic (Ganesh, 1992; Brown and Devito, 1993).The utilization of the dye as a sole source of nitrogen may detoxify the parent compounds or there intermediates. Detoxification of the azodyes by microbial cultures or laccase was attributed to the conversion of azo-nitrogen to non-toxic metabolites (Chivukula and Renganathan, 1995; Abadulla *et al.,* 2000).

The initial step of biodegradation of azo dyes is a reductive cleavage of the azo group under anaerobic conditions this reaction is catalyzed by a variety of biological systems and leads to the accumulation of aromatic amines (Ganesh, 1992; Brown and Devito, 1993). It is thought that under anaerobic conditions azo dyes can interact rather non-specifically with the compounds of the electron transport chain and thereby serve as artificial electron acceptors. Molecular oxygen inhibits this reaction since the electron do not reach the azo dyes when the natural acceptor is present (Wuhrmann *et al.,*1980) and since the postulated intermediates of the reaction hydrazine and /or the azo anion free radical, are reoxidized by oxygen. Exceptions to the oxygen sensitivity of the biological azo reduction have been represented by the azoreductases from the rat liver cytosol (Huang *et.al.,* 1979) and Orange II Azoreductase from *Pseudomonas* KF46. Azoreductase,the enzyme characterized in the present report is another oxygen insensitive Azoreductase.

In the present study the Azoreductase from moderately halotolerant B*acillus megaterium* was purified by a combination of ammonium sulphate precipitation, anion exchange chromatography, and the specific activity of purified enzyme was 6 U. Nachiyar *et al*. (2005) reported a specific activity of 23 U for Azoreductase they purified from *Pseudomonas aeruginosa.* However a comparison of activity values from the literature is difficult since most studies are based on decolourization of different azo dyes. The molecular weight of the Azoreductase being purified from *Bacillus megaterium* was determined to be 65 kDa (approximately). Previously molecular weights for azo reductases have been reported to be 30 kDa from *Pseudomonas* KF46 (Zimmermann *et al.,* 1982), 85 kDa from *Staphylococcus aureus* (Huizong *et al.,* 2005), 61.6±1.4 from *Bacillus* sp. Strain SF (Jurgen *et al.*, 2004) 29 kDa from Pseudomonas aerugenosa (Nachiyar *et al.*, 2005) 21.5 kDa from *Bacillus cereus* (Pricelius *et al.,* 2007). The optimum temperature and pH range for the activity of the Azoreductase being studied here was found to be between 30^0C and 45^0C pH 7–8 respectively, which is similar to the temperature and pH optima found in the literature (Hu, 2001; Huizhong *et al.,* 2005).

Conclusion

Finally based on the present study it can be concluded that Halophilic microflora can be a good source for the degradation of azo compounds present in the industrial effluents which are polluting the aquatic life as well as are equally harmful to animals and human beings. Halophiles have greater metabolic diversity and are able to easily survive in the hypersaline wastewaters effluents being discarded by the industries in which non-halophilic microflora can not survive and thus the effluent has first to be treated in order to make the effluent suitable for the growth of non-halophiles, which is a bit costlier and time consuming process, in such cases halophiles can be directly used without any pretreatment of the effluents. Azoreductase purified from *Bacillus megaterium* here is oxygen insensitive and thus treatment of effluents can be performed in very less cost at the industrial site as compared to the anaerobic treatment which requires large input.

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