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Purification and Characterization of Azoreductase from *B. licheniformis*

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ABSTRACT

Dye decolorizing bacteria was isolated and identified from textile effluent samples. The isolate was identified as Bacillus licheniformis by 16S rDNA sequencing. Azoreductase, an enzyme responsible for catalyzing the reductive cleavage of azo bonds was purified from B.licheniformis. The enzyme was purified by a combination of ammonium sulphate precipitation, ion exchange and affinity chromatography. The optimum pH and temperature of purified enzyme was found to be 7 and 20°C. The molecular mass of purified enzyme determined by SDS-PAGE was found to be 18 kDa.

***Key words**: Azoreductase, B.licheniformis, optimization, purification.*

INTRODUCTION

Synthetic dyes are widely used in textile, paper making, color printing, food, leather and cosmetic industries. The three most common group of dyes are azo, anthraquinone and phthalocyanine which are considered to be toxic to human beings and environment as well[1,2]. The azo dyes possess a complex aromatic molecular structure together with their intense color and toxicity makes them difficult to remove or biodegrade[3].

The nature of dyes are designed in such a way that they are resistant to light, water and oxidizing agents and hence they cannot be treated by conventional processes. Large quantities of these dyes are disposed as industrial effluent into the environment which inturn affects the photosynthetic activity of hydrophytes by impeding light penetration and posing serious threat to aquatic life[4]. Therefore the degradation or removal of dyes from effluents before discharge has become a great environmental challenge. In recent times biological processes are receiving more attention since they are cost effective and ecofriendly[5].

Azoreductase are class of enzymes that catalyzes the cleavage of azo bond (-N=N-) to produce colorless aromatic amine products. Various studies have been conducted on bacterial azoreductase, thus suggesting its application for bioremediation[6].

The present study was focussed on isolation and identification of dye decolorizing bacteria from effluents and purifying the enzyme azoreductase from bacteria.

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MATERIALS AND METHODS

Sample collection

Samples were collected from textile effluent water from Erode located in Kaveri river, Karunkalpalayam, India. Samples were collected in sterilized screw capped bottles and transported to the laboratory in an ice bucket.

Isolation and screening of dye decolorizing bacteria

4 different bacteria were isolated from textile effluent samples by serial dilution and streak plate method on Nutrient agar. Identification was done by colony, cellular, morphological characters and performing biochemical tests following Bergey's Manual of Systematic Bacteriology[7].

The high decolorizing bacteria was screened by performing a decolorization assay with Reactive blue 36 using UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) at its respective λ_{max} (502 nm). The bacteria showing highest percentage of decolorization was used for further studies. The percentage decolorization was calculated using the following equation

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance value - final absorbance value}}{\text{Initial absorbance value}} \times 100$$

Molecular identification of isolate

Isolation of genomic DNA from the bacterial isolates was done following the method from Experimental techniques in bacterial genetics[8]. The PCR primers used to amplify 16S rDNA fragments were bacteria specific primers[9]. A forward primer F27 (5'-AGAGTTTGATCMTGGCTCAG - 3') and a reverse primer R1492 (5'-ATAGGYTACCTTGTTACGACT - 3'). The reaction mixture consisted of 10pmol of each primer, 5µl from colony suspension as template DNA, 12.5µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA). The PCR reaction was set initial denaturing at 95ºC for 5 min, followed by 30 cycles of 30 sec of denaturing at 95ºC , 30 seconds of annealing at 50ºC and 2 min of elongation at 72ºC with final extension at 72ºC for 10 min for first set. The PCR product (1400 bp) was cleaned using a Qiagen DNA gel extraction Kit (QIAGEN, CA) according to directions of manufacturer. 16S rDNA sequences were initially analyzed at NCBI server using BLAST tool and corresponding sequences were downloaded. Evolutionary history was inferred using the Neighbor–joining method[10].

Assay of Azoreductase

The assay for azoreductase activity was done following the procedure described by Maier *et al.*, 2004[11]. Assays were carried out in cuvettes with total volume of 1ml Ultraspec 2100 UV-VIS Spectrophotometer (Amersham Biosciences). The reaction mixture consists of 400 µl of sample and 20µl Reactive blue 36 (500mg/l). The reaction was started by addition of 200µl of NADH (7mg/l) and was monitored photometrically at 502 nm at different time intervals. The linear decrease of absorption was used to calculate the azoreductase activity. One unit of azoreductase can be defined as the amount of enzyme required to decolorize 1µmol of acid per minute.

Purification of azoreductase from isolated bacteria

Crude enzyme preparation

A single colony of the bacterial isolates were grown in 100 ml of BHI (Brain Heart Infusion) broth containing 200 ppm of Reactive Blue 36. The culture was incubated at 37^{o}C till it reaches the OD value of 0.9 at 600nm (late log phase). The cells were harvested at 6000rpm for 10 min at 4^{o}C. The bacterial pellet was resuspended in 50 mM potassium phosphate buffer, pH-7 and the final concentrations of 1mg/ml of lysozyme and 10µl/ml DNase were added, and the sample was incubated at 37°C for 20minutes. The lysate was clarified by centrifugation at 10,000 for 30 minute at 4°C and the pellet was discarded. Ammonium sulfate was added at 4°C to the clarified cell extract and EDTA was added to a final concentration of 0.5mM. All subsequent purifications steps were carried out at 4°C.

Ammonium sulphate and ion exchange chromatography was carried out for further purification following the method of Misal *et al*., 2011[12]. The final step of purification was performed by affinity chromatography adopting the method of Maier *et al*., 2004[11].

pH and temperature optima of purified azoreductase

The effect of pH on azoreductase activity was determined by incubating the reaction mixture at pH ranging from 4-9. The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 20ºC to 40ºC in 50mM potassium phosphate buffer (pH 7). The relative activity of azoreductase at each temperature and pH were determined. The reaction was initiated by the addition of 0.1mM NADH.

Protein estimation

The protein concentration of azoreductase was estimated following the method of Lowry *et al.*, 1951[13] using BSA as standard protein.

SDS-PAGE gel electrophoresis

The SDS-PAGE analysis of purified azoreductase was carried out with 5% stacking and 12.5% resolving polyacrylamide gel following the method by Laemmli, 1970[14]. Protein bands were stained with Coomassie brilliant blue R-250.

Results and Discussion

Identification and phylogenetic position of isolated bacteria

Bacteria that could efficiently decolorize the reactive dyes were isolated from the textile effluent sample in a dyeing industry. The identification of the isolate was done on the basis of 16S rDNA sequences. The isolate was identified as *Bacillus licheniformis* by BLAST analysis at NCBI server. Gen bank accession number of the isolated strain was obtained (KP136793). Figure. 1 shows the phylogenetic position of *B.licheniformis* with other closely related strains. Anuradha *et al*., 2014[15] had isolated *B.subtilis* and *P.aeruginosa* from the effluents of dyeing industry.

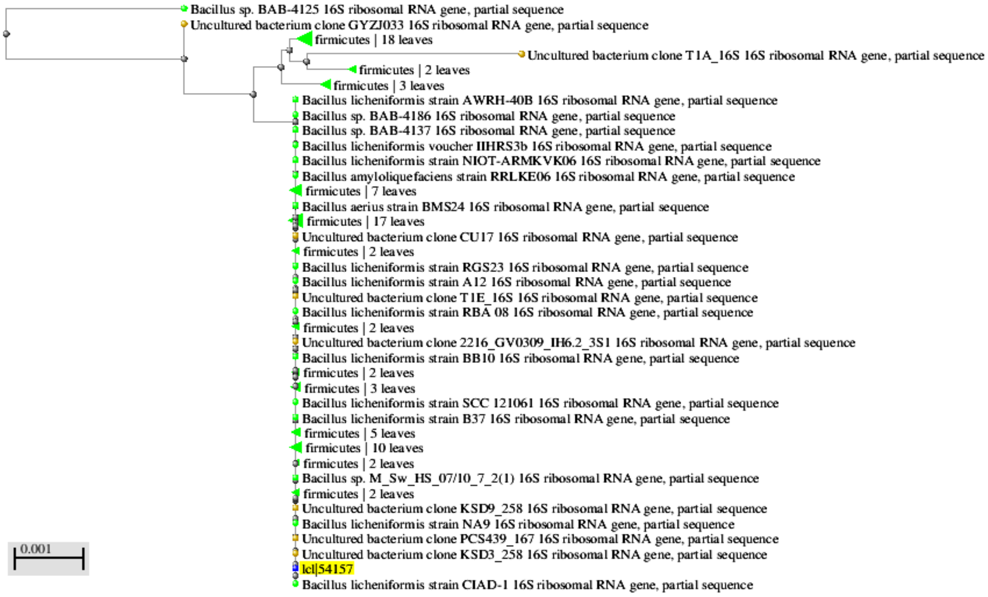

Fig. 1: Phylogenetic relationship of *Bacillus licheniformis* by Neighbor-joining method based on 16S rDNA gene sequences

Purification of azoreductase from *B.licheniformis*

A total amount of 580.73mg of protein was obtained from the crude extract of *B.licheniformis*. During each step of purification the activity was noted. It was found that the specific activity of the enzyme was increasing at every step of purification. The results are presented in table 1. Similar trend was reported by earlier studies[16.17]. Enzyme activity in crude extract was found to be 0.12U/mg and increased to 5.31U/mg at the final purification process. The effort was successfully made to achieve 86 fold purification with 31% recovery (approximately) and 5.31U/mg of total specific activity. Maier *et al.*,2004[11] had purified azoreductase from *Bacillus* strain SF by a 3 step purification (Ammonium sulphate precipitation, anion exchange chromatography, affinity chromatography) and the overall purification level was 250 fold. Protein separation by SDS-PAGE resulted in a single band equivalent to molecular mass of 18kDa approximately as shown in figure 2.

Table 1: Purification of azoreductase from *B. licheniformis*

Fraction	Total protein (mg)*	Total activity (U)*	Specificactivity ((U/mg protein/min)*	Yield %*	Purification factor (fold increase)*
Crude extract	580.73±61.18	23.59±0.27	0.12±0.05	100±0.00	1.29±0.08
Ammonium sulphate	203.90±1.49	24.73±0.08	0.012±0.28	95.29±0.86	9.27±0.59
DEAE Cellulase	7.20±0.73	15.78±0.68	2.191 ±0.66	60.80±0.92	75.73±0.28
Affinity chromatography	1.53±0.14	8.13±0.97	5.313±.0.49	31.32±0.29	86.06±.6.07

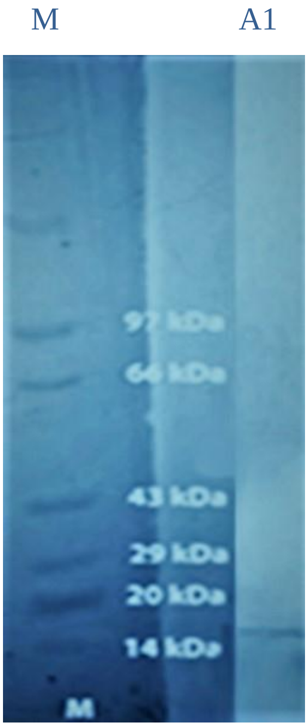

Figure 2: Molecular weight determination of azoreductase by SDS-PAGE.

(M- Molecular weight marker. A1- Purified azo reductase)

Determination of optimum pH and temperature for enzyme activity

The specific activity of enzyme at various pH was calculated and the results are presented in table 2. The highest enzyme activity was 15.52 U/ml noted at pH 7. But further increase in pH to 9, the activity declined slowly (6.05 U/ml). The lowest enzyme activity was 4.22 U/ml at pH 4. Thus in the present study pH 7 was found to be ideal for azoreductase activity. This is closely supported by the results of Saranraj *et al*., 2014[18]., Maier *et al*., 2004[11].

Table 2: Effect of pH on azoreductase activity

pH value	Activity U/ml
4	4.22±0.66
5	5.28±0.71
6	10.43±0.37
7	12.52±0.56
8	9.18±0.75
9	6.05±0.32

Determination of optimum temperature for enzyme activity

Effect of different temperature ranging from (20-40ºC) on azoreductase activity was studied. The results are shown in table 3. The optimum temperature was found to be 20ºC with an activity of 10.35U/ml. Further increase in temperature to 30, 35, 40ºC the activity of the enzyme was decreased gradually. Studies by Jahir Kahn (2011)[19] states that the optimum pH and temperature range for activity of azoreductase purified from *B.megateruim* was 30-45ºC , pH 7-8.

Table 3: Effect of temeperature on azoreductase activity

Temperature(ºC)	Activity U/ml
20	10.35±2.25
25	9.49±0.48
30	10.20±1.64
35	7.18±0.48
40	6.38±0.48

CONCLUSION

Azoreductase purified from bacteria (*B.licheniformis*) in the effluent sources could be efficiently used for decolorization process of azo dyes containing industrial wastewater under optimal conditions of pH 7 at 20ºC. However the physiological role and gene regulation of azoreductase in decolorization process remains to be elucidated.

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