ISSN: 2155-6199

Research Article Open Acces

Keywords Azoreductase; Nitroaromatic; Biotransformation; Biodegradation

## Introduction

Nitro aromatic compounds are major group of environmental pollutants released into the environment exclusively from anthropogenic sources. ey are mainly produced from incomplete combustion of petroleum and natural gasses. Nitro compounds are mostly utilized as synthetic intermediate in chemical, pharmaceutical industries. Some of them are routinely used in industrial solvents, dyes, agrochemicals and explosives. e direct discharge of these compounds as an industrial waste into the ecosystem is harmful to the biological system as well as human beings. Many of these compounds have mutagenic and carcinogenic potential. ese compounds can be converted to nontoxic compounds in the environment by microorganisms [1,2]. Nitro aromatic compounds such as nitrobenzene, nitrophenol, nitrotoluene and nitrobenzoate are common precursors for the synthesis of complex synthetic and industrial nitrogen containing aromatic organic compounds. Upon ingestion of nitro compounds into the human body it can be converted to toxic metabolic intermediates. ese metabolic intermediates can be further converted into the non-toxic compounds by various enzyme systems including some oxidases and reductases [3].

It was previously shown that the nitro aromatic compounds can be easily reduced under anaerobic conditions to aromatic amines by di erent kinds of microorganisms [4]. Complete mineralization of the nitro aromatic compounds under aerobic condition has also been demonstrated in several bacterial strains. Moreover, the involvement of reductase enzymes in the aerobic/anaerobic reduction mechanism of the nitro aromatic compound remains to be noteworthy. e reduction is mediated by single or two-electron system in the organisms. Single electron reduction reactions of nitro aromatic compounds are catalyzed by avin containing reductases such as NADPH: cytochrome P-450 reductase, ferredoxin: NADP reductase and bacterial oxygen-sensitive nitroreductases [5-7]. It was also well known that the two-electron reduction of nitro aromatic compounds to nitroso (NO) compounds and, subsequently, to hydroxylamines is speci cally catalyzed by bacterial oxygen-insensitive nitroreductases [7,8]. Some of the speci c nitroreductases have been isolated and characterized from anaerobic human intestinal bacteria [9].

Azoreductases are widely present in the microorganisms which can speci cally catalyze the reduction of azo (-N=N-) bond and $NO_2$ group of the complex organic compound. e reduction and / or biodegradation of toxic azo dyes by microorganisms have been extensively studied, and the primary role of azoreductases is explored [10,11]. Ra and Cerniglia (1993) demonstrated that the azoreductase and nitroreductase activities were indistinguishable [12]. e azoreductase catalyze the reduction of azo dyes in presence of avin and/or nicotinamide adenine dinucleotide/ nicotinamide adenine dinucleotide phosphate (NADH /NADPH) as an electron equivalent [13,14]. In our previous reports, we demonstrated that some of the nitro aromatic compounds can also be reduced by neutrophilic azoreductase by two electron reaction mechanism to its respective amines [15,16].

Alkaliphilic microorganisms are mostly ignored due to their rare occurrence and complex growth conditions. ese microorganisms can grow in highly alkaline conditions and can also tolerate the elevated temperature. Many alkaliphilic bacterial strains have been isolated and proven to be vital for the biotechnological and industrial applications. e alkaliphiles are largely known for its highly stable proteolytic and hydrolytic enzymes; these enzymes have wide industrial applications

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Received October 05, 2014; Accepted January 28, 2015; Published January 30, 2015

Citation: Misal SA, Humne VT , Lokhande PD, Gawai KR (2015) Biotransformation of Nitro Aromatic Compounds by Flavin-Free N9i-.c9Bzoru

amaranth dye as a substrate. e activity assay for each cofactor was performed separately in 1 ml reaction mixture contained 0.25 mM cofactor (NADH/ NADPH/ $FADH_2$ /$FMNH_2$), 0.05 mM amaranth dye and 50 µl of enzyme solution in 100 mM sodium phosphate bu er (pH 7.4). Change in the absorbance of amaranth dye at 520 nm was monitored by UV-visible spectrophotometer at 37°C.

e e ect of various metal ions on azoreductase activity was assayed with various metal ions such as $MgSO_4$, $MnSO_4$, $FeCl_3$, $CuSO_4$, and $HgCl_2$ and di erent concentration of EDTA, SDS. e activity assay was performed with 2 mM of metal ions and keeping other components similar as described above.

## Reduction of nitro aromatics

e reduction reaction was carried out at 37°C, in 20 ml of reaction mixture contained 1 mM nitro aromatic compounds, 1 mM NADH and 2 ml of enzyme solution (2 mg/ml) in 0.1 M sodium phosphate bu er (pH 7.4). e reaction was started with the addition of NADH and was monitored with constant stirring for 12 hrs. e absorbance of the reaction mixture was measured at each hour by UV-visible spectrophotometer. Immediately, a er 12 h, the 20 ml reaction mixture was diluted with 20 ml of DCM. e dissolved organic compounds were recovered from DCM and separated on silica gel column chromatography. e purity of the transformed products were checked by thin layer chromatography with hexane/ethyl acetate (4:1, v/v) and visualized under UV light. e pure transformed products were analyzed by IR and NMR spectroscopy.

# Results and Discussion

## Puri cation of azoreductase from *B. badius* D1

e avin-free NADH-azoreductase has been puri ed from *B. badius* D1 by two-step procedure summarized in Table 1. e puri ed azoreductase appeared to be a single band on SDS and native-PAGE corresponding to a molecular mass of approximately 43 kDa (Figure 1a). Moreover, the single peak was obtained during the size exclusion chromatography elution of azoreductase corresponding to the molecular size of 43 kDa (Figure 1b). It suggests the monomeric nature of azoreductase. Previously, azoreductase was reported to be a monomeric in nature from *Pseudomonas sp.*, *Bacillus sp.* bacterial strains. Furthermore, the homodimer and homotetramer form of azoreductase was shown from *Shigella dysenteriae* type 1 and *Staphylococcus aureus* respectively [13,19,24,25].

## Characterization of azoreductase

e e ect of pH, temperature on azoreductase activity and thermal stability is already described [13]. e optimum activity of the puri ed enzyme was observed at pH 7.4 and 60°C. is enzyme has wide substrate speci city including mono and di azo dyes. e substrate speci city was further studied with some nitro aromatic compounds.

## Analysis of cofactor requirements and substrate speci city of azoreductase

Some azoreductases are avin containing, or they require avins as a cofactor for electron transfer [26,27]. ese types of azoreductases are categorized as avin dependent azoreductases. In the present study, thorough analysis by TLC and UV-visible spectroscopy signi ed that the puri ed enzyme does not contain avin as a cofactor. In addition, the externally added reduced avins did not enhance the azoreductase activity (Table 2a). It clearly demonstrates that this azoreductase is neither avo-protein nor avin dependent. e avin-free monomeric

| Puriýcation step | Total protein (mg) | Total activity (Units) | Speciýc activity (U/mg) | Puriýcation fold | % Yield |
|---|---|---|---|---|---|
| Cell lysate | 1871 | 929 | 0.49 | 1 | 100 |
| 80% $(NH_4)_2SO_4$ precipitation | 743 | 655 | 0.88 | 1.8 | 71 |
| Sephadex G-100 size exclusion chromatography | 27 | 138 | 5.11 | 10.4 | 15 |

Table 1: 3 X U L ¿ F D W L R Q V X P P D U \ R I B. badius D1 H G X F W D V H

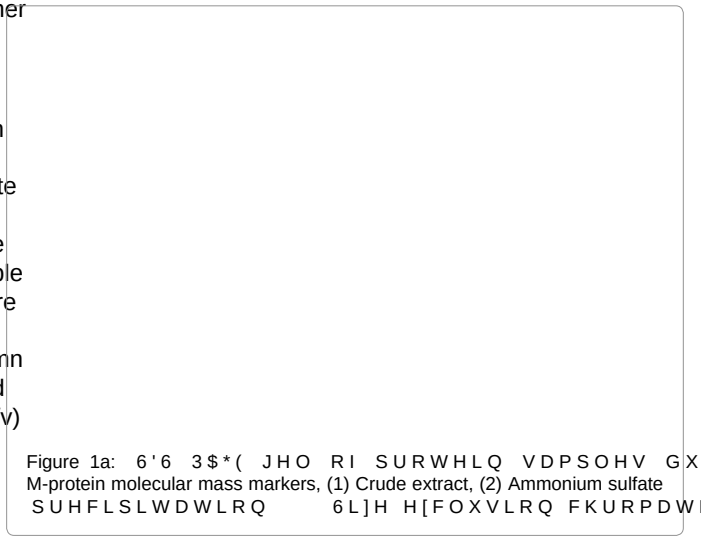

Figure 1a: 6 ' 6 3 $ * ( J H O R I S U R W H L Q V D P S O H V G X U L Q M-protein molecular mass markers, (1) Crude extract, (2) Ammonium sulfate S U H F L S L W D W L R Q 6 L ] H H [ F O X V L R Q F K U R P D W R J U

Figure 1b: 0 R O H F X O D U P D V V H V W L P D W L R Q R I S X U L ¿ H G P R O H F X O D U P D V V V W D Q G D U G V D P \ O D V H N ' D 150 kDa, bovine serum albumin: 66 kDa, carbonic anhydrase: 29 kDa, and lysozyme: 14 kDa) were used. Kav is calculated for each standard marker protein by the formula: Kav 9 H í 9 R 9 W íKav values are then plotted versus the log of protein molecular mass to make a standard curve. (o: $ ] R U H G X F W D V H " 3 U R W H L Q V W D Q G D U G V

| Sr. No | Cofactor | (µM) | (U/mg protein) |
|---|---|---|---|
| 1 | NADH | 1.02 ± 0.07 | 16.5 ± 2.05 |
| 2 | NADPH | 6.12 ± 0.35 | 13.2 ± 1.2 |
| 3 | $FADH_2$ | 4.71 ± 0.81 | 10.8 ± 1.75 |
| 4 | $FMNH_2$ | 4.14 ± 0.76 | 10.3 ± 1.42 |

The Km and Vmax values were determined by Lineweaver-Burk plot. ± is the standard deviation of the mean of three independent experiments.

Table 2a: Cofactor requirement for azoreductase activity.

Citation: Misal SA, Humne VT, Lokhande PD, Gawai KR (2015) Biotransformation of Nitro Aromatic Compounds by Flavin-Free NADH-Azoreductase. J Bioremed Biodeg 6: 272. doi:

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18. = L P P H U P D Q Q