# PURIFICATION AND CHARACTERIZATION OF NITROREDUCTASE FROM RED ALKALIPHILIC BACTERIUM *Aquiflexum* sp. DL6

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*Biochemistry Division, Department of Chemistry, University of Pune, Pune 411 007, India*
*e-mail: krgawai@chem.unipune.ac.in*
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Nitroaromatic compounds are toxic to living organisms. Most of them exhibit human mutagenic and carcinogenic potential. Biotransformation and bioremediation processes can convert these compounds into non-toxic compounds. Acclimatization of bacterial strain *Aquiflexum* sp. DL6 with nitro-aromatics resulted in significant induction of nitroreductase (EC 1.5.1.34). The enzyme was purified by the combination of DEAE-cellulose and Sephadex G-100 column chromatography with 80-fold purification and 22% yield. Molecular weight of purified nitroreductase was estimated to be 29 kDa by SDS-PAGE. The enzyme characteristics were explored by varying the pH and temperatures, and the optimum activity was found at pH 9.5 and 40°C. It was revealed that the substrate specificity of nitroreductase of *Aquiflexum* sp. DL6 was wide for the most of the tested nitro-aromatic compounds. The kinetic parameters like Michaelis constant and velocity maxima were determined with o-nitrophenol and NADH as substrates.

Nitro-aromatics compounds are found as potential environmental pollutants representing their wide use as explosives, pesticides, dyes, polyurethane foams, pharmaceuticals and plastics, etc. [1–5]. They are stable and persistent in the environment and toxic to living organisms. Its exposure poses many health hazards by affecting reproductive and central nervous systems, heart and liver, many eventually leading to death [6–9]. Moreover, its reduced intermediates also exhibit mutagenic and carcinogenic potentials in humans [2, 3, 10–11].

Most reports suggested the physico-chemical methods for removal of nitroaromatic compounds from waste water. Use of microorganisms for removal of these compounds is the best and cost effective approach [12]. Microorganisms can transform the nitro aromatic compounds by reducing the aromatic ring by means of the monooxygenase and dioxygenase enzyme. Monooxygenase and dioxygenase insert a single oxygen atom or two hydroxyl groups into the aromatic ring, respectively, which lead to an elimination of nitro group. In addition, nitroreductase is able to carry out the reduction of the nitro group to the corresponding amino derivatives [13].

In biotransformation, enzymes which catalyze the reduction of nitro aromatic compounds are termed as nitroreductases. The sensitivity of a particular nitroreductase towards oxygen has been used to classify these enzymes into oxygen-sensitive and insensitive type. The mechanism underlying the observed oxygen sensitivity of certain nitroreductases was found to involve the re-oxidation of the one-electron reduced nitroanion radical to the parent compound with the concomitant formation of superoxide [14] while, the oxygen-insensitive enzymes catalyze an obligatory two-electron reduction of this substrate [15–17].

More recently, studies have discovered partial reduction of the nitro group to a hydroxyl amino derivative which eventually releases nitrogen as ammonia. The reductive pathway requires one mole of an oxygen and one mole of NADH to convert nitro aromatic compound to important metabolic intermediates and release ammonia [4, 5, 13].

Alkaliphiles are the microorganisms that grow optimally at pH above 9.0 but cannot or slowly grow near neutral pH values. Differences between internal and environmental pH across the plasma membrane of the cell lead to rapid transportation of ions and substances. Accordingly, the cell keeps the intracellular pH in the range between 7.0 and 8.5 in order to thrive in alkaline environments [18–20]. Various enzymes isolated and well characterized from alkaliphiles were azoreductase, pullulanases, alkaline amylases and cellulases, xylanases and pectinases, etc. [21–23].

However, a thorough exploration of the biochemical and kinetic properties of nitroreductase purified from alkaliphiles has not been well focused in the literature. The aim of the study was to purify and carry out of preliminary characterization of a nitroreductase from red alkaliphilic bacterium *Aquiflexum* sp. DL6.

## MATERIALS AND METHODS

**Chemicals.** Nutrient agar, EDTA, DTT, NADH and nitro compounds were obtained from SRL (India). DEAE-cellulose and Sephadex G-100 were pur-

chased from Sigma, (USA ) and Pharmacia Fine Chemicals (Sweden), respectively. All other chemicals were of the highest grade of purity and commercially available.

**Microorganism and growth conditions.** The *Aquiflexum* sp. DL6 strain was collected from alkaline Crater Lake of Lonar (India). Isolation of pure strain of a microorganism was done by serial dilution and plate methods. The *Aquiflexum* sp. DL6 bacterial strain was identified by 16S rRNA method described earlier [23] (data not shown). The sequence of the 16S rRNA gene of the strain *Aquiflexum* sp. DL6 is available on NCBI database (GenBank ID: JF812063).

*Aquiflexum* sp. DL6 bacterial strain was grown in the cultivation medium containing (g/l): yeast extract — 5.0, peptone — 5.0 and sodium chloride — 5.0 with trace elements (mg/l): $KH_2PO_4$ — 300, $Na_2HPO_4$ — 980 and $MgSO_4$ — 10. The pH was adjusted to 9.0. Flasks containing 100 ml of a medium were inoculated with 5 ml of microorganism suspension and incubated at 37°C. After sufficient growth for 24 h, nitro compounds were added from the stock solutions, with final concentration 1 g/l.

**Crude extract preparation.** After 24 h incubation, the bacterial cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C, washed with physiological saline (0.85% NaCl) and thrice with 0.1 M sodium phosphate buffer (pH 7.4). The pellet was suspended in 50 ml of the same buffer containing 1 mM EDTA, 1 mM DTT, 1 mM lysozyme and 20% glycerol $(v/v)$. Cells were disrupted at 4°C by sonication for 30 seconds, 6 times with 70% outputs using a Sartorius labsonic (Germany). Cell debris was removed by centrifugation at $10,000 \times g$ for 15 min at 4°C. The supernatant obtained constituted the crude bacterial extract and was used for further studies.

**Estimation of protein.** Concentration of protein at each step of purification was checked by Lowry's method using BSA as a standard. Protein concentration in the chromatographic fractions was monitored by measuring absorbance at 280 nm.

**Assay of nitroreductase activity.** The activity of nitroreductase was determined spectrophotometrically at 37°C using a UV-visible spectrophotometer, Jasco V 630 (Japan) by monitoring a decrease of $OD_{410}$ based on the procedure described by Bryant et al. with slight modifications [15]. The reaction mixture 3 ml contained 0.1 M sodium phosphate buffer (pH 7.4), 1 mM NADH and 0.2 mM substrate o-nitrophenol (**ONP**) and 100 μl of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 mM of ONP per min. All experiments were performed at least in triplicates.

**Purification of nitroreductase from *Aquiflexum* sp. DL6.** The bacterial culture of *Aquiflexum* sp. DL6 was incubated with various nitro compounds for the specific induction of nitroreductase enzyme. After the ac-

climatization for 72 h at 37°C for various nitro compounds, nitroreductase was isolated and purified with the combination of following procedures at 4°C.

*Ammonium sulfate precipitation.* Solid ammonium sulfate was added with constant stirring to the crude extract over a period of 12 h at 4°C to get 40% of the salt saturation. The resulting precipitate was separated by centrifugation at 4°C and $15,000 \times g$ for 20 min and the supernatant saturated up to 80% with solid ammonium sulfate. The resulting precipitate was collected and dissolved in a minimal volume of 0.1 M phosphate buffer (pH 7.4). The protein solution was dialyzed against the same buffer at 4°C for 24 h.

*Ion-exchange chromatography.* After dialysis, the solution was loaded on pre-equilibrated DEAE–cellulose column (2 × 30 cm) with equilibrating buffer 0.1 M phosphate buffer, pH 7.4. The protein was eluted with a linear gradient of NaCl (0–500 mM) in the same buffer. Fractions of 5 ml were collected, those fractions showed higher nitroreductase activity were pooled and reverse dialyzed against solid sucrose at 4°C to reduce the volume.

*Molecular exclusion chromatography.* The reverse dialyzed enzyme preparation was applied to a Sephadex G-100 column (2 × 30 cm) equilibrated with 2 bed volumes of equilibrating buffer. The enzyme was eluted by the same buffer at a flow rate of 6 ml/h. Fractions of 3 ml were collected and those that showed higher nitroreductase activity were pooled and reverse dialyzed against solid sucrose at 4°C to concentrate the protein.

**Characterization of purified nitroreductase from *Aquiflexum* sp. DL6.** SDS-PAGE was made with 4.5% stacking and 10% resolving polyacrylamide gels as described by Laemmli [24]. Electrophoresis was performed with at least 20 μg of protein samples per well and 20 μg of protein molecular weight markers in a Bangalore Genei Midi Vertical Unit (India) at constant voltage of 100 V for 1 h. To check the homogeneity of nitroreductase, native PAGE was performed without SDS according to the method of Laemmli. Protein molecular mass standards (Bio Lit, India) were used. Gels were stained for proteins with silver [25].

*Determination of optimal pH and temperature of purified nitroreductase.* The influence of pH on the nitroreductase activity was studied over a wide range of pH (from 3.6 to 10.7) using a mixture of different buffers adjusted to the same ionic strength 100 mM (carbonate, sodium phosphate, and sodium acetate). At each pH the enzyme was incubated with buffer for 30 min at 37°C, and residual activity was checked with UV-visible spectrophotometer.

Effect of temperature on activity of nitroreductase was studied by detecting the nitroreductase assay at temperature range from 10 to 90°C in 100 mM sodium phosphate buffer (pH 7.4).

*Determination of thermal stability.* The purified enzyme samples were pre-incubated with 100 mM phos-

**Table 1.** The purification of the *Aquiflexum* sp. DL6 nitroreductase

| Purification step | Total protein, mg | Total activity, U | Specific activity, U/mg protein | Purification, -fold | Yield, % |
|---|---|---|---|---|---|
| Crude extract | 519.87 | 346.42 | 0.67 | 1 | 100 |
| (NH$_4$)$_2$SO$_4$ precipitation | 46.50 | 154.73 | 3.33 | 4.99 | 45 |
| DEAE-cellulose | 3.00 | 92.84 | 30.95 | 46.19 | 27 |
| Sephadex G-100 | 1.40 | 74.79 | 53.61 | 80.45 | 22 |

phate buffer (pH 7.4) at different temperatures between 10 to 90°C for 30 min, brought to room temperature and the enzyme activity was measured.

*Inhibition studies of nitroreductase activity.* The effect of EDTA, SDS and metal salts such as MgCl$_2$, ZnSO$_4$, AgNO$_3$, CuSO$_4$ and HgCl$_2$ on nitroreductase activity was explored. The purified enzyme from *Aquiflexum* sp. DL6 was incubated with different concentrations of EDTA, SDS (0.2 to 2 mM) and metal salts for 30 min at 37°C followed by the measurement of residual activity under the standard assay conditions.

*Kinetic studies of puried nitroreductase.* Initial velocities of the enzymatic reactions were performed by varying the concentration of one substrate, o-nitrophenol (from 0.05 to 1 mM) or NADH (from 0.05 to 1 mM), while the concentration of the second substrate was kept constant. Michaelis constants ($K_M$) and maximal velocity ($V_{max}$) for the reduction of o-nitrophenol and the oxidation of NADH by the puried nitroreductase from *Aquiflexum* sp. DL6 were determined from Lineweaver—Burk plots.

*In vitro transformation of nitro compounds by nitroreductase from Aquiflexum sp.* The reaction mixture 3 ml contained 0.1 M sodium phosphate buffer (pH 7.4), 1 mM NADH and 0.2 mM nitro compounds and 0.2 ml of the purified enzyme solution. The reactions were monitored with constant stirring in water bath at 37°C, and the decrease of OD$_{410}$ was measured at every 30 min.

## RESULTS

**Purification of nitroreductase from Aquiflexum sp. DL6.** Acclimatization of bacterial strain *Aquiflexum* sp. DL6 with nitro-aromatics for 72 h resulted in significant induction of nitroreductase. Total 520 mg of protein was obtained from crude extract of disrupted cells of *Aquiflexum* sp. (11.34 g wet weight). In the purification steps of the enzyme, more than 90% of contaminating proteins were eliminated during ammonium sulfate precipitation. The sample was then dialyzed to remove the salts and loaded on DEAE cellulose column; all the fractions were collected and checked for its activity. The concentrated enzyme solution applied to Sephadex G-100 yielding approximately 80-fold purification (Table 1). The molecular

weight of nitroreductase was estimated by native and SDS-PAGE and found to be ~29 kDa (Fig. 1).

**Characterization of purified nitroreductase from *Aquiflexum* sp. DL6.** *Effect of pH and temperature on the nitroreductase activity.* The effect of pH on purified nitroreductase from *Aquiflexum* sp. DL6 was determined at various pH from 3.6 to 11.2 at 37°C and the optimum pH was found to be 9.5 (Fig. 2).

Effect of temperature was studied by incubation of assay mixture of nitroreductase from 10 to 90°C. Optimum enzyme activity was observed around 40°C and further increase in temperature showed a decrease in activity, the enzyme activity retained more than half of its total activity at 60°C (Fig. 3).

Thermal stability was determined by incubating the purified enzyme with phosphate buffer at various temperatures. It was found that the nitroreductase from *Aquiflexum* sp. DL6 had maximum thermal stability at 40°C.

*Inhibition studies of nitroreductase activity.* Influence of metal ions on nitroreductase activity was tested with addition of equimolar concentration of metal
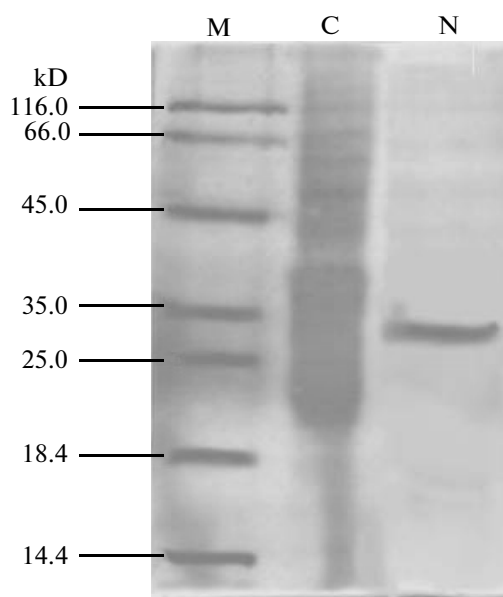


**Fig. 1.** SDS-PAGE pattern of the purified nitroreductase from *Aquiflexum* sp. DL6 (lane N). Lane M — protein molecular markers, C-crude extract.
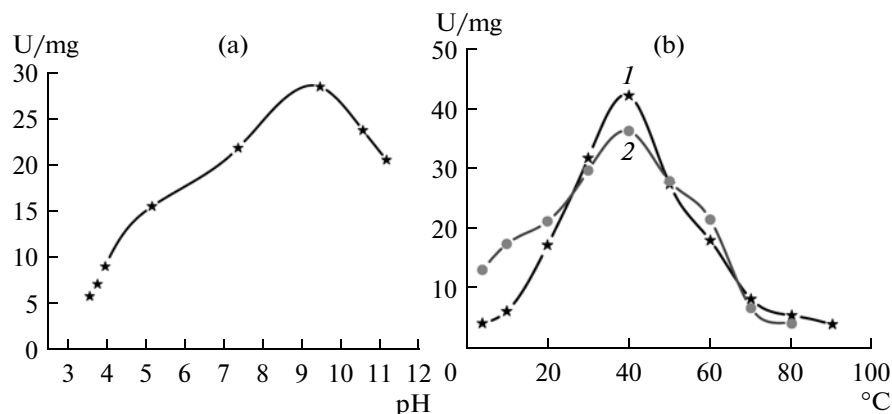
**Fig. 2.** Effect of pH (a) and temperature (b) on the *Aquiflexum* sp. DL6 nitroreductase activity. *1* — effect of temperature; *2* — thermal stability.
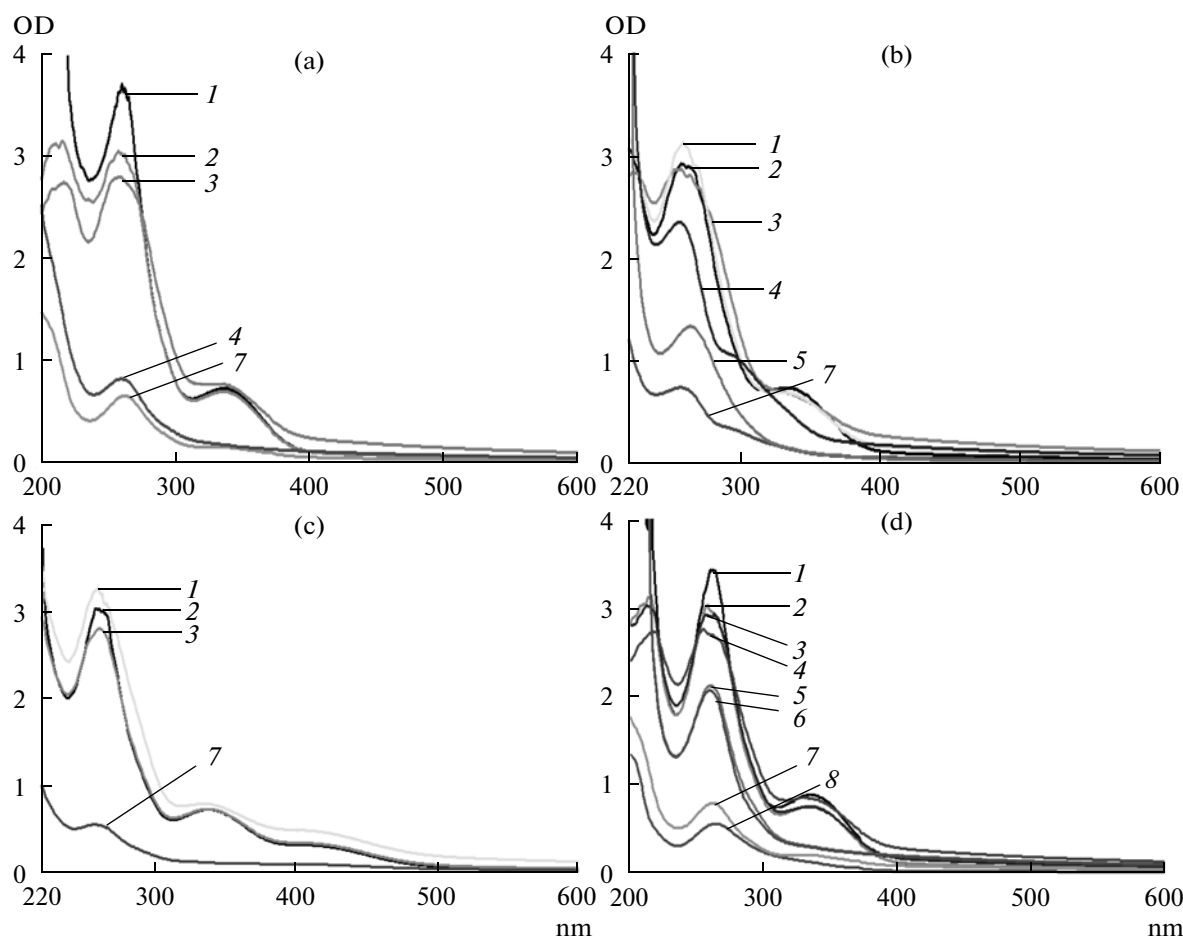


**Fig. 3.** UV-visible spectra of *in vitro* reduction of nitro compounds by the purified *Aquiflexum* sp. DL6 nitroreductase in the presence of 1 mM NADH. a — 3-nitrobenzaldehyde, b — m-nitrobenzoic acid, c — o-nitroaniline, d — m-nitrotoluene. *1* — 0 min, *2* — 30 min, *3* — 1 h, *4* — 2 h, *5* — 5 h, *6* — 12 h, *7* — 24 h , *8*— 48 h of incubation.

salts. Addition of 2 mM AgNO$_3$ completely inhibited the activity of nitroreductase while ZnSO$_4$, HgCl$_2$ and CuSO$_4$ taken in the same concentration inhibited enzyme activity from 50 to 68%. MgCl$_2$ did not inhibit the nitroreductase activity. Addition of EDTA and SDS in the reaction mixture at the concentration 0.2

to 2.0 mM resulted in concentration-dependent decrease in the nitroreductase activity, at 2.0 mM concentration of both EDTA and SDS had shown around 45% decrease in the activity (Table 2).

*Substrate specificity of nitroreductase.* To determine the substrate specificity of nitroreductase, the activity

of the enzyme was determined by using 7 different nitro-aromatic compounds as a substrate at equimolar concentrations. The assay conditions were identical and ONP was considered as a control. The *Aquiflexum* sp. DL6 nitroreductase also effectively reduced other nitro compounds such as p-nitrophenol, 3-nitrobenzaldehyde, 4-nitrobenzoic acid, o-nitroaniline, m-nitrotoluene and 1-chloro-2-nitrobenzene. After addition of p-nitro phenol or 4-nitro benzoic acid more than 80% of nitroreductase activity was revealed. In the presence of 3-nitrobenzaldehyde or 1-chloro-2-nitrobenzene more than 50% of enzyme activity was found but o-nitroaniline or m-nitrotoluene showed less than 50% activity.

*Kinetic studies of nitroreductase.* The $K_M$ and $V_{max}$ (velocity maxima) was obtained by measuring the enzyme activity by varying the concentrations of one of the substrates or NADH and keeping the concentration of the second substrate fixed. In case of ONP, the $K_M$ and $V_{max}$, values were 1.28 mM and 12.5 U, respectively. While, in case of NADH, the $K_M$ and $V_{max}$ were found to be 0.35 mM and 56 U, respectively for 0.2 mM ONP.

*In vitro reduction of some nitro compounds with the purified nitroreductase.* The nitro reduction potential of the purified nitroreductase from *Aquiflexum* sp. DL6 has been examined by incubating 4 nitro aromatic compounds like 3-nitrobenzaldehyde, m-nitrobenzoic acid, o-nitroaniline, o-nitrotoluene. The decrease in absorbance and shift in peak position in some cases resulted in formation of aromatic amines (Fig. 4). During this *in-vitro* transformation, NADH served as an electron donor.

## DISCUSSION

There is no available data on the nitroreductase from alkaliphiles and especially from red *Aquiflexum* sp. in literature. We purified the enzyme from *Aquiflexum* sp. DL6 by three-step procedure resulting in 80-fold purification and 22% at final step. A single band was observed in native and SDS-PAGE indicating that the nitroreductase active form might be a monomer. The purified *Aquiflexum* sp. DL6 enzyme was found capable of reducing nitroaromatic compounds under aerobic conditions indicating that the mechanism involved an obligatory two electron transfer from NADH. Thus, this enzyme could be classified as an oxygen-insensitive nitroreductase [15].

The nitroreductase of *Aquiflexum* sp. DL6 had optimum pH at around 9.5; however, it demonstrated comparable activity over the range of pH 7 to 11. Commonly, it is the ability of alkaliphilic microorganisms to live in high pH conditions by successfully lowering the pH across the cell wall and membrane keeping the intracellular pH near to neutral values. We observed that the nitroreductase from *Aquiflexum* sp. DL6 had the temperature optimum at 40°C. Temperature optima for different nitroreductases reported in the literature ranged from 25

**Table 2.** Influence of inhibitors on the *Aquiflexum* sp. DL6 nitroreductase activity

| Inhibitors | Concentration, mM | Residual nitroreductase activity, % |
|---|---|---|
| Control (NADH) | 2.0 | 100 |
| EDTA | 0.2 | 91 |
| | 0.6 | 81 |
| | 1.0 | 80 |
| | 1.4 | 78 |
| | 1.8 | 58 |
| | 2.0 | 49 |
| SDS | 0.2 | 79 |
| | 0.6 | 72 |
| | 1.0 | 66 |
| | 1.4 | 61 |
| | 1.8 | 57 |
| | 2.0 | 55 |

to 50°C [26, 27]. The purified enzyme from *Aquiflexum* sp. DL6 was found thermally stable about 75% at 60°C. Hence, this might be the most thermostable nitroreductase ever reported. The substrate specificity of the enzyme was tested with 7 different nitro compounds. The rate of decolorization or reduction changed depending on structure of the compound. Purified nitroreductase from *Aquiflexum* sp. DL6 was able to utilize all the nitro compounds used as a substrate with different efficiency according to the substituted groups on the aromatic ring present in structure that coincided with similar data in the literature [1, 9, 28, 29].

The majority of the reported nitroreductases are known to be flavoproteins [29]. For *Aquiflexum* sp. DL6 enzyme, the flavin was not detected in thin layer chromatography and any significant peak did not shown between 350−500 nm in UV-visible spectrum, indicating the absence of a bound flavin to the enzyme.

In conclusion, the purified nitroreductase from *Aquiflexum* sp. DL6 differed from those described previously from other bacteria. The enzyme could utilize NADH as cofactor and electron donor. $AgNO_3$ could inhibit the nitroreductase activity completely. It exhibited wide substrate specificity for nitro aromatic compounds and optimal activity at high alkaline conditions. The revealed enzyme properties might have economic potential in many industrial and biotechnological processes, specifically, agriculture, food, detergents, textile, leather and paper industries. It is expected that numerous nitroreductases will be found in alkaliphiles in the coming years and they will be used in novel biocatalytic processes and biotransformation reactions.

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