

Characterization of *Thiomonas delicata* arsenite oxidase expressed in *Escherichia coli*

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Abstract Microbial arsenite oxidation is an essential biogeochemical process whereby more toxic arsenite is oxidized to the less toxic arsenate. *Thiomonas* strains represent an important arsenite oxidizer found ubiquitous in acid mine drainage. In the present study, the arsenite oxidase gene (*aioBA*) was cloned from *Thiomonas delicata* DSM 16361, expressed heterologously in *E. coli* and purified to homogeneity. The purified recombinant Aio consisted of two subunits with the respective molecular weights of 91 and 21 kDa according to SDS-PAGE. Aio catalysis was optimum at pH 5.5 and 50–55 °C. Aio exhibited stability under acidic conditions (pH 2.5–6). The V_{\max} and K_m values of the enzyme were found to be $4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and 14.2 μM , respectively. SDS and Triton X-100 were found to inhibit the enzyme activity. The homology model of Aio showed correlation with the acidophilic adaptation of the enzyme. This is the first characterization studies of Aio from a species belonging to the *Thiomonas* genus. The arsenite oxidase was found to be among the acid-tolerant Aio reported to date and has the potential to

be used for biosensor and bioremediation applications in acidic environments.

Keywords Acidic tolerance · Arsenite oxidase · Molecular modeling · Recombinant expression · *Thiomonas delicata*

Introduction

Arsenite oxidase catalyzes the oxidation of arsenite to arsenate by a two-electron transfer. Arsenite oxidase consists of two heterologous subunits; a large catalytic subunit (AioA) with bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor and a 3Fe-4S iron sulfur cluster, and a small subunit (AioB) with Rieske 2Fe-2S center. Arsenite oxidase is a member of the dimethylsulfoxide (DMSO) reductase family of molybdoenzyme which is a family of different prokaryotic redox enzymes that function in electron transport chain (Romão 2009).

Arsenite oxidizing bacteria have been isolated from diverse arsenic contaminated environments, such as cattle-dipping fluids, hot springs, mine tailing and drainage water. As an ancient bioenergetic enzyme which utilizes arsenite, a highly toxic metalloid as substrate, characterization of arsenite oxidase is of special interest for bioremediation and biosensor construction. To date, there are two extensively characterized arsenite oxidase which origin from a chemolithoautotrophic *Alphaproteobacteria Rhizobium* sp. NT-26 and heterotrophic *Betaproteobacteria Alcaligenes faecalis*. Both bacteria grow optimally at slightly alkaline pH 8, which can be categorized as alkaline-tolerant bacteria.

In mining environments, particularly acid mine drainage (AMD), inorganic As(III) are widespread as a result from the bioleaching of arsenic-bearing minerals. Under low pH

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condition, arsenite oxidizers from these extreme environment are commonly isolated with acid-tolerant properties with an optimum growth pH near neutrality with the exception of *Thiomonas* strains (Hallberg and Johnson 2003; Battaglia-Brunet et al. 2006; Katayama et al. 2006; Duquesne et al. 2008; Bryan et al. 2009; Arsène-Ploetze et al. 2010), that grow optimally around pH 5. Microbial metabolism in AMD causes natural attenuation process to occur whereby *Thiomonas* strains are suggested to have acted as the arsenite oxidizer (Casiot et al. 2003; Duquesne et al. 2003; Morin et al. 2003; Bruneel et al. 2006; Battaglia-Brunet et al. 2011; Bertin et al. 2011). *Thiomonas* strains are therefore essential for maintaining the arsenic biogeochemical cycle in AMD. Oxidation of As(III) plays a pivotal role in the natural bioremediation since it could contribute to an improved immobilization of arsenic in helping to mitigate arsenic contamination. Freel et al. (2015) showed that several phylogenetic groups of *Thiomonas* strains populated along Regious creek AMD for more than a decade even though in low abundance. Since the arsenic genomic islands are identified to have evolved differentially in the closely related *Thiomonas* strains, there are differences in the arsenite oxidation capability in the arsenic-rich environment (Freel et al. 2015).

Thiomonas delicata DSM 16361, isolated from Cheni gold mine, shows high arsenite oxidizing capability of 4 mg As(III) l⁻¹ h⁻¹ which grows optimally at pH 4–7 as a moderate acidophile (Battaglia-Brunet et al. 2006). Most interestingly, purification and detailed characterization of Aio from facultative chemolithoautotrophic arsenite oxidizing *Thiomonas* strains have yet to be described. The characterization of the arsenite oxidase is of great importance as we could further explore the enzymatic features that contribute to the irreplaceable role of *Thiomonas* strains as an arsenite oxidizer in AMD. This could facilitate the engineering of enzymes and aid in construction of robust biosensor for monitoring arsenic in acid mining effluent. Therefore, in this study, by performing heterologous expression and purification of the arsenite oxidase from *T. delicata*, we characterized the arsenite oxidase in terms of its biochemical properties. Homology modeling of the enzyme was constructed to support the experimental findings. To the best of our knowledge, this is the first characterization study of arsenite oxidase from a chemolithoautotrophic betaproteobacterium.

Materials and methods

Bacterial strains and growth conditions

Thiomonas delicata DSM 16361 was obtained from German Collection of Microorganisms and Cell Cultures

(DSMZ) and grown in R2A medium (pH 7.2). Recombinant strains were grown aerobically at 37 °C in Luria-Bertani broth supplemented with 100 µg ampicillin ml⁻¹.

Gene cloning and construction of the recombinant plasmid

Genomic DNA was extracted by using Wizard Genomic DNA Purification kit (Promega). Based on the sequence of *Thiomonas* 3As arsenite oxidase gene (GenBank accession no. FP475956.1), oligonucleotide primers 5'-GGAGGC-CATATGACCGAAAAAGTATCGCGTCGC-3' and 5'-GGAAAGCTTTCACGATTCAAACCCCGTACATGCG-3' were designed to amplify the region of full length arsenite oxidase gene (*aioBA*) using Kapa HiFi Polymerase (Kapa Biosystem). With *Nde*I and *Hind*III restriction sites incorporated (underline), the amplified *aioBA* was cloned into pET21a (Novagen). According to Van Lis et al. (2012), a His-tag was introduced using PCR with forward primer using 5'-TAAGCCCATCACCGTCAGG-3' and reverse primer 5'-GTGGTGGTGGTGGTGGTGCAG-CACGTTGGCCTGGCG-3' using as template the *aioBA*-pET21a construct. The construct *aioBA*-His-pET21a was then transformed into *E. coli* strain C43. The recombinant plasmid was confirmed by DNA sequencing. The sequence of the *aioBA* cloned from *T. delicata* DSM 16361 has been submitted to the GenBank database under the accession number of KX792110.

Heterologous expression of *T. delicata* Aio in *E. coli* and purification of the recombinant enzyme

The expression of recombinant Aio was performed in ZYM-5052 autoinduction medium (Studier 2005) including 100 µg ampicillin ml⁻¹ and 1 mM Na₂MoO₄ for 48 h in 20 °C at 180 rpm. Purification of Aio was performed from the total soluble fraction of recombinant *E. coli* cells. The cells were harvested and resuspended in cold binding buffer (20 mM NaH₂PO₄, pH 7.4, 500 mM NaCl, 20 mM Imidazole) and lysed by using Qsonica Sonicator Q700 (amplitude 50%, 1 s pulse on 1 s pulse off for 3 min). Unlysed cells were removed by centrifugation at 10,000×g for 10 min, and the lysate was subsequently ultracentrifuged at 110,000×g for 1 h 30 min to remove membrane fraction. The resulting supernatant was collected, filtered (0.22 µm filters, Merck Millipore) and then loaded onto HisTrap HP 1 ml column (GE Healthcare), with flow rate set at 0.5 ml min⁻¹ for optimal protein loading. The column was equilibrated and washed with binding buffer with flow rate 1 ml min⁻¹ before eluted in elution buffer (20 mM NaH₂PO₄, pH 7.4, 500 mM NaCl, 500 mM Imidazole) using imidazole gradient (20–500 mM). The fraction containing active enzyme was

pooled and dialyzed against 50 mM MES buffer pH 5.5. The precipitated protein was then removed by centrifugation at $10,000\times g$ for 5 min. The supernatant was concentrated with Vivaspın 20 (MWCO 10,000 Da) centrifugal concentrator and loaded onto a HiLoad 16/60 Superdex 200 pre-equilibrated with 50 mM MES buffer pH 5.5, 150 mM NaCl. Flow rate was set at 0.3 ml min^{-1} . All chromatography steps were carried out at room temperature (20–25 °C) using ÄKTApriime plus (GE Healthcare Life Sciences). Freshly purified enzyme was then concentrated for activity assay and SDS-PAGE was carried out according to method from Laemmli (1970).

Enzyme assays

Arsenite oxidase activity was assayed according to the method from Anderson et al. (1992) in 50 mM MES buffer (pH 5.5) with 60 μM of an artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) and 200 μM arsenite. Reduction of DCPIP was monitored spectrophotometrically at 600 nm at 25 °C for 5 min ($\epsilon_{600\text{ pH }5.5}$ experimentally determined at $10\text{ mm}^{-1}\text{ cm}^{-1}$) using 100 Vis Spectrophotometer (Buck Scientific). Protein concentration was determined by Bradford assay with bovine serum albumin as standard.

The kinetic study of the expressed enzyme was carried out using $0.5\text{ }\mu\text{g ml}^{-1}$ and varying concentrations of arsenite (5–500 μM) in 50 mM MES (pH 5.5) at 25 °C, and the results were analyzed using Michaelis–Menten enzyme kinetic model using GraphPad Prism 7. One unit of Aio activity was defined as the amount of enzyme to oxidize 1 μmol of arsenite per min under the assay conditions.

The effect of pH on Aio activity was investigated at 25 °C in 50 mM citrate–phosphate buffer (pH 2.5–4), MES buffer (pH 5–6), sodium phosphate (pH 7–8) and glycine–NaOH (pH 9–10). The pH-dependent stability was measured by incubating Aio (0.03 mg ml^{-1}) in 50 mM of buffer (pH 2.5–10) for 16 h at 4 °C prior to measurement of the residual enzymatic activity. The effect of temperature on Aio activity was examined in 50 mM MES (pH 5.5) between 25 and 70 °C. Thermal stability was determined by measuring the residual activity after incubating Aio at temperatures between 25 and 70 °C after 1 h.

The effect of different metal ions and chemical agents towards enzyme activity was investigated. The enzyme (0.01 mg ml^{-1}) was assayed in the presence of 10 mM metal ions (K^+ , Li^+ , Ca^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} and Zn^{2+}) and anions (NO_2^- , SO_4^{2-} and Cl^-). The effects of different surfactant concentrations (SDS and Triton X-100) and chemical reagents (EDTA and urea) on enzyme stability were examined with the additive in the reaction mixture for the assay. The enzyme activity without any additive was taken as 100%.

Statistical analysis

All the experiments were conducted in triplicates measurements. The means were compared by ANOVA at 5% significance level. The SPSS statistical package (IBM SPSS Statistics 23) was used for statistical evaluations.

Bioinformatics analysis

The homology model of arsenite oxidase from *T. delicata* was generated using the SWISS-MODEL workspace with arsenite oxidase crystal structure from *A. faecalis* (PDB: 1G8K chains A and B) as template. The Poisson–Boltzmann electrostatic potentials were calculated using the PDB2PQR and APBS. The structural superimposition and graphical representation of the structure model was prepared in UCSF CHIMERA software.

Results and discussion

T. delicata arsenite oxidase gene cloning and analysis

A total of 3093 bp gene cluster comprising of both *aioB* and *aioA* was amplified successfully from genomic DNA of *T. delicata* DSM 16361. Nucleotide BLAST search of both *aioB* and *aioA* revealed high homology with Aio gene from a number of *Thiomonas* strains, with both genes sharing highest sequence identity of 99% to *Thiomonas* sp. CB2. Compared to previously characterized Aio, the deduced amino acid sequence of AioA (841 amino acid residues) display sequence identity of 65, 63, 62, 45% from *Hermiimonas arsenicoxydans*, *A. faecalis*, *Ralstonia* sp. 22 and *Rhizobium* sp. NT-26, respectively. The deduced amino acid sequence AioB (179 amino acid residues) shared sequence identity of 61, 58, 56, 41% from *A. faecalis*, *Hermiimonas arsenicoxydans*, *Ralstonia* sp. 22 and *Rhizobium* sp. NT-26, respectively.

Expression of Aio in *E. coli* and purification of the recombinant enzyme

Under the control of T7 promoter in plasmid pET-21a, the *aioBA* was expressed in *E. coli* C43 using autoinduction, where lactose acts as the inducer for protein expression. Recombinant proteins were purified by sequential Ni–NTA and gel filtration chromatographic steps. Purity of Aio was checked by the limit of electrophoresis and estimated to be 90% pure (Fig. 1). Similar to other arsenite oxidases (Ellis et al. 2001; Santini and vanden Hoven 2004; Duquesne et al. 2008; Prasad et al. 2009), the size of the large subunit arsenite oxidase (AioA) was around 91 kDa while small

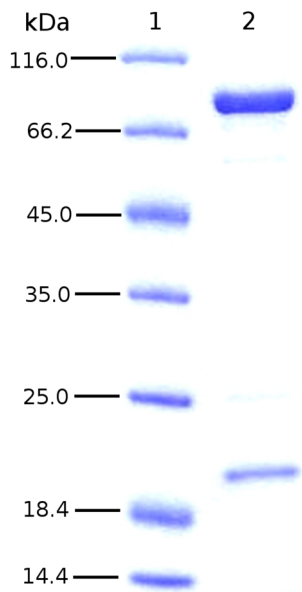


Fig. 1 SDS-PAGE gel of purified Aio stained with Coomassie Blue R350. Lane 1 Protein molecular weight marker; lane 2 *T. delicata* Aio

subunit (AioB) was 21 kDa without removing the signal peptide.

Characterization of recombinant Aio

By utilizing DCPIP as the artificial electron acceptor, the recombinant Aio showed typical Michaelis–Menten kinetics with substrate arsenite, with V_{\max} of $4 \pm 0.07 \mu\text{mol arsenite oxidized min}^{-1} \text{mg}^{-1}$ of proteins and K_m $14.2 \pm 1.03 \mu\text{M}$ (Fig. 2). K_m of the recombinant Aio was found to be higher than Aio from *A. faecalis*, but lower than *Arthrobacter* sp. 15b and NT-26 (Table 1). High K_m indicated the lower affinity of the arsenite oxidase towards arsenite substrate. As for the V_{\max} , the *T. delicata* Aio displayed a higher specific activity than the other arsenite oxidases.

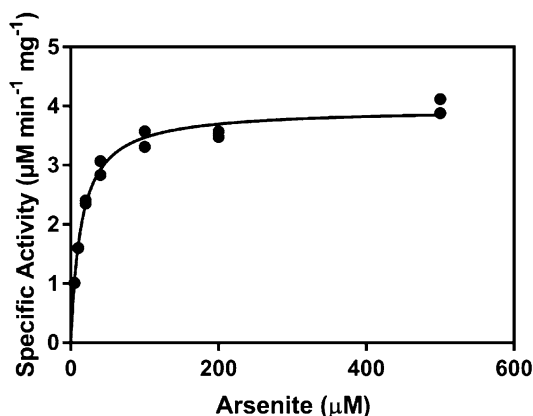


Fig. 2 Kinetic plot obtained with recombinant *T. delicata* Aio with substrate arsenite

The purified Aio displayed a typical bell-shaped pH–activity relationship with maximum activity at pH 5.5, which is the same as Aio NT-26 (Santini and vanden Hoven 2004) and >80% relative activity between pH 5 and 6 (Fig. 3a). However, after incubation for 16 h at 4 °C, the pH stability profile was shifted where the enzyme retained high activity at pH 2.5–4 and >80% relative activity between pH 3 and 6. Beyond pH 7, there was a drastic reduction in the enzyme activity. At pH 7–9, the activity preserved was almost 60%, while at pH 10, only 30% activity was retained (Fig. 3b). Since *T. delicata* has the ability to grow in wide pH range from pH 4 to 7, it is possible for Aio to develop acid-stable properties to tolerate the pH fluctuation at the transition zones of acidic mine waters.

Optimum activity of Aio was found at 50 °C, but statistically at par with 55 °C. Relatively high activity was noticeable at 40 and 55 °C (Fig. 3c). Aio retained nearly 45% of its initial activity after incubation at 60 °C for 1 h (Fig. 3d). Even though there is a slight drop in the optimum temperature as compared with Aio from *A. faecalis* and NT-26 (60 °C), the Aio is still considered rather thermostable. These results showed that Aio is an acid-tolerant and thermostable enzyme.

Purified Aio was tested with a variety of cations, anions and chemical agents which might influence the enzyme activity. The selected metal ions and chemical agents showed no apparent inhibition to the Aio activity with more than 80% residual activity retained as shown in Table 2. Both Co^{2+} and Zn^{2+} did not exhibit obvious inhibition to Aio activity as to *Arthrobacter* sp. 15b Aio (Prasad et al. 2009). Co^{2+} and Zn^{2+} strongly inhibited *Arthrobacter* sp. 15b Aio where only 1.5 and 1.8% arsenite oxidation activity was retained after treatment (Prasad et al. 2009). By comparison, *T. delicata* Aio was tolerant to Co^{2+} and Zn^{2+} , retaining 84 and 88% residual activity, respectively. This heavy metal tolerance of *T. delicata* Aio is possible, whereby genes conferring resistance to zinc and cobalt were found in the genome of *Thiomonas* sp. 3As (Arsène-Pløetze et al. 2010). The addition of 10 mM sulphate anion (SO_4^{2-}) did not inhibit *T. delicata* Aio as well. This ability to tolerate high sulphate anions might be explained by the presence of this anion in AMD formed via the oxidation of pyrite that could lead to an increased ability of *T. delicata* Aio to resist sulphate. Surfactant SDS and Triton X-100 were observed to inhibit the activity of Aio with residual activity of 60 and 19%.

Homology modeling analysis

To explore the structural basis underlying the differences in enzymatic properties, a homology model was built using *A. faecalis* Aio crystal structure was used as a template by

Table 1 Characteristic of *T. delicata* Aio compared with Aio from other mesophilic organisms

Source	K_m (μM)	V_{max} (U mg^{-1})	References
<i>Thiomonas delicata</i>	14.2	4	This study
<i>Alcaligenes faecalis</i>	8	2.88	Anderson et al. (1992)
<i>Arthrobacter</i> sp. 15b	26	2.45	Prasad et al. (2009)
<i>Rhizobium</i> sp. NT-26	61	2.4	Santini and vanden Hoven (2004)

One unit of enzyme activity is defined as the amount of enzyme necessary to oxidize 1 μmol of arsenite per min using DCPIP as electron acceptor under the assay conditions

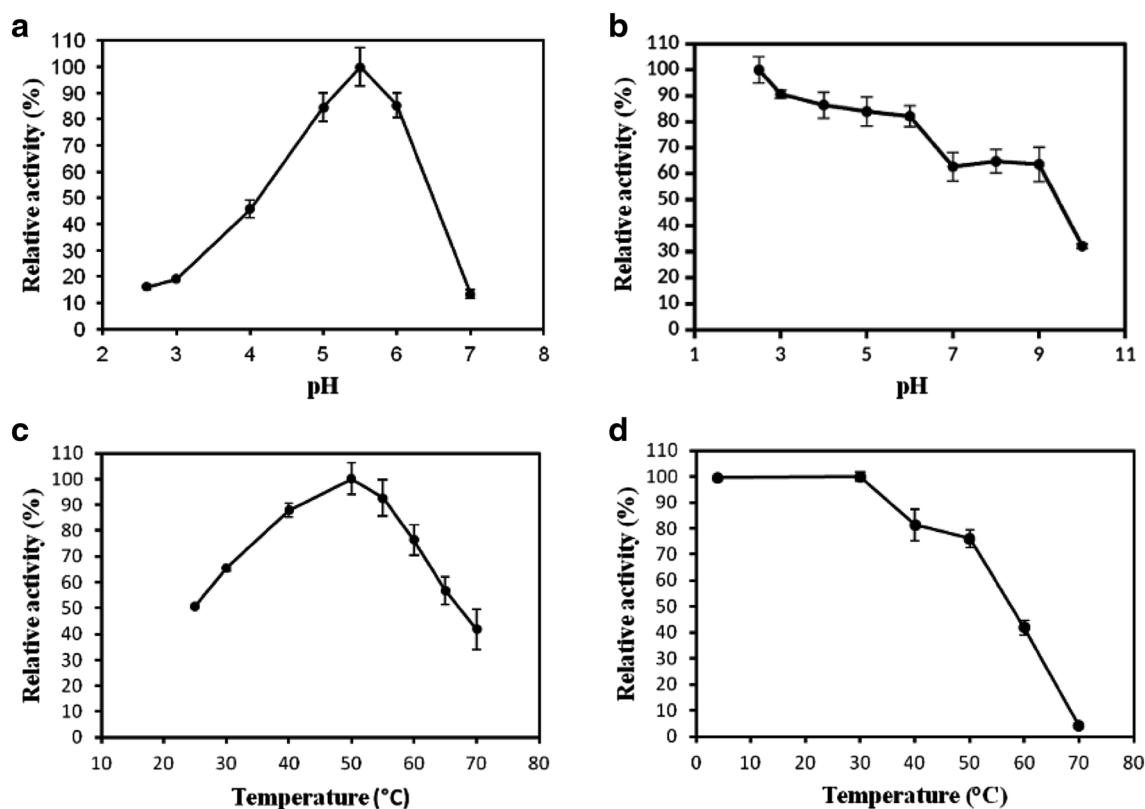


Fig. 3 Effects of pH and temperature on the activity of purified Aio. **a** pH–activity profile; **b** pH stability relationship; **c** temperature activity dependence; **d** temperature stability profile. The effect of pH on Aio activity was investigated at 25 °C in 50 mM buffer (pH 2.5–7). The 100% relative activity was $3.1 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$. pH stability studies were carried out by incubating enzymes (0.03 mg ml^{-1}) in 50 mM buffers (pH 2.5–10) at 4 °C for 16 h prior to measurement of residual activity. The 100% relative activity was

$3.5 \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$. The effect of temperature on Aio activity was examined in 50 mM MES (pH 5.5) from 25 to 70 °C. 100% relative activity was $3.4 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$. The temperature–stability relationship was studied after incubation at different temperature (10–70 °C) for 1 h in 50 mM MES (pH 5.5). 100% relative activity was $3.7 \pm 0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$. Data points and error bars represent mean and standard error of at least three assays, respectively, $p < 0.05$

SWISS-MODEL. The reliability of the model was supported by its high sequence identity of 64% and with QMEAN4 score of -2.16 . Comparison of the homology model and template showed high similarity in overall fold as well as active site residues. Notable difference between the template and model was observed on the surfaces of the enzyme (Fig. 4). One of the distinct features of Aio is the existence of a 15-residue-longer loop near the catalytic cleft. The loop region is unique in all *Thiomonas* strain.

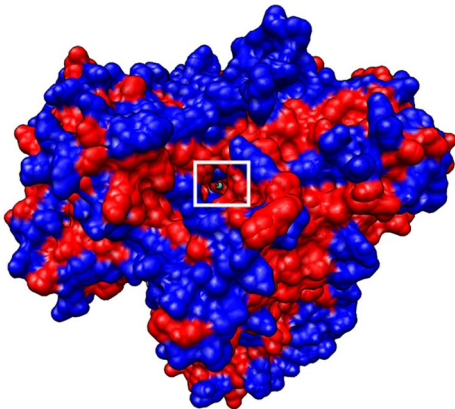
This could contribute to the difference in enzyme stability and flexibility, where the longer surface loop can lead to an increase of possible amplitude of the movement between the secondary structures.

In extreme environments, the nature of protein surfaces plays essential roles to maintain the protein stability. Located either at periplasm or membrane attached, Aio is likely to be exposed to pH of the extracellular environment. A study from Wilks and Slonczewski (2007) demonstrated

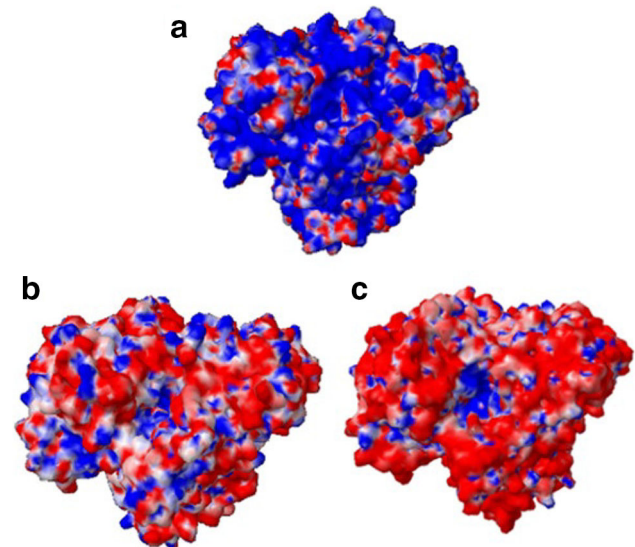
Table 2 Effects of metal ions, anions and chemicals on arsenite oxidase activity

	Relative activity (%)
Control	100 ± 5.1
Metal ions (10 mM)	
K ⁺	91.9 ± 4.2
Li ⁺	104.6 ± 4.9
Ca ²⁺	94.9 ± 1.3
Co ²⁺	83.8 ± 7.5
Ni ²⁺	89.9 ± 7.4
Mn ²⁺	101 ± 1.8
Zn ²⁺	87.8 ± 3.4
Anions	
NO ²⁻	94.4 ± 8.9
SO ₄ ²⁻	93.9 ± 1.8
Cl ⁻	100.2 ± 4.9
Chemicals	
Urea (10 mM)	100.3 ± 1.7
EDTA (10 mM)	91.3 ± 1.8
SDS (2 mM)	60.9 ± 8.5
Triton X-100 (0.1%, v/v)	19.5 ± 1.7

Values are represented by mean and standard error of at least three assays. 100% relative activity = $3.9 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$

**Fig. 4** Space-filling homology model of *T. delicata* Aio. Active site of the enzyme is boxed. Conserved residues between the *T. delicata* Aio and *A. faecalis* structure are red; different residues are blue

that the periplasm of gram negative bacteria do maintain the same proton concentration as the surrounding environment even though the cytoplasm is maintained at near neutral pH. Therefore, by calculating the electrostatic potential data of the surfaces of Aio (Fig. 5), it was revealed that the surfaces of *T. delicata* Aio are rich with positively charge residues, while *A. faecalis* and NT-26 Aio have more negatively charged surface residues. This observation might contribute to the pH stability of *T. delicata* Aio, whereby penetration of hydronium ions inside a structure of predominantly basic protein surface can be

**Fig. 5** Electrostatic rendering of the solvent-accessible surface of **a** *T. delicata* Aio, **b** *A. faecalis* 1G8K and **c** NT-26 4AAY. Computed at pH 7 with red (−5 kT/e) and blue (5 kT/e)

avoided at low pH as shown in an acid-resistant citrate synthase (Francois et al. 2006).

Conclusion

The characterization of Aio from a species belonging to the *Thiomonas* genus was described for the first time. The high activity of *T. delicata* DSM 16361 Aio coupled with acid-stable and metal tolerance properties suggests that the enzyme has potential to be used for biosensor and bioremediation applications in acidic environments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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