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ARTICLE

Rapid catalytic oxidation of As(III) to As(V) by using *Bacillus* spore–2,2,6,6-tetramethylpiperidine-1-oxyl system

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Oxidation of As(III) to As(V) is a critical process in the treatment of contaminated water. We found 95% As(III) (10 mg L^{-1}) could be rapidly oxidized to As(V) by laccase–2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) system in 1 h. Basing on this finding, we used *Bacillus subtilis* spore instead of laccase for As(III) oxidation with the same effect because the former had plenty of CotA–laccase on their surface. The catalytic ability of CotA protein and spore was confirmed by expressing CotA protein and knocking out *cotA* gene from wild-type spore. Both laccase– and spore–TEMPO systems have similar oxidation rate constants, Michaelis–Menten constant, and maximal velocity owing to the formation of oxoammonium cation of TEMPO in the presence of dissolved oxygen. Several other laccase mediators such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic-acid) (ABTS), acetosyringone (AS), 1-hydroxybenzotriazole (HBT), 2-hydroxybutyl acrylate (HBA), violuric acid (VLA), 4-oxo-TEMPO, 4-amino-TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO benzoate, and 4-hydroxy-TEMPO coupled with spore for As(III) oxidation were also investigated in detail. Spore–TEMPO system exhibited the highest oxidation efficiency and tolerated the addition of 10 mg L^{-1} Al^{3+}, Ti^{4+}, Cu^{2+}, K^{+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}. Both laccase and spore recovered via ultrafiltration and centrifugation, respectively, could be reused for at least five cycles. The advantages of spore-based system include eco-friendliness, easy operation and storage, low cost, recyclability, sustainability, and without the need for professional training and enzyme purification. These findings may show promising implications in developing a new eco-friendly and cost-effective technology for the treatment of arsenic-containing water.

Introduction

As a serious global problem, arsenic pollution in drinking water has recently raised public awareness and scientific concern[1] and has affected 150 million people in over 70 countries.[2] World Health Organization provisions a guide limit value in drinking water at 10 μg L^{-1}, and the U.S. Environmental Protection Agency provides a new standard in the range of 2–20 μg L^{-1}.[3, 4] In natural water environments, As(III) and As(V) are the major species, and As(III) is more toxic than As(V).[5] Moreover, As(III) is more mobile in natural waters and less efficiently removed in adsorption/coagulation processes than As(V) because the former appears predominately in a fully protonated form (H_3AsO_3) below pH 9.2, whereas the latter occurs as a mixture of $H_2AsO_4^-$ and $HAsO_4^{2-}$ at circumneutral pH values.[6] Therefore, the oxidation of As(III) to As(V) is a critical process in the treatment of contaminated water.[7] Many oxidants and technologies for the oxidation of As(III) have been investigated. For example, some radicals, such as hydroxyl radical,[8] superoxide,[9, 10] and sulfate radical,[11] are considered as new potential species for As(III) oxidation instead of the classical ones,[12] such as chlorine, ozone, potassium permanganate, and persulfate. Some catalytic oxidation systems, such as Fenton reaction,[13] metal activated persulfate,[14] and TiO_2 photocatalytic systems[15, 16] have been applied to As(III) oxidation. Energy such as light[11, 15], ultrasonic waves,[17, 18] and electricity[19] has been used to enhance As(III) oxidation. Each of these processes and oxidants has advantages and disadvantages. For example, chemical oxidants such as Fenton reagent, hydrogen peroxide, chlorine, and manganese oxide have high oxidation efficiency but may generate secondary pollution. Electrochemical peroxidation and photocatalytic oxidation are environment friendly but have a low oxidation efficiency and cannot be extensively used in a natural environment.[20, 21] Therefore, developing efficient As(III) oxidation processes is needed to remove stringent arsenic.

Enzymatic catalysis is a promising green method with reasonable consumption of energy and reagents for disposing emerging contaminants because of its high specificity and efficiency. Peroxidase- and laccase-catalyzed enzymatic reactions have been successfully used for the removal of several organic pollutants, such as phenols,[22, 23] alcohols,[24] and dichlorophen[25], from water. However, the enzymatic reaction on the transformation of heavy metals such as arsenic has been much less explored. Mao et al.[26] recently suggested to remove As(III) from water via reactions mediated by laccase with the presence of oxygen. According to their report, no As(III) transformation was observed during the first 10 h of reaction. However, after 10 h, the reaction of As(III) was rapidly

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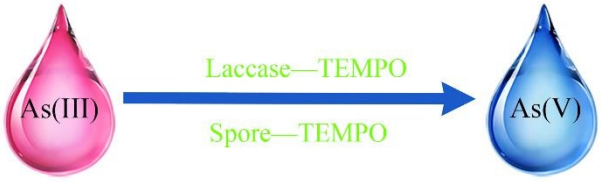
accelerated, and 85.7% removal was achieved at 48 h. Unfortunately, our findings were different from their repeated results. In addition, shortcomings, including the non-recyclability of laccase in the solution and the long reaction time, still limit its practical application.

Bacillus spores are a remarkable phase in the life cycle of *Bacillus*. These spores can survive for several years and also can be recycled via fermentation. In this regard, spores expressing certain enzymes, proteins, or peptides on their surface have been presented as a stable, simple, and safe new tool for the biospecific recognition of target analytes, the biocatalytic production of chemicals, and the delivery of biomolecules with pharmaceutical relevance.[27] The engineered spores have been used for whole-cell biosensor,[28, 29] biocatalysis,[30] surface display of recombinant proteins for bioanalysis and biotechnological application,[31] and as vehicles for vaccines and therapeutic agents[32, 33]. Considering the presence of laccase (i.e., CotA protein) on the surfaces of *Bacillus subtilis* (*B. subtilis*) and *Bacillus amyloliquefaciens* spores, we successfully used wild-type bacterial spores as a whole-cell biosensor in antioxidant capacity[34] and phenol assays[35]. Furthermore, we proposed a pioneering biological method using inactivated bacterial spores to prepare functional monodisperse microparticles, which have been successfully used in immunoassays,[36, 37] catalysis,[38] pH sensing,[39] DNA analysis,[40] and selective enrichment of phosphorylated proteins[41]. Therefore, these spores have advantages, vast potential research value, and application prospect in different fields.

In this work, we found for the first time that laccase–TEMPO (a commercially available and stable N-oxy radical) system could rapidly oxide As(III) to As(V). Based on this finding, *B. subtilis* spores were utilized as a catalysis for As(III) oxidation because of the presence of CotA protein on the their surface. The effects of surface CotA protein, and several mediators such as ABTS, AS, HBT, HBA, VLA, 4-oxo-TEMPO, 4-amino-TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO benzoate, and 4-hydroxy-TEMPO on As(III) oxidation were also investigated in detail. This work may contribute to the understanding of the main mechanisms of As(III) oxidation and may aid in the development of a new biological As(III) decontamination method driven by spores. Our proposed spore-based method exhibits several advantages, such as easy operation and storage, low cost, recyclability, sustainability, and without the need for professional training and enzyme purification.



Scheme 1. Spore–TEMPO and laccase–TEMPO used in the As(III) oxidation.

Experimental

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Materials

B. subtilis was provided by State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Wuhan, China). Taq DNA polymerase, restriction endonucleases and the DNA markers were purchased from Takara Biotechnology Co., Ltd (Dalian, China). The synthesized primers were acquired from Tsingke Biotechnology Co., Ltd (Wuhan, China). Protein markers were obtained from Transgen Biotechnology Co., Ltd (Beijing, China). Bacterial Genemic DNA Extraction kit and Plasmid Purification Kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Sodium arsenite ($NaAsO_2$) was obtained from Xiya Regent Co., Ltd (Chengdu, China). Standard stock solution of As(V) was obtained from National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials (Beijing, China). Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (St. Louis, MO, USA). ABTS was purchased from Aladdin Biochemistry Technology Co., Ltd (Shanghai, China). TEMPO and TEMPO-derivatives, containing 4-oxo-TEMPO, 4-amino-TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO benzoate, and 4-hydroxy-TEMPO were obtained from TCI Development Co., Ltd (Shanghai, China). AS, HBT, HBA and VLA used in this work were obtained from Yuan Ye Biotechnology Co., Ltd (Shanghai, China). The reagent was diluted and dissolved using ultrapure H_2O (18.2 MΩ) prepared from a model Cascada IX laboratory ultrapure H_2O system from Pall Co., Ltd (Washington, NY, USA).

Methods

Preparation of Spore. *B. subtilis* was grown in Luria-Bertani (LB) solid medium supplemented with 0.25 mmol L^{-1} Cu^{2+} at 37 °C for 4 d[42]. Spores scraped off from the medium were washed three times with deionized water, and then stored at 4 °C. The number of spores was determined with bacterial colony counting method under plating serial dilutions on LB plates[43, 44].

Activity Assays of Laccase. The activities of laccase, spores and CotA were determined by monitoring the oxidation of ABTS using a UV-visible (UV-vis) spectrophotometer (DU780, Beckman Coulter, USA) at 420 nm (ε=3.6×10^4 M^{-1} cm^{-1}) with 0.5 mM ABTS as the substrate in acetic acid buffer (0.1 mol L^{-1}, pH 4.5) at 37 °C.[45] In briefly, 20 μL sample and 10 μL of 1 mmol L^{-1} ABTS were added into 170 μL, measured at 420 nm. One unit of laccase activity was defined as the amount of laccase required to oxidize 1 μM of ABTS per min.

As(III) Oxidation by Spore–TEMPO. As(III) oxidation by spore–TEMPO was conducted with 2 mL centrifuge-tube containing 1 mmol L^{-1} TEMPO and 7.48×10^7 CFU mL^{-1} spores for 10 mg L^{-1} As(III) at pH 4.5 and incubated at 55 °C for 1 h, terminated by centrifuge at 12 000 rpm for 2 min, and the supernatant was detected using molybdenum blue method.[46, 47] Briefly, 200 μL of the withdrawn sample solution was added into 1.8 mL of mixture solution, containing 100 μL of sulfuric acid (5 mol L–1), 100 μL of molybdate reagent solution (3.51%) and 50 μL of ascorbic acid (1%), incubating at 60 °C for 15 min. The concentration of As(V) was measured using UV-vis spectrophotometer at 840 nm.

Cloning, Expression, and Purification of CotA. *cotA*, encoding *B. subtilis* CotA protein, was amplified by PCR using *B. subtilis* chromosomal DNA as the template with the primers *cotA*-F (5'-GCTGGATCCATGACACTTGAAAAATTTGTGG-3') and *cotA*-R (5'-GCTCTCGAGTTATTTATGGGGATCAGTTAT-3'). The PCR conditions: 7 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C, followed by 72 °C for 10 min. The PCR program (1542 bp) was digested with *XhoI* and *BamHI*, and then cloned into pet28a to generate pet28a-*cotA* recombinant plasmid. Finally, the recombinant plasmid was transformed into *Escherichia. coli* (*E. coli*) Rosetta (DE3) and result was confirmed by sequence analysis.

E. coli Rosetta (DE3) carrying pet28a-*cotA* plasmid was inoculated into LB medium with 50 μg L^{-1} kanamycin and grown at 37 °C, 180 rpm until an optical density at 600 nm of 0.5 was reached. Then 0.1 mmol L^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.25 mmol L^{-1} $CuSO_4$ were added into the medium and culture conditions were adjusted to 25 °C, 140 rpm. The *E. coli* Rosetta (DE3) was Incubated for a further 5 h until the density at OD_{600} of *E. coli* almost was 2.5. Then switching off the shaking function for microaerobic conditions, the *E. coli* Rosetta (DE3) was harvested after a further 24 h[48, 49]. The cells were harvested using a high-speed centrifuge (12 000 rpm, 5 min, 4 °C) and resuspended with 10 mmol L^{-1} imidazole binding buffer. Cell was disrupted using Low Temperature Ultrahigh Pressure Continuous Flow Cell Disrupter and the cell debris removed by centrifugation (12 000 rpm, 20 min, 4 °C). The CotA protein purification was preformed using a Ni-NTA sefinose resin kit. The CotA protein was analyzed by SDS-PAGE with 30% polyacrylamide running gel, performed in a DYCZ-24DN mini double vertical electrophoresis system.

The Knockout of cotA Gene. Homologous double exchange was used to knocked out the *cotA* gene yielding *cotA* knock-out strain (Mt-strain) in this work according to our previous works[34]. In brief, we amplified the upstream and downstream regions of target locus using *B. subtilis* genomic DNA as a template and primer pair (F: 5'-CCGCTCGAGCCCGACAAACTTGCCTCT-3', R: 5'-CGGGGTACCTTAAAAGCTTTTTCCTGGTTTA-3'). The upstream and downstream were connected to the pDG780 vector respectively, and the recombination plasmid was transformed into *B. subtilis*. Finally, the transformant was screened using ampicillin and kanamycin resistance. The positive transformants showed resistance to kanamycin and susceptibility to ampicillin and kanamycin. ABTS assay was used to determine whether the cotA gene was deleted from *B. subtilis* genome successfully.

Results and discussion

As(III) Oxidation by Laccase–TEMPO

Laccases belong to the multi-copper family of oxidases with four copper ions in two catalytic centers and uses oxygen as an electron acceptor.[50] Laccases are accessible and inexpensive and can efficiently work in water under mild reaction conditions. Through coupling with electron transfer mediators, such as ABTS, benzotriazole, or syringaldehyde, laccase has been effectively used for the oxidation of phenolic compounds toward benzylic alcohols and aromatic amines.[51] Accordingly, laccase-mediator systems are ideal candidates for sustainable chemical and oxidoreductive biotechnological applications. Among these systems, laccase–TEMPO catalytic system is the most employed due to TEMPO accessibility and high compatibility with laccases.[52]

As(III) cannot be converted to As(V) when only laccase or TEMPO is present. This conversion reaction was catalyzed and accelerated in laccase–TEMPO system as detected using high-performance liquid chromatography–hydride–generation–atomic fluorescence spectroscopy[53] (HPLC–HG–AFS) (SA-10, Beijing Titan Instruments, China); 97% As(III) conversion to As(V) was achieved in 1 h (Fig. 1a) because the substrate of laccase could be effectively explored via the ancillary action of low molecular weight electron transfer agents (i.e., mediators) in the presence of dissolved oxygen.[54] The reactions of laccase–TEMPO and laccase–TEMPO–As(III) were characterized by UV-vis spectrophotometer from Shimadzu Corporation (Japan) (Fig. 1b). When the TEMPO was mixed with laccase, the absorbance at 245 nm decreased, whereas that at 300 nm increased, indicating that TEMPO was transferred to its oxoammonium ions via the laccase–oxygen reaction.[55, 56] In the laccase–TEMPO–As(III) reaction, the characteristic spectral features at 245 nm increased, but those at 300 nm decreased gradually, indicating that the oxoammonium ions were reduced to TEMPO again. The kinetic parameters of the laccase–TEMPO-catalyzed As(III) oxidation were analyzed (Fig. 1c), and the oxidation reaction reached equilibrium in 1 h. The oxidation rate constant (k, s^{-1}) was calculated using Equation 1 as follows:

$$\boldsymbol{Q_t = Q_e(1 - \exp(-kt))} \quad (1)$$

Where Q_t (mg L^{-1}) is the oxidation capacity of As(III) by oxidant at time t (min), and Q_e (mg L^{-1}) is the equilibrium oxidation capacity. The oxidation kinetics was fitted by a pseudo-first-order model (R^2= 0.999) with k = 3.12 s^{-1}.

Michaelis–Menten reaction kinetics was also assessed (Fig. 1d). Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were calculated using Equation 2 as follows:

$$\boldsymbol{V = (V_{max}\ [S])/(K_m + [S])} \quad (2)$$

Where V is the reaction velocity and [S] is the substrate concentration. V_{max} and K_m values were calculated using nonlinear regression and were estimated to be 3.21×10^{-3} mmol L^{-1} min^{-1} and 0.033 mmol L^{-1}, respectively.

After oxidation reaction, the laccase was collected through ultrafiltration to remove the buffer solution and ions such as TEMPO and its oxoammonium ion with arsenic. The laccase still maintained high activity for As(III) oxidation after five recycles (Fig. 1e). Previous works indicate that the oxoammonium ion of TEMPO and arsenic can interact with laccase and lead to the inactivation of the latter.[57, 58] Our results might be due to the high oxidation efficiency with short reaction time and the low

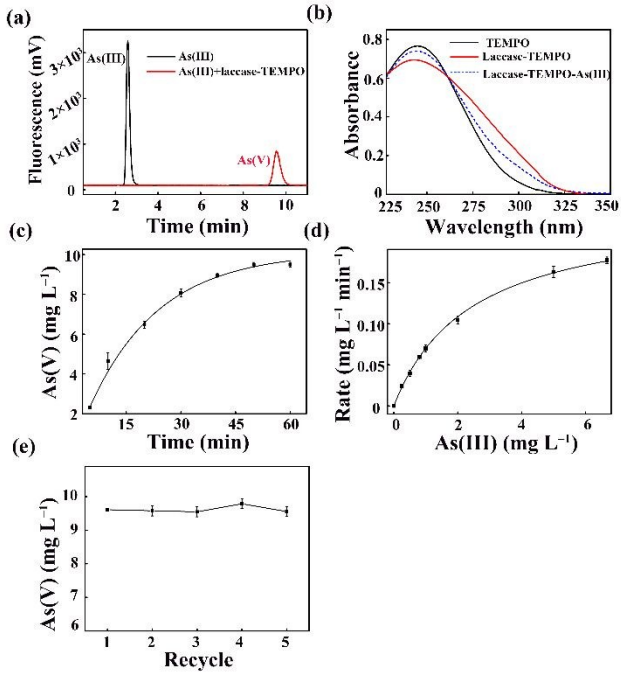

Fig. 1 Oxidation of As(III) by laccase–TEMPO. (a) Chromatogram of As(III) oxidation by laccase–TEMPO. Conditions: 45 U L^{-1} laccase, 1 mmol L^{-1} TEMPO, 10 mg L^{-1} As(III), pH 4.5, 40 °C, 1 h reactive time. (b) UV-vis monitoring of TEMPO and TEMPO oxoammonium in the As(III) oxidation reaction by laccase-TEMPO. Conditions: 1 U mL^{-1} laccase, 0.5 mmol L^{-1} TEMPO, pH 4.5, 40 °C. (c) The kinetic of As(III) oxidation by laccase–TEMPO. Conditions: 45 U L^{-1} laccase, 1 mmol L^{-1} TEMPO, 10 mg L^{-1} As(III), pH 4.5, 40 °C. (d) Michaelis–Menten reaction kinetics of As(III) oxidation by laccase-TEMPO. Conditions: 45 U L^{-1} laccase, 1 mmol L^{-1} TEMPO, pH 4.5, 40 °C, 0-6.667 mg L^{-1} As(III), 10 min reactive time. (e) The recyclability of laccase in the As(III) oxidation. Conditions: 1 U mL^{-1} laccase, 1 mmol L^{-1} TEMPO, pH 4.5, 40 °C, 1 h reactive time. The error bars represent SE of the means. The data represent means ±SE (n=3).

concentration of TEMPO, which was beneficial to keep the activity and recycling application of laccase.

As(III) Oxidation by Spore–TEMPO

Bacterial spores have CotA–laccase on their surface and could survive under environmental challenges (e.g., wet, dry heat, UV, gamma radiation, and oxidizing agents).[59] The use of spores instead of commercial laccase has several advantages, including low cost, long life, reproducibility due to their ability to cycle between the spore and germinating states, and without the need for professional training and enzyme purification. To prove this concept, we expressed the recombinant CotA protein in the cell cultures of *E. coli* system. The results of SDS-PAGE and Western blot analysis (Fig. 2a, b) indicated that a specific band at the molecular weight of 65 kDa belongs to CotA protein, thus confirming that the CotA protein of *B. subtilis* was successfully cloned and expressed. The recombinant protein was then purified and tested using ABTS assay. The colorless ABTS was converted into blue colored $ABTS^{\bullet+}$ in the presence of recombinant protein (Fig. 2c), thereby confirming that the recombinant protein has laccase activity. Furthermore, the ability of the recombinant protein to oxidize As(III) to As(V) was confirmed using HPLC–HG–AFS (Fig. 2d). The similar UV-vis spectral characteristic of CotA–TEMPO (Fig. 2e) and laccase–TEMPO (Fig. 1b) revealed that these As(III) oxidation reactions have a similar reaction mechanism. The *cotA* was then knocked out from the genome of *B. subtilis* by using homologous double exchange to yield *cotA* Mt-strain. Loss of laccase activity was confirmed by adding mutant spores (Mt-spores) to the ABTS solution because the solution remained colorless and did not generate $ABTS^{\bullet+}$ (Fig. 2f). On the contrary, the colorless ABTS was also converted into blue-colored $ABTS^{\bullet+}$ in the presence of wild-type spore similar to that in the recombinant CotA protein. These results indicate that the CotA protein induces the spore to show laccase activity.

Bacterial spores, Mt-spores, Mt-spore–TEMPO, and spore–TEMPO were respectively mixed with As(III) solution to test their ability to oxidize As(III) to As(V) (Fig. 2g). As(V) formation

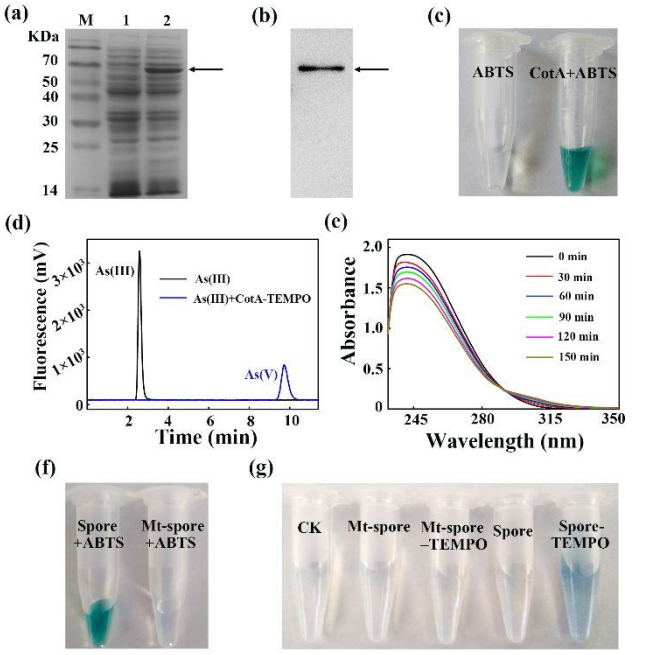

Fig. 2 The mechanism determination of spore–TEMPO used for As(III) oxidation. SDS-PAGE analysis (a) and Western blot analysis (b) of CotA protein of *B. subtilis*, Lane M, marker; lane1, lane 2, before and after cell extract of IPTG-induced Rosetta (DE3); (c) ABTS assay used to determinate the laccase activity of CotA. (d) Chromatogram of As(III) oxidation by CotA-TEMPO. Conditions: 45 U L^{-1} CotA, 1 mmol L^{-1} TEMPO, 10 mg L^{-1} As(III), pH 4.5, 40 °C, 1 h reactive time. (e) UV-vis monitoring of TEMPO and TEMPO oxoammonium in the reaction of CotA and TEMPO. Conditions: 1 U mL^{-1} CotA, 1mmol L^{-1} TEMPO, pH 4.5, 40 °C. (f) ABTS assay used to determinate the laccase activity of spore and Mt-spore. (g) Molybdenum blue method analysis of As(III) oxidation by Mt-spore, Mt-spore–TEMPO, spore, and spore–TEMPO. CK means only in the presence of As(III). Conditions: 7.48×10^7 CFU mL^{-1} spore or Mt-spore, 1mmol L^{-1} TEMPO, 10 mg L^{-1} As(III), pH 4.5, 55 °C, 1 h reactive time.

was detected using molybdenum blue method after the spores

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Table 1 Comparison of As(III) oxidation by Spore–TEMPO and other oxidants

Oxidants	Q_m (mg L^{-1})	Time (h)	Conditions	Ref.
TiO_2/H_2O_2	22.5	4	30 mg L^{-1} As(III), 0.5 g L^{-1} TiO_2, 2 mmol L^{-1} H_2O_2, pH 2.5	60
UV/ $Ti(SO_4)_2$	0.198	0.35	0.2 mg L^{-1} As(III), 5 mg L^{-1} $Ti(SO_4)_2$, pH 5	15
Resin-supported nanosized zero-valent iron	4	8	5 mg L^{-1} As(III), 500 mg L^{-1} nZVI, 140 rpm, pH 6.5, 25 °C	61
Immobilized bacteria	0.92	1	1 mg L^{-1} As(III), 1.6 × 10^{10} cell, 260 mg L^{-1} NH_4-N, pH 7, 20 °C	62
Wolffia/ phyllosphere bacteria	0.1	168	0.1 mg L^{-1} As(III), 14 h light every day, pH 5.5, 25 °C	63
Gammaproteobacterial/ graphite electrode	0.42	24	15 mg L^{-1} As(III), polarized graphite electrode, 22-24 °C	64
Fe(III)/ sulfite	0.46	0.5	0.5 mg L^{-1} As(III), 0.1 mmol L^{-1} Fe(III) and Na_2SO_3, visible light, pH 6	11
Microbial fuel cells	0.2	168	0.2 mg L^{-1} As(III), power density of MFCs was 752.6 ± 17 mW m^{-2}, 22 ± 2 °C	65
Spore–TEMPO	9.5	1	10 mg L^{-1} As(III), 7.48 × 10^7 CFU mL^{-1} spore, 1 mmol L^{-1} TEMPO, pH 4.5, 55 °C	This work

were removed from the reaction system via centrifugation. This detection method is cheaper and more convenient than HPLC–HG–AFS because the formation of As(V) could be observed with the naked eye. Only the color of spore–TEMPO–As(III) system turned blue, indicating that only wild-type spores can catalyze TEMPO and oxidize As(III) to As(V) due to the presence of CotA–laccase on their surface.

The oxidation of 10 mg L^{-1} As(III) was used as a model to evaluated spore–TEMPO– As(III) catalysis reaction system in the following reaction conditions: pH in the range of 3–9 and temperature in the range of 40 °C–70 °C (Fig. 3). This system exhibited high oxidation efficiency within a wide range of pH and temperature because CotA–laccase is an intrinsically highly thermostable and pH-stable enzyme.[66] The maximal oxidation efficiency of As(III) was obtained at pH 4.5 and 55 °C. This developed method was significantly superior over many other systems using different materials for As(III) oxidation (Table 1).

The ability of several laccase-mediators such as ABTS, AS, HBA, HBT, VLA, 4-oxo-TEMPO, 4-amino-TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO benzoate, and 4-hydroxy-TEMPO to oxidize As(III) was investigated and compared with that of TEMPO by adding them into the spore–As(III) solution. These mediators with different chemical structures (Fig. 4) represent the following three different reaction mechanisms underlying laccase catalytic reaction: via an ionic oxidation that is suggested for TEMPO and TEMPO derives,[24] via electron transfer that is suggested for ABTS,[67] or via a hydrogen atom transfer that is suggested for AS, HBA, HBT, and VLA, which

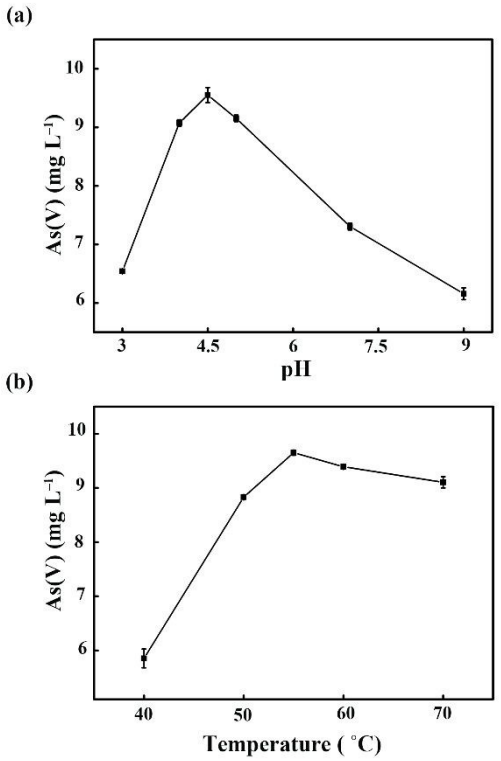

Fig. 3 The effect of pH (a) and temperature (b) in As(III) oxidation by spore–TEMPO. Conditions: 7.48×10^7 CFU mL^{-1} spore, 1 mmol L^{-1} TEMPO, 10 mg L^{-1} As(III), 1 h reactive time. The error bars represent SE of the means. The data represent means ±SE (n=3).

requires substrates with relatively weak C-H bonds[50, 68, 69]. Approximately 0.418 mL min^{-1} O_2 was also added into the spore–As(III) solution to evaluate its effect for As(III) oxidation. As shown in Table 2, except for CotA–laccase, the redox potentials of O_2 and laccase-mediators are higher than that of As(V). After 1 h, 95%, 34%, 32%, 4.8%, 25%, and 20% of As(III) was oxidized to As(V) by using TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO, 4-oxo-TEMPO, 4-amino-TEMPO, and 4-hydroxy-TEMPO benzoate, respectively. However, no transformation of As(III) was observed when excessive O_2, AS, HBA, HBT, and VLA were used for 10 h. ABTS exhibited low transformation rate (10%) during 10 h. These results indicate that the mechanism underlying the reaction between mediator and substrate is the key for As(III) oxidation.

Furthermore, the transformation efficiency of TEMPO, 4-methoxy-TEMPO, 4-hydroxy-TEMPO, 4-oxo-TEMPO, 4-amino-TEMPO, and 4-hydroxy-TEMPO benzoate increased to 100%, 95%, 95%, 8.9%, 89%, and 46%, respectively, in 10 h, indicating that the conversion rate of As(III) could be improved by prolonging the reaction time. The difference of TEMPO and TEMPO derives for As(III) oxidation is due to the different stability of oxoammonium cations under acidic condition[70] and redox potential[71]. That is, the high redox potential of TEMPO-analogous compounds compared with CotA–laccase leads to slow oxoammonium cation formation, which may require a long time to oxidize As(III) to As(V).[72] Therefore, TEMPO with the lowest redox potential and stable oxoammonium cation has the highest oxidation efficiency.

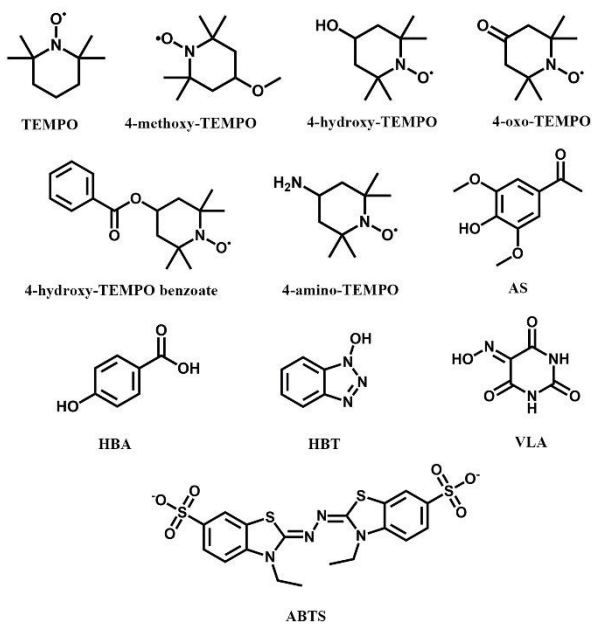

Fig. 4 The chemical structure of mediators used in this work.

Table 2 Oxidation of As(III) using spore–mediators system

	As(III) oxidation (%)		Redox potential
	1 h	10 h	(vs NHE)
As(V)	–	–	0.56[73]
O_2	–	–	2.42[73]
TEMPO	95	100	0.75, 0.74[70]
4-methoxy-TEMPO	34	95	0.80[72]
4-hydroxy-TEMPO	32	95	0.80[72]
4-oxo-TEMPO	4.8	8.9	0.78[72]
4-amino-TEMPO	25	89	0.89[72]
4-hydroxy-TEMPO benzoate	20	46	1.00[74]
ABTS	< 1	10	0.69 and 1.10[75]
AS	–	–	0.60[76]
HBA	–	–	0.90[77]
HBT	–	–	1.09[75]
VLA	–	–	0.92[75]
CotA from *B. subtilis*	–	–	0.46[78]

4-methoxy-TEMPO and 4-hydroxy-TEMPO have moderately oxidation efficiency, though their redox potentials are higher than that of TEMPO possibly because their oxoammonium cations can stabilize under the reaction condition. Moreover, both of them have similar oxidation efficiency owing to their same redox potential. The oxidation efficiency of 4-hydroxy-TEMPO benzoate was lower than those of TEMPO, 4-methoxy-TEMPO, and 4-hydroxy-TEMPO owing to its highest redox potential. The low oxidation efficiency of 4-amino-TEMPO might be mainly due to its unstable oxoammonium cations at pH 4.5 and its high redox potentials. Among TEMPO-analogous compounds, 4-oxo-TEMPO has the lowest oxidation efficiency because its oxoammonium cations decomposed irreversibly into nonradical species at pH above 3.5.[70]

The kinetics and Michaelis–Menten reaction kinetics of As(III) oxidation by spore–TEMPO system were also investigated (Fig. 5a, b). The oxidation reaction achieved equilibrium in 1 h and was fitted by a pseudo-first-order model (R^2= 0.995). The k value was 2.46 s^{-1}. A K_m of 0.04 mmol L^{-1} and a V_{max} of 3.10×10^{-3} mmol L^{-1} min^{-1} were also obtained. These parameters are similar to those of commercial laccase, which further confirmed that spores and laccase both have similar catalysis mechanism.

The effects of adding inorganic metal ions (e.g., Al^{3+}, Ti^{4+}, Cu^{2+}, K^{+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}) with the same concentration for 10 mg L^{-1} As(III) oxidation were investigated (Fig. 5c). Except for Mn^{2+}, these tested metal ions did not affect As(III) oxidation. The oxidation efficiency of As(III) slightly decreased in the presence of Mn^{2+} possibly because a part of spore–laccase was consumed to oxidize Mn^{2+} to Mn^{3+}.[79, 80] These results indicate that the spore–TEMPO system for As(III) oxidation has preferable tolerance to metal ions.

After each use, the spores were collected through centrifugation and washed twice for the next cycle of oxidation. The As(III) oxidation efficiency did not significantly decreased after fifteen cycles (Fig. 5d). This finding is similar to that obtained by using commercial laccase and strongly suggests

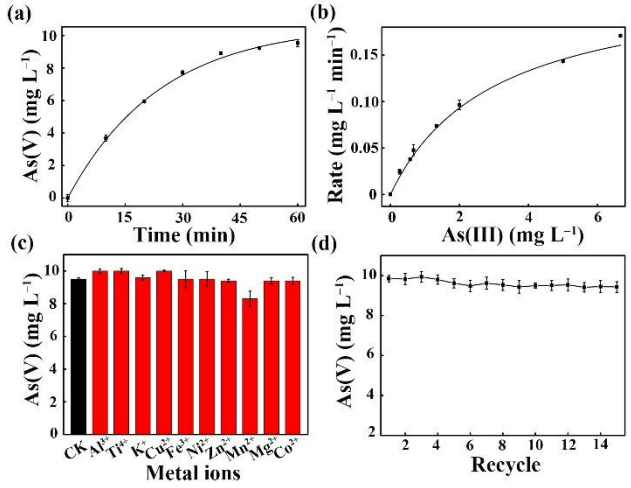

Fig. 5 As(III) oxidation used by spore–TEMPO. (a) The kinetic of As(III) oxidation by spore–TEMPO. Conditions: 7.48×10^7 CFU mL^{-1} spore, 1 mmol L^{-1} TEMPO, pH 4.5, 55 °C, 10 mg L^{-1} As(III). (b) Michaelis–Menten reaction kinetics of As(III) oxidation by spore–TEMPO. Conditions: 7.48×10^7 CFU mL^{-1} spore, 1 mmol L^{-1} TEMPO, pH 4.5, 55 °C, 0-6.667 mg L^{-1} As(III), 10 min reactive time. (c) The effect of metal ions (10 mg L^{-1}) in the oxidation of As(III) by spore–TEMPO. CK means As(III) oxidation in the absence of metal ions. (d) The recyclability of spore in the As(III) oxidation. Condition: 7.48×10^7 CFU mL^{-1} spore, 1 mmol L^{-1} TEMPO, pH 4.5, 55 °C, 10 mg L^{-1} As(III). The error bars represent SE of the means. The data represent means ±SE (n=3).

that spores have good recyclability in As(III) oxidation. Previous works have demonstrated that TEMPO could be effectively recovered and reused by selective adsorption onto a hydrophobic resin[81] or heterogenization of TEMPO derivatives on a solid support[82-87] to address the practical hurdle of the TEMPO price. Accordingly, the recovery and reuse of TEMPO will carry out in our lab in the future work.

Conclusions

In this work, we revealed for the first time that laccase–TEMPO and spore–TEMPO systems are both eco-friendly systems for As(III) oxidation. Laccase or spores can catalyze and oxidize TEMPO to form oxoammonium cations in the presence of oxygen, which can oxidize As(III) to As(V). According to this mechanism, several TEMPO derives also exhibited different abilities for As(III) oxidation, whereas other laccase mediators show minimal evidence for As(III) oxidation. Mediators have different mechanisms for the reaction between mediator and As(III). Using spores instead of laccase has several advantages, including low cost, easy to collect, reuse, and store and does not need professional training and enzyme purification. All these results imply that the spore–TEMPO system is a cost-effective process for arsenic removal in contaminated waters.

Conflicts of interest

The authors declare no competing financial interest.

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Rapid catalytic oxidation of As(III) to As(V) by using *Bacillus* spore–2,2,6,6-tetramethylpiperidine-1-oxyl system

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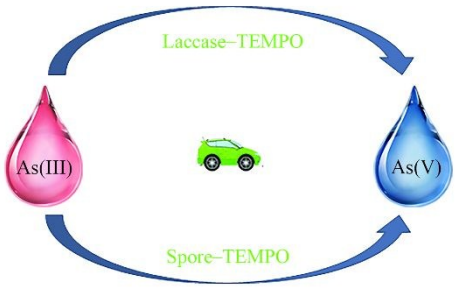

A rapid and green oxidation of As(III) to As(V) was proposed using *Bacillus* Spore–TEMPO system.