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Sustainable Endospore-Based Microreactor System for Antioxidant Capacity Assay

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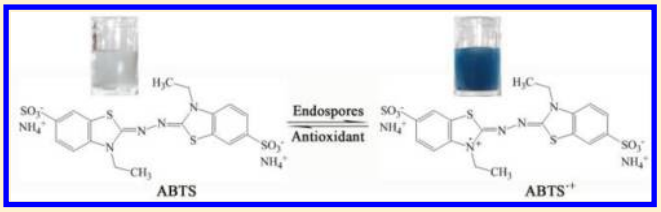
Supporting Information

ABSTRACT: A novel endospore-based microbial method for "post-additional" antioxidant capacity assay was developed. The technique was based on oxidation and catalysis of the 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) by *Bacillus subtilis* 168 endospores in the presence of dissolved oxygen. Coat protein A (CotA), which belongs to the endospore coat, was expressed, purified, and assessed for its ability to oxidize ABTS into the ABTS$^{\bullet+}$ radical cation. The wild-type endospore necessary for oxidizing ABTS into ABTS$^{\bullet+}$ radical cation was confirmed by knocking out the *cotA* gene from *B. subtilis* 168 by homologous double exchange. Findings revealed that the catalytic activity of the endospores may be attributed to the presence of the CotA protein. The use of endospores instead of purified enzymes to prepare ABTS$^{\bullet+}$ greatly reduced the assay cost and eliminated the need to purify and store of enzymes. The self-life of the radical cation was kept stable for at least 12 days without addition of a stabilizer and laccase inhibitor. This behavior enables the large-scale preparation of ABTS$^{\bullet+}$. The antioxidant capacities of the individual antioxidants and fruit samples were easily quantified and compared using the proposed method. The developed technique can be further developed as a high-throughput screening technique for antioxidants.



Oxidation reactions, which involve free radicals and other reactive species, are important in human physiology and food industry.[1,2] However, environmental and behavioral stressors (e.g., pollution, sunlight exposure, cigarette smoking, and excessive alcohol consumption) produce excess free radicals that result in oxidative stress. Oxidative-stress products directly damage biomolecules or initiate chain reactions that cause extensive damage to cellular structures. Antioxidants protect biological and food systems from the harmful effects of oxidative stress.[3] The mechanism by which antioxidants achieve their functions include reacting with free radicals, chelating catalytic metals, and scavenging reactive oxygen species.[4] As such, analytical methods for the antioxidant capacity assay of various molecules have received significant research interest.

Several assays based on electrochemical, fluorescence, chemiluminescence, and spectrophotometric detection have been used to measure the antioxidant capacities of antioxidants.[5−7] Among these methods, colorimetric assays have attracted considerable attention because they require simple instrumentation (e.g., UV−vis spectrophotometer) and simple screening analysis (e.g., by the naked eye). Several colorimetric methods for antioxidant capacity assay have been developed.[8−21] The most widely used colorimetric method is the ABTS-based Trolox equivalent antioxidant capacity (TEAC) assay.[12,13] This method was previously suggested as a standardized method for determining antioxidant capacity.[6] Originally, TEAC assay was performed in inhibition mode. In this mode, a mixture of ABTS, the sample, and hydrogen peroxide is produced. The reaction is initiated by addition of metmyoglobin into this mixture, and the solution absorbance is read after a fixed time. Thereafter, the inhibition percentage is determined by comparison of the results with a blank assay. However, the use of inhibition mode is limited because antioxidants react with oxidizing agents themselves, which could lead to overestimation of antioxidant capacity.[14] Thus, "post-addition" techniques have been proposed to improve the TEAC assay protocol;[15,16] these techniques are expected to minimize the interference of compounds with oxidants during radical formation and prevent the possible overestimation. In these proposed techniques, ABTS is oxidized to generate blue-colored ABTS$^{\bullet+}$ with maximum absorption at 414, 645, and 734 nm in the presence of proper oxidants. Antioxidants are added after radical formation. These antioxidants quench ABTS$^{\bullet+}$ and convert it into a colorless product (i.e., ABTS). The degree of decolorization reflects the amount of ABTS$^{\bullet+}$ that is scavenged, and results may be directly monitored by spectrophotometric detection.[6,17] The antioxidant capacity, which is based on the ability of antioxidants to decolorize the ABTS$^{\bullet+}$, is commonly evaluated using the TEAC value. This parameter is assigned to all compounds that scavenge ABTS$^{\bullet+}$ by comparing the scavenging capacity to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

According to the literature, ABTS$^{\bullet+}$ is generated either by chemical or enzymatic reactions. However, chemicals are

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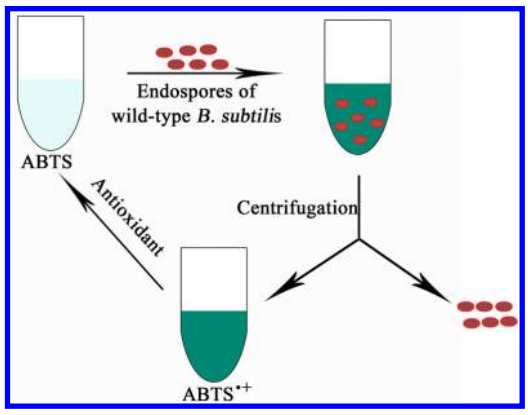
generally generated using high temperatures [e.g., 60 °C for 2,2′-azobis(2-amidinopropane) HCl] or extended periods of time (e.g., overnight for potassium persulfate).[17,18] Enzymes have been applied in colorimetric antioxidant assays because they exhibit several advantages over chemical oxidants. Enzymes are eco- and exhibit high substrate specificities and high efficiencies under mild conditions. The original enzyme-ABTS-based antioxidant assay was developed by investigating the effect of antioxidants on metmyoglobin and H_2O_2-mediated ABTS oxidation.[12] In some studies, a horseradish peroxidase (HRP) was used instead of metmyoglobin[13] because the fast-reacting antioxidants also reduce the ferryl myoglobin radical. A major disadvantage of the postaddition method is that $ABTS^{\bullet+}$ is a metastable radical; thus, the radical quickly decays into a colorless product through disproportionation in the presence of redundant ABTS or H_2O_2, leading to decreased absorption signals.[18] For instance, $ABTS^{\bullet+}$ is only stable for about 2 d at 0 to 4 °C in the presence of redundant ABTS (e.g., 50-fold).[19] This property does not meet the requirements of high-throughput detection and long-term monitoring of test samples. An appropriate concentration of H_2O_2 is therefore generally required for the ABTS−H_2O_2 system. Laccase-based methods for antioxidant determination have been developed to overcome this drawback.[20,21] Such methods have been proposed because generating $ABTS^{\bullet+}$ using laccase is easy and does not require additional oxidizers, with the exception of dissolved oxygen (O_2). However, laccase activity is not inhibited during antioxidant determination. Reduced ABTS reoxidizes and interferes with subsequent antioxidant capacity assays. Thus, the laccase step must be limited to avoid interference during antioxidant measurement.[21]

Protein enzymes that include metmyoglobin, HRP, and laccase also exhibit several disadvantages, including high cost, nonrecyclability, sensitivity of catalytic activity to environmental conditions, poor stability because of denaturation, and difficult preparation and purification. In the present study, a novel endospore-based system for antioxidant capacity assay was proposed. This method utilizes the "post-addition" technique as well as $ABTS^{\bullet+}$ and is based on the presence of coat protein A (CotA), which belongs to the outer spore coat of *Bacillus subtilis*.[22−24] CotA is a bacterial laccase that oxidizes ABTS to colored $ABTS^{\bullet+}$ in the presence of dissolved O_2. Scheme 1 shows the procedure of the proposed antioxidant capacity assay. First, the wild-type endospores of *B. subtilis* 168 are added to the ABTS solution to generate $ABTS^{\bullet+}$. The endospores are then removed from the solution to avoid disproportionation reaction and maintain the stability of the $ABTS^{\bullet+}$ solution, which is a requirement in high-throughput detection and long-term monitoring of samples. The antioxidants are added to the $ABTS^{\bullet+}$ solution, and the antioxidant capacity is quantified by reducing the absorption signals of $ABTS^{\bullet+}$, which transforms back into colorless ABTS. The developed method was used to determine the TEAC values (i.e., antioxidant capacities) of various antioxidants and food samples. The TEAC values obtained were comparable with those obtained from a commercial assay method.

Scheme 1. Schematic of the Antioxidant Capacity Assay of *Bacillus subtilis* 168 Endospores



EXPERIMENTAL SECTION

Materials. Taq DNA polymerase, restriction endonucleases, and other modifying enzymes were obtained from Takara (Takara Biotechnology (Dalian) Co., Ltd. Japan). All primers were synthesized by Tsingke Bio Tech Co., Ltd. (Wuhan, China). The DNA markers and protein molecular-size markers, competent cells [*E. coli* DH5α and *E. coli* BL21 (DE3)], and Ni-NTA Sefinose resin kit were obtained from Sangon Biotech (Shanghai, China). The DIG High Prime DNA Labeling and Detection Starter kit I was obtained from Roche Diagnostics (Shanghai), Ltd. (Shanghai, China). ABTS, quercetin, Trolox, glutathione, ascorbic acid, uric acid, α-tocopherol, and β-carotene were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO). The T-AOC assay kit used was from Beyotime Institute of Biotechnology (Jiangsu, China). A stock solution of 10.0 mmol L^{-1} ABTS was prepared using ultrapure H_2O. Trolox and other compounds were dissolved in appropriate solvents to produce 10.0 mmol L^{-1} of antioxidant stock solutions. Trolox and quercetin were dissolved in methanol. Glutathione and ascorbic acid were dissolved in ultrapure H_2O. Uric acid was dissolved in 1.0 mol L^{-1} of NaOH, and α-tocopherol was dissolved in 95% ethanol. β-carotene was dissolved in chloroform. The stock solutions were stored at temperatures between 0 and 4 °C. Working solutions of the antioxidants were prepared daily by diluting the stock solutions with the corresponding solvents. Trolox, glutathione, ascorbic acid, and uric acid were diluted with PBS solution (pH 7.4). Quercetin and α-tocopherol were diluted with 95% ethanol, and β-carotene was diluted with *N*,*N*-dimethylformamide (DMF). Fruit juices (i.e., cherry tomato, grape, peach, orange, and kiwifrit) for analysis were purchased at a local supermarket. The solutions were filtered and diluted with H_2O to reduce their concentrations to the assay range proposed in this work. Ultrapure H_2O (18.2 MΩ cm) was produced using a model Cascada IX laboratory ultrapure H_2O system (Pall Co., Ltd.). The pH of all buffer solutions was measured using a PB-10 pH meter (Sartorius AG, Germany). All of the reagents were of analytical grade and purchased from commercial suppliers.

Cloning of the *cotA* Gene. Genomic DNA from *B. subtilis* 168 was extracted using phenol/chloroform/isopentanol extraction and precipitated with ethanol. The *cotA* gene was amplified using a polymerase chain reaction (PCR) with the forward primer 5′-ATT*GGATCC*GATGACACTT-GAAAAATTTG-3′ (*Bam*HI) and the reverse primer 5′-CAT*AAGCTT*TTATTTATGGGGATCAGT-3′ (*Hin*dIII). The PCR program of the 5334 PCR system (Eppendorf, Germany) utilized was as follows: 10 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 48 °C and 2 min at 72 °C, and a final extension at 72 °C for 7 min. The PCR fragments (1542 bp) were digested using *Bam*HI and *Hin*dIII and then cloned in the expression vector pET28b(+) (Novagen), which carries the

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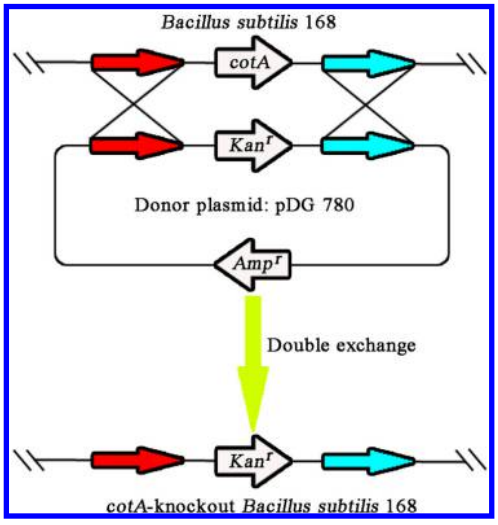
strong bacteriophage T7 promoter and terminator. *cotA* cloned in pET28b(+) was transformed into *E. coli* DH5α for cloning, and results were confirmed using sequence analysis.

Expression and Purification of CotA Protein. The plasmid recovered from *E. coli* DH5α was transformed into *E. coli* BL21 (DE3) for expression. *E. coli* BL21 (DE3) cells were grown in 5 mL of Luria−Bertani (LB) medium supplemented with kanamycin (25 μg mL^{-1}) at 37 °C and 180 rpm overnight. The prepared culture was then diluted to 1:100 with fresh LB medium containing kanamycin (25 μg mL^{-1}). Subsequently, the culture was incubated using a Platform Constant Temperature (Shanghai Jing Hong Laboratory Instrument Co., Ltd., Shanghai, China) instrument at 37 °C and 180 rpm for 3 h. A DU800 UV/vis spectrophotometer (Beckman Coulter, USA) was used to detect the optical density (OD) of the *E. coli* BL21 (DE3) cells. Expression was induced at a density (OD_{600}) of 0.6 by addition of 1.0 mmol L^{-1} of isopropyl-β-D-thiogalactoside (IPTG) to the culture at 18 °C and centrifugation at 180 rpm for 15 h. The cells were harvested using a high-speed centrifuge (Hitachi high-speed centrifuge CR21GIII, Tokyo, Japan) at 4 °C and 8000 rpm for 10 min and then resuspended in native binding buffer (pH 7.4). Cell disruption was performed by sonicating the cells on ice using a Sonifier sonicator (Branson, MO). The cell debris was removed through centrifugation (12000 rpm, 20 min, 4 °C), and the supernatant was incubated for 15 min at 70 °C. Denatured proteins were removed through centrifugation (12000 rpm, 20 min, 4 °C). Purification was performed using a Ni-NTA Sefinose resin kit (Sangon Biotech). The protein content was determined by Bradford assay, using bovine serum albumin as the standard. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed in a DYCZ-24DN mini double vertical electrophoresis system (LIUYI Instrument Factory, China) using 30% polyacrylamide running gel.

***CotA* Gene Knockout.** Homologous double exchange was used to knock out the *cotA* gene (Scheme 2). A 700 bp sequence upstream of *cotA* was used as the upstream homology arm, and a 900 bp sequence downstream of *cotA* was used as the downstream homology arm sequence. The pDG780, which has ampicillin and kanamycin resistance genes, was used as the suicide vector. The primer pair F: 5′-TGC*GAGCTC*TTTATCGTCCTCACAGCCG-3′ (*Sac*I) and R: 5′-TGC*TCTAGA*TTTCATCTGTCCTTATCTAAAT-3′ (*Xba*I) was used to amplify the upstream arm, and the primer pair F: 5′-CCG*CTCGAG*CCCGACAAACTTGCCTCT-3′ (*Xho*I) and R: 5′-CGG*GGTACC*TTAAAAGCTTTTTCCTGGTTTA-3′ (*Kpn*I) was used to amplify the downstream arm. The PCR fragment of the upstream arm was digested using *Sac*I and *Xba*I. The fragment was then subsequently connected to the pDG780 vector that had been previously digested with the same enzymes. Sequence analysis confirmed the cloning procedure. Positive transformants were screened using ampicillin and kanamycin resistance. The cloning process of the downstream arm was similar to that of the upstream arm. The pDG780 vectors carring the upstream and downstream homologous arms were transformed into *B. subtilis* 168 to provide it with the *cotA*-knockout mutant strain. The mutant strain was screened using kanamycin resistance, and results were determined using cloning PCR. The morphologies of the endospores were observed using a JEM-100CX II transmission electron microscope (Japan Electronics Co., Ltd., Japan).

Scheme 2. Schematic of Homologous Double Exchange



Southern Blot Analysis. Southern blot analysis was performed using a DIG High Prime DNA Labeling and Detection Starter kit I (Roche). Genomic DNA was isolated and digested to different size fragments using the restriction endonuclease *Kpn*I. The fragments were separated via 0.8% gel electrophoresis (25−50 V for 12−16 h). The result was analyzed using an Alpha Imager Mini gel imaging system (Silicon Valley). The DNA fragments were then transferred to a nylon membrane through capillary action. The purified PCR product of a *cotA* fragment was used as the template after denaturation. Probes prepared through random primer synthesis were marked with digoxin (DIG). Hybridization was performed overnight in a DIG Easy Hyb. Finally, the resulting product was obtained using the NBT/BCIP chromogenic reaction.

Preparation of Endospores. The bacterial culture was routinely grown at 37 °C in LB solid medium for at least 7 d. Endospores scraped off from the medium were washed twice with distilled H_2O and then stored in distilled H_2O at room temperature. Viable cell counts were determined by plating the endospores on 1.5% LB solid agar medium, and colonies were counted after overnight incubation.

Preparation of ABTS$^{\bullet+}$. ABTS$^{\bullet+}$ (40 mL 0.3 mmol L^{-1}) was prepared by adding 4.0 mL of wild-type *B. subtilis* 168 endospores (7.48×10^9 CFU mL^{-1}) to 36.0 mL of 0.33 mmol L^{-1} ABTS solutions at room temperature (pH 3.0). The endospores were then centrifuged for 40 min to separate them from the blue-green ABTS$^{\bullet+}$ solution. The ABTS$^{\bullet+}$ solution was stored in the dark at 4 °C until use. The pH of the ABTS$^{\bullet+}$ solution was adjusted to 7.4 before the antioxidant capacity assay was performed.

Electron Paramagnetic Resonance (EPR) Analysis. EPR analysis of ABTS$^{\bullet+}$ was performed at room temperature in a JES-FA200 spectrometer (JEOL Ltd.). EPR measurements were conducted using a radiation of 9.15 GHz (X band) with a modulation frequency of 100 kHz, a sweep width of 10 mT, modulation width of 0.1 mT, center field of 326.544 mT, scan time of 60 s, time constant of 0.03 s, and microwave power of 5 mW.

Antioxidant Capacity Assay. The proposed antioxidant capacity assay method was based on the capacity of a sample to scavenge ABTS$^{\bullet+}$ compared with a standard antioxidant (Trolox) in a dose−response curve. The ABTS$^{\bullet+}$ stock solution was diluted with 5 mmol L^{-1} of phosphate buffered saline

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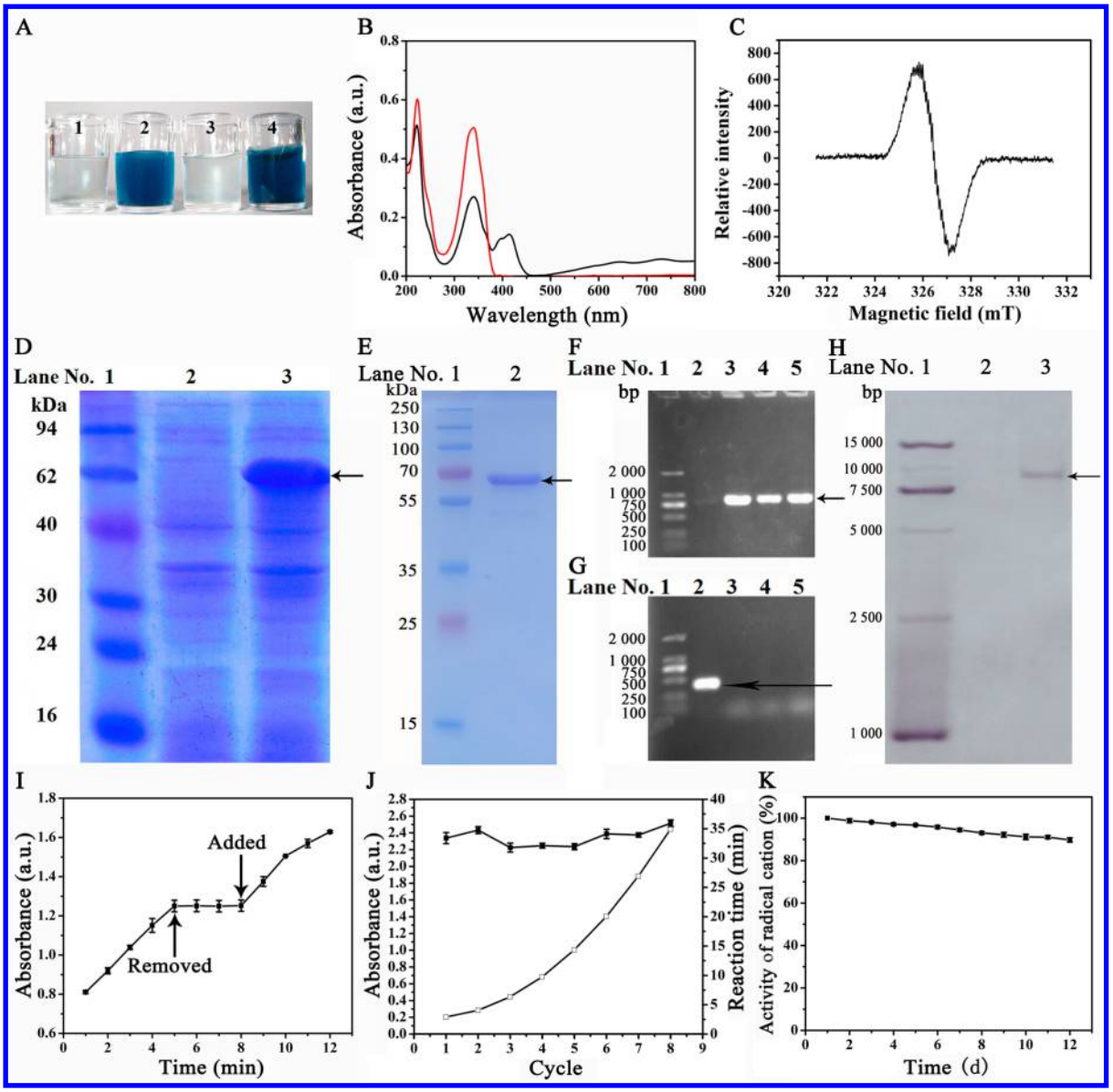

Figure 1. (A) Color reaction: (1) H_2O + ABTS, (2) endospores of wild-type *B. subtilis* + ABTS, (3) endospores of *cotA* knockout *B. subtilis* + ABTS, and (4) recombinant CotA protein + ABTS. (B) UV−vis spectra of ABTS (red line) and its oxidation product ($ABTS^{\bullet+}$; black line). (C) EPR spectral analysis of $ABTS^{\bullet+}$ cation radical. (D) SDS−PAGE results of cell extracts: lane 1, molecular-size marker; lane 2, cell extract of *E. coli* BL21 (DE3) transformed into pET28b(+); lane 3, cell extracts of *E. coli* BL21 (DE3) transformed into pET28b(+)/*cotA*. The arrow represents the CotA protein; (E) SDS−PAGE results of the purified CotA protein: lane 1, molecular-size marker; lane 2, purified CotA protein. The arrow represents the CotA protein; (F) agarose gel electrophoresis of colony PCR products amplified using primers of the kanamycin resistance gene: lane 1, DL2000 DNA marker; lane 2, colony PCR product of wild-type *B. subtilis* 168; lanes 3−5, colony PCR products of the positive *cotA*-knockout mutant strain. The arrow represents the kanamycin resistance gene. (G) Agarose gel electrophoresis of colony PCR products amplified using primers of part of the *cotA* gene: lane 1, DL2000 DNA marker; lane 2, colony PCR product of wild-type *B. subtilis* 168; lanes 3−5, colony PCR products of the positive *cotA*-knockout mutant strain. The arrow represents the *cotA* gene. (H) Chromogenic reaction results of Southern blot: lane 1, DL15000 DNA marker; lane 2, genomic DNA digestion products of *cotA*-knockout mutant strain; lane 3, genomic DNA digestion products of wild-type *B. subtilis* 168. The arrow represents the *cotA* gene. (I) Response of ABTS against endospores: Conditions: 4.0 mL of endospores (7.48×10^9 CFU mL^{-1}) added to 36.0 mL of ABTS (0.5 mmol L^{-1}), pH 4.5. (J) Recyclability of endospores (■: absorbance, □: reaction time); conditions: 4.0 mL of endospores (7.48×10^9 CFU mL^{-1}) reacted with 36.0 mL of ABTS (0.33 mmol L^{-1}), pH 4.5. (K) Stability of $ABTS^{\bullet+}$ solution. $ABTS^{\bullet+}$ radical cation solution was stored at 4 °C. Data are presented as (mean ± SD) (error bars), $n = 3$.

(PBS) to pH 7.4. The final absorbance of the diluted solution was 1.0 ± 0.02 at 414 nm[19,25] (i.e., an $ABTS^{\bullet+}$ concentration of approximately 0.1 mmol L^{-1}). A reaction time of 6.0 min was selected in subsequent antioxidant assays. These parameters were selected based on the TEAC values determined in previous studies.[19,26−28] The absorbance difference between the reaction and the blank groups was recorded as ΔAb. $ABTS^{\bullet+}$ scavenging activity was calculated using the formula: $(\Delta Ab_a - \Delta Ab_p)/\Delta Ab_a \times 100$, where ΔAb_a and ΔAb_p represent ΔAb in the absence and presence of samples, respectively. Finally, antioxidants that scavenged $ABTS^{\bullet+}$ were assigned with TEAC values by comparing their scavenging activities with that of Trolox.[15] A BioTek Synergy HT Multi microplate reader

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(Vermont) was used to measure all optical absorptions. All assays were performed in triplicate at room temperature.

RESULTS AND DISCUSSION

Characterization of the Endospore-Based Microbial System. Addition of the endospores of *B. subtilis* induced laccase activity. The endospores also oxidized the laccase substrate ABTS to generate blue-green $ABTS^{\bullet+}$, which exhibits maximum absorption at 414, 645, and 734 nm, as previously described (Figure 1, panels A and B).[6,19,29] The formation of $ABTS^{\bullet+}$ was further confirmed by EPR analysis (Figure 1C). A protein band with a molecular weight of 65 kDa was observed during SDS−PAGE (Figure 1D). The presence of this protein reveals that the CotA protein of *B. subtilis* 168 was successfully cloned and expressed. The recombinant CotA protein was purified (Figure 1E) and then added to the ABTS solution. The ability of the recombinant CotA protein to oxidize ABTS to $ABTS^{\bullet+}$ was confirmed (Figure 1A).

The *cotA* gene was knocked out from the genome of *B. subtilis* 168 through homologous double exchange and the colony PCR product of the wild-type *B. subtilis* 168 was analyzed and compared with that of the *cotA*-knockout mutant strain. A PCR product of the expected 794 bp size (Figure 1F; lanes 3−5), which was a fragment of kanamycin resistance gene, was obtained with DNA from *cotA*-knockout mutant strain. A middle fragment of *cotA* (500 bp) was present in the product of wild-type *B. subtilis* 168 (Figure 1G; lane 2). No fragment of *cotA* gene was observed in product of *cotA*-knockout mutant strain (Figure 1G; lanes 3−5). Southern blot results further revealed the absence of the *cotA* gene from the genomic DNA of the mutant strain and the presence of the *cotA* gene (8588 bp) in the genomic DNA of the wild-type strain (Figure 1H). These findings indicate that the *cotA* gene was successfully replaced by the kanamycin resistance gene in the mutant strain. While the endospores of the mutant strain formed slower than those of the wild-type strain, the morphology of these endospores did not obviously change after *cotA* knockout (Figure S-1 of the Supporting Information).

Loss of laccase activity was confirmed by adding mutant endospores to the ABTS solution. In this experiment, the solution remained colorless and did not generate $ABTS^{\bullet+}$ (Figure 1A). These results indicate that the CotA protein is the key for $ABTS^{\bullet+}$ formation. The reaction may be switched off or on by removing and reinserting the endospores after brief intervals (Figure 1I). The easy workup of the reaction products after withdrawing the wild-type endospores was ensured. Prompt formation of $ABTS^{\bullet+}$ was captured in the reaction profile. The recyclability of wild-type endospores was then investigated to determine their practical application (Figure 1J). The same endospores performed the same oxidation reaction for each succeeding run. After each use, the endospores were precipitated through centrifugation and then washed for the next cycle of catalysis. The endospores were successfully recycled and reused for at least eight successive reactions, although extended reaction times of cycles were necessary to achieve the same $ABTS^{\bullet+}$ yields. This finding may be attributed to the gradual loss of activity of the CotA protein because of repeated use and washing. CotA-laccase activity remained stable for at least 1 month when the endospores were stored in distilled H_2O at room temperature (Figure S-2 of the Supporting Information). The CotA-laccase activity of the endospores retained about 44.7% of their initial activity after 5 month. These endospores, although partly lost CotA-laccase activity, also could be used to form $ABTS^{\bullet+}$ and achieved the same yields with longer reaction time than the use of the initial endospores. The CotA-laccase of the endospores showed higher values compared with the initial findings after incubation at 50−80 °C over a certain time range (Figure S-3 of the Supporting Information). This behavior may be attributed to loosening the CotA-laccase structure after high-temperature incubation, which then increases laccase activity compared with initial value.[24] Loss of laccase activity was not observed after 6 h at temperatures varying from 50 to 60 °C. The half-life of inactivation at 80 °C was 4.5 h. This remarkable property shows that the CotA-laccase of endospores exhibited high thermostability. Moreover, wide-type endospores exhibiting partial loss of CotA-laccase activity regained their full CotA-laccase activity in favorable environmental conditions because of "sporulation-germination" cycles. These endospores survive most environmental challenges (e.g., extreme temperatures, desiccation, and exposure to solvents and noxious chemicals) for extended periods of time up to millions of years.[30] This behavior not only reduces the cost of detection but also eliminates the strict demand of enzyme storage because of the higher hydrolytic and thermal stability of the endospores compared with that of natural enzymes.

The $ABTS^{\bullet+}$ stock solution was prepared by mixing 4.0 mL of wild-type *B. subtilis* 168 endospores (7.48×10^9 CFU mL^{-1}) and 36.0 mL of 0.3 mmol L^{-1} ABTS diluted with phosphate/disodium hydrogen phosphate buffer (optimization of the formation of $ABTS^{\bullet+}$, Figure S-4 of the Supporting Information). Absorption signals of the solution at 414 nm ($\varepsilon = 3.6 \times 10^4$ mol^{-1} L cm^{-1}) were achieved to a platform after 40 min, which indicates that the formation of $ABTS^{\bullet+}$ was completed (Figure S-4B of the Supporting Information). The endospores were then centrifuged to separate them from the $ABTS^{\bullet+}$ solution. The $ABTS^{\bullet+}$ stock solution stored in the dark at 4 °C, without the use of the laccase inhibitor (e.g., methanol)[21] and stabilizers (e.g., adenosine triphosphate),[31] exhibited only a nominal decrease in efficacy from 100.0% to 90.0% after 12 d (Figure 1K). This finding reveals that the interference of redundant ABTS (or H_2O_2)[18] and laccase[21] in the antioxidant capacity assay and disproportionation of $ABTS^{\bullet+}$ are effectively eliminated. That is, the $ABTS^{\bullet+}$ radical cation was stable for at least 12 d. The lifetime of the radical cation was significantly longer than that of the HRP−H_2O_2 system (2 d)[19] and was comparable to that of the G-quadruplex DNAzyme catalyzed ABTS−H_2O_2 system.[31] Wild-type *B. subtilis* 168 endospores were analogous to a "factory" consisting of numerous CotA-laccase molecules, which can be massively produced through cell culturing. Compared with current engineered microorganism-based microbial biosensors, the present endospores are simple, inexpensive, and can be extracted using simple techniques. These endospores can endure extreme environmental conditions, have no need for nutrients, and can be stored and easily cycled between the vegetative endospore and germinated living cell states.[32,33] Thus, the developed method exhibites the inherent advantages of microbial sensors, such as low cost, easy fabrication, sensitivity, and stability comparable with those of enzyme sensors, and no requirement of enzyme purification.[34,35] These advantages are beneficial for the large-scale preparation of $ABTS^{\bullet+}$, the high-throughput detection, and long-term monitoring of test samples.

A more generally used chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was selected to make a

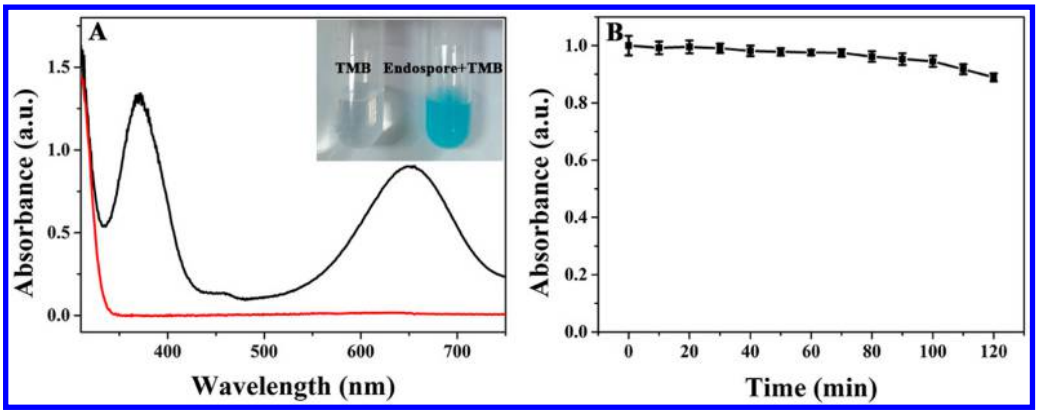

Figure 2. (A) UV−vis spectra of TMB (red line) and TMB oxidation (black line). Inset: the photograph of TMB solution and the mixture of TMB and endospores. (B) Stability of $TMB^{\bullet+}$ solution. $TMB^{\bullet+}$ radical cation solution was stored at 4 °C. Data are presented as (mean ± SD) (error bars), n = 3.

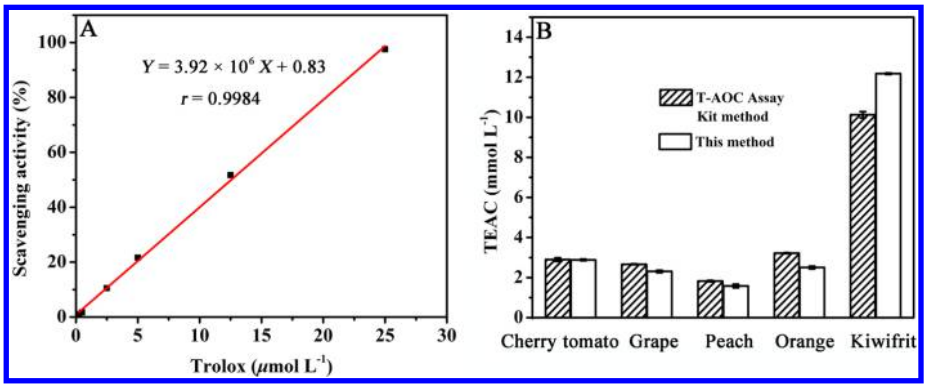

Figure 3. (A) Calibration curve of $ABTS^{\bullet+}$ scavenging activity in different concentrations of Trolox and (B) comparative TEAC values (mmol L^{-1}) of some fresh fruit juices measured using a T-AOC assay kit and the proposed method. The data are presented as (mean ± SD) (error bars), n = 3.

comparison study with ABTS. The oxidation of TMB by endospores in acid buffer produces a blue color (Figure 2A), with major absorbance peaks at 370 and 652 nm. The product was further analyzed using an Agilent 6540 ultrahigh-definition accurate-mass quadrupole time-of-flight liquid chromatography/mass spectrometry system (LC−MS) and generated a peak at 2.2 min with a molecular weight of 241.17 (Figure S-5A of the Supporting Information). These properties are consistent with those of the $TMB^{\bullet+}$ radical cations,[36−38] which demonstrates the catalytic activity of endospores for the $TMB^{\bullet+}$ formation in the presence of O_2.

Oxidation conditions, such as pH and reaction time, were optimized (Figure S-6 of the Supporting Information). Optimal endospore activity in the TMB substrate was observed at pH 4.0. Absorbance at 652 nm decreased with increases in pH, and significant TMB oxidation was not observed at pH values up to 7.0 (Figure S-6A of the Supporting Information). $TMB^{\bullet+}$ radical cations were stable for approximately 2 h at pH 4.0 (Figure 2B) but unstable at pH values higher than 6.0. The blue solution turned yellow within 1 min after the pH was adjusted to 7.0. Absorbance increased as the reaction time increased from 10 to 30 min (Figure S-6B of the Supporting Information), and the highest absorbance were obtained at 30 min. Absorbance then decreased with further increases in reaction time. During this time, the color of the reaction solution gradually changed from blue to yellow-green and then to yellow with maximum absorbance at 450 nm. The yellow product was further analyzed using LC−MS (Figure S-5B of the Supporting Information). On the basis of our experimental results as well as findings from previous studies,[36−38] we believe that the peak at 2.2 min may be attributed to the di-imine (i.e., TMB^{2+}).

Antioxidant Capacity Assay for Individual Antioxidants and Real Samples. Trolox, a standard widely used in antioxidant assays, was used as the model antioxidant in this study. The ability of the proposed method to scavenge $ABTS^{\bullet+}$ was investigated to test the feasibility of the method in evaluating antioxidant capacity. Absorption signals decreased with increasing antioxidant concentration. As well, the color of the sensing system faded and finally disappeared. This behavior suggests that the method can be used for antioxidant screening using only the naked eye. Thus, high-throughput screening of potential antioxidants may be performed via a simple process.

Antioxidant concentration-dependent $ABTS^{\bullet+}$ scavenging activity was investigated under various conditions, and dose−response curves were obtained by adding different concentrations of antioxidant compounds to the premixed $ABTS^{\bullet+}$ solution. The $ABTS^{\bullet+}$ scavenging activity of Trolox exhibited good linearity over a relatively broad concentration range from 1.07×10^{-2} to 25.00 μmol L^{-1} (Figure 3A). The linear equation for the calibration graph of Trolox was $y = 3.92 \times 10^6 x + 0.83$, where y is the $ABTS^{\bullet+}$ scavenging activity and x is the Trolox concentration. The correlation coefficient (r) in this experiment was 0.9984. This result indicates that the proposed method can be used to quantify individual antioxidants. The limit of detection (LOD) for Trolox was 3.21×10^{-3} μmol L^{-1} (3σ), lower than that detected by other ABTS-based antioxidant assay methods.[39] Repeatability, which is expressed

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Table 1. Linear Equations, Correlation Coefficients(r), Linear Ranges, Limits of Detection (LOD), and TEAC Values of Several Antioxidants (n = 3)

antioxidants	linear equation and correlation coefficients	linear range (μmol L^{-1})	LOD (μmol L^{-1})	TEAC, this method	TEAC, reference
ascorbic acid	$y = 1.30 \times 10^6 x + 1.32$ (r = 0.9990)	3.28×10^{-2} − 75.00	9.83×10^{-3}	1.41	1.60[19]
uric acid	$y = 3.80 \times 10^6 x + 1.20$ (r = 0.9935)	8.17×10^{-3} − 25.00	2.45×10^{-3}	4.57	2.60[19]
glutathione	$y = 1.04 \times 10^6 x + 8.89$ (r = 0.9929)	0.50−90.00	1.72×10^{-2}	1.07	1.09[40]
quercetin	$y = 8.54 \times 10^6 x + 4.24$ (r = 0.9911)	7.33×10^{-3} − 10.00	2.20×10^{-3}	5.16	4.95[40]
α-tocopherol	$y = 1.68 \times 10^6 x - 4.94$ (r = 0.9987)	0.25−60.00	4.03×10^{-2}	0.93	0.97[16]
β-carotene	$y = 5.88 \times 10^6 x + 0.04$ (r = 0.9984)	1.43×10^{-2} − 15.00	4.29×10^{-3}	2.04	2.14[41]

as the relative standard deviation (RSD %), was 2.4%. This parameter was evaluated using 5.0 μmol L^{-1} of Trolox variability data over 20 replicates (n = 20). Results indicate that the proposed method exhibits excellent repeatability. Interferences caused by some related substances were further investigated. Experiments were performed by accurately mixing a specimen with Trolox; here, the $ABTS^{\bullet+}$ scavenging activity relative to 25.0 μmol L^{-1} Trolox was used as the reference standard. Potential interfering compounds such as Cu^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, SO_4^{2-}, CO_3^{2-}, NO_3^-, HCO_3^-, I^-, glucose, D-fructose, D-mannose, lactose, sucrose, and soluble starch, did not interfere with the assay under 1.0×10^{-4} mol L^{-1}. Moreover, casein, bovine hemoglobin, and human serum albumin did not show significant interference below 1.0×10^{-4} g mL^{-1}. Thus, the developed method is free from interferences from other substances in food extracts or plasma.

Several pure (e.g., hydrophilic antioxidants, such as ascorbic acid, uric acid, and glutathione) and lipophilic (e.g., α-tocopherol, quercetin, and β-carotene) antioxidants were analyzed using the proposed method. The TEAC values of the antioxidants were calculated using the $ABTS^{\bullet+}$ scavenging activity obtained relative to Trolox at the same concentration.[15] Linear equations, r, linear ranges, LOD, and TEAC values of the antioxidants were obtained, and results are listed in Table 1. Findings indicate that the TEAC values of the detected antioxidants were comparable with those obtained using other methods.[16,19,40,41]

The pure antioxidants were analyzed using $TMB^{\bullet+}$ radical cations at pH 4.0. Similar to the TEAC values obtained in the $ABTS^{\bullet+}$-based method, the TEAC values of the antioxidants were calculated using the $TMB^{\bullet+}$ scavenging activity obtained relative to Trolox at the same concentration. As shown in Table S-1 of the Supporting Information), the studied compounds exhibited lower antioxidant capacity than those previously obtained.[16,19,40,41] That is, the use of $ABTS^{\bullet+}$ radical cations for antioxidant capacity assay is more accurate and reliable than the use of $TMB^{\bullet+}$ radical cations. Moreover, $ABTS^{\bullet+}$ radical cations produced using the developed method present more advantageous characteristics than $TMB^{\bullet+}$ cations and may thus be reliably used for antioxidant capacity assay at pH 4.0. First, the long lifetime of $ABTS^{\bullet+}$ radical cations meets the requirements of high-throughput detection and long-term sample monitoring. Second, $ABTS^{\bullet+}$ radical cation can be used over a wide pH range without perturbing the samples' pH because of the antioxidant capacity depending on pH. Thus, $ABTS^{\bullet+}$ radical cation can be used to study effects of pH on antioxidant mechanisms.[42,43]

The proposed method was also successfully applied to several fresh fruit juices, in which ascorbic acid is an essential component. The antioxidant activity in kiwi fruit was significantly higher than those of other fruits, and peach exhibited the minimum antioxidant activity (Figure 3B). Good agreement between the results obtained from the developed method and those obtained when the same samples were analyzed using an alternative commercial method (T-AOC assay kit) was observed. This finding demonstrates the high potential use of the endospore-based method for analyzing antioxidant activity in real samples. The reliability of the method was examined through a recovery experiment (Table S-2 of the Supporting Information). Recoveries ranging from 95.7% to 107.5% were obtained after standard addition. These results demonstrate that the sensor can be applied in real samples, which indicates potential use in practical applications.

CONCLUSIONS

A novel endospore-based microbial method for antioxidant capacity assay was proposed in this study. This method utilized a "post-additional" mode. Blue-green $ABTS^{\bullet+}$ was produced through an $ABTS-O_2$ reaction catalyzed by wild-type endospores of *B. subtilis* instead of enzymes. The developed method exhibited several advantages, such as easy operation, storage, and widespread application among others. Moreover, enzyme purification was not necessary. The life span of the radical cation was extended 6-fold by the proposed method; thus, large-scale preparation of $ABTS^{\bullet+}$ is possible. This finding meets the requirements for high-throughput detection and long-term monitoring of tested samples. The developed method was successfully used to detect the TEAC values of several antioxidant compounds and real samples, thereby confirming its potential practical application. As screening results can be analyzed by the naked eye, the use of expensive instruments is unnecessary.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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