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Sulfate reduction and heavy metal removal by a novel metal-resistant sulfate-reducing bacterium: mechanism and optimization

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ABSTRACT

Simultaneous removal of carbon, sulfate and heavy metal was observed, by a novel metal-resistant *Enterococcus avium* strain BY7 sulfate reducing bacterium. Morphology of strain BY7 was observed by scanning electron microscopy, which was a long rod-shaped. Strain BY7 was a heterotrophic sulfate reducer which was able to use sucrose, glucose, citrate, ethanol, acetate and lactate as carbon source. Sulfate, sulfite and elemental sulfur could be utilized to produce sulfide by strain BY7. Elemental sulfur was produced as intermediate product during sulfate reduction. The optimal sulfate reduction by strain BY7 was observed with initial pH value of 8.0 under 30°C, respectively. Tolerance to acidic condition and resistance to metal were observed by strain BY7. Heavy metals could be removed with high efficiency (>90%), with an order of $Ni^{2+} > Pb^{2+} > Cu^{2+} > Fe^{3+} > Cd^{2+}$. When multicomponent metals were present, synergistic enhancement for metal removal was achieved. Because of the tolerance to acidic condition and resistance to metal, strain BY7 sulfate reducing bacteria might be used as an engineering bio-reagent for heavy metal removal and sulfate desalination.

Keywords: Sulfate-reducing bacteria; *Enterococcus avium*; Heterotroph; Heavy metal removal; Metal resistant

1. Introduction

Metal pollution has been a crucial global environmental problem, which mainly came from metal mining, agriculture irrigation, metal-containing industry, and other related human activities [1,2]. Heavy metals were recognized as pollutants with high ecological significance, since they were not biodegradable, tended to accumulate and could be involved in food chain and cause health problem to living organisms and humans. Heavy metal pollution was caused not only by the commonly used metals but also caused by rare metals. Amongst all, lead, chromium, arsenic, cadmium, mercury are the five major heavy metal pollutants in China [1].

Due to simultaneous removal of sulfate and heavy metal, microbial sulfate reduction became an attractive alternative to traditional physical and chemical method, and was used in metal-containing wastewater and soil treatment [3–6]. During sulfate reduction process, sulfate (electron acceptor) was anaerobically converted to sulfide, by using organic compounds (heterotrophic) or H_2 gas (autotrophic) as electron donor. Heavy metal was removed as metal sulfide precipitation; thus, metal with low solubility product

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constant for metal sulfide could be removed. The parameters of pH, carbon and sulfur source, temperature, inhibition of heavy metals and microbial species could affect sulfate reducing and heavy metal removal processes [4,7,8]. Metal-resistant sulfate-reducing bacteria (SRB) showed great potential for metal treatment in application [9,10].

As far as known, most of the mesophilic sulfate reducers belonged to the genera of Deltaproteobacteria and Clostridia according to 16S rRNA gene sequences [11–15]. Many thermophilic sulfate-reducing bacteria were found within Nitrospirae [16], and sulfate reducers affiliated with Euryarchaeota and Crenarchaeota were also reported [17,18]. However, there were some novel sulfate-reducing bacteria isolated recently, *Citrobacter* sp. [19] and *Citrobacter freundii* SR 10 [6] which were isolated from wastewater, and *Acinetobacter calcoaceticus* SRB4 [20] which was isolated from river sediment ecosystem. These three SRBs belonged to Gammaproteobacteria, which enlarged our knowledge about unknown SRB. There might be more unknown SRB in nature ecosystems awaiting to be investigated, and the environmental contribution of SRB might be larger than we assumed [21].

Recently, we isolated a strain of *Enterococcus avium* strain BY7 SRB from an anaerobic wastewater treatment system of a chemical production factory in Zhaoqing, Guangdong province, China, which does not belong to the traditional SRB group [22]. To further explore the application potential of strain BY7 SRB, process optimization and metal resistance were investigated in this study. Optimization on growth and sulfate reduction of strain BY7 were performed, with the presence of different carbon and sulfur source, under variable pH value and temperature. Heavy metal removal and toxicity to strain BY7 were evaluated in both individual solution (Cu, Cd, Cs, Ni, Pb and Fe) and metal mixture (Cu, Fe and Cr). The compositions of sulfur compounds and metal sulfide precipitations were identified by X-ray photoelectron spectroscopy (XPS). Thus, this study gave a reference for potential application of strain BY7, as an attractive engineering bacteria in sulfate-containing heavy metal bioremediation.

2. Materials and methods

2.1. Culture and medium composition

Strain BY7 SRB was previously isolated in our laboratory [22], from a colophony chemical factory in Deqing, Guangdong province, P.R. China, which produces pinene and terpineol from colophony, a large amount sulfuric acid was used to achieve acidic condition. BY7 bacterium was also conserved in Guangdong Microbial Culture Collection Center (GDMCC1.1349). Medium (SRM) used in this study was adapted from Zhang et al. [6] with pH value of 7.5. Composition of SRM was summarized in Table S1. All medium used were autoclaved for 30 min under 120°C.

2.2. Growth of strain BY7 with different carbon and sulfur source

Growth of strain BY7 bacteria was monitored in triplicates over time, with SRM listed in Table S1. Doubling time (t_d) and maximum specific growth rate (μ_m) were calculated as Gu [23] reported. Liquid samples were collected every 3 h for analysis of OD600, sulfate and COD.

Chemicals listed in Table 1 were investigated as carbon source for growth and sulfate reduction of strain BY7, in presence of 1,200 mg L^{-1} sulfate, concentration of all followed substrates were balanced to 20 gCOD L^{-1}, except bicarbonate (40 g L^{-1}). Head space was filled with nitrogen gas when organic compounds were used as carbon source. When bicarbonate was introduced as carbon source, H_2 mixed gas (H_2/N_2 5/95) was used in head space. Samples were collected every 3 h for analysis of OD600 and sulfate. For quantification of sulfur reduction activity with various sulfur sources, ammonium iron sulfate was introduced to medium to collect metal sulfide precipitation, as previously reported [24].

In presence of 20 gCOD L^{-1} lactate, different sulfur sources (20 mM) were studied as electron acceptor, for growth and sulfate reduction of strain BY7, as shown in Table 1 (nitrogen in as head space). OD600 was monitored every 3 h, all samples were used for quantification of precipitation at the end experiment. The obtained precipitations were collected for XPS analysis of S2p.

2.3. Influences of pH value and temperature on strain BY7

To verify the effect of initial pH value on growth and sulfate reduction of strain BY7 bacteria, SRM with 12,000 mg L^{-1} COD and 1,200 mg L^{-1} sulfate concentration was used, respectively. Initial pH value of mediums was adjusted to 2–12, respectively (see Table 1 for details). To study the influence of initial pH value on the growth and sulfate reduction of strain BY7 bacteria, medium as described above was used, with initial pH value of 7.5,

Table 1
Details of experiment setup

Variables	Details of each variable											
Carbon	Sucrose	Acetate	Glucose		Citrate		Lactate		Ethanol		Bicarbonate	
Sulfur	Sodium sulfate	Sodium sulfite	Magnesium sulfate		Elemental sulfur		Potassium persulfate		Sodium dodecyl sulfate (SDS)			
pH	2	3	4	5	6	7	7.5	8	9	10	11	12
Temperature	10°C	15°C	20°C		25°C		30°C		35°C		40°C	45°C
Heavy metal	Cu^{2+}	Cr^{3+}	Cd^{2+}		Fe^{3+}		Ni^{2+}		Pb^{2+}		Mixture (Cu^{2+}, Cr^{3+}, Fe^{2+})	

incubation temperatures were controlled from 10°C to 45°C in biological incubator, respectively.

2.4. Metal removal by strain BY7

To evaluate metal removal by strain BY7, SRM was used with 12,000 mg L^{-1} COD and 1,200 mg L^{-1} sulfate in the medium, respectively. Final concentration of 1 mM Cu^{2+} (64 mg L^{-1}), Cr^{3+} (52 mg L^{-1}), Cd^{2+} (112 mg L^{-1}), Fe^{3+} (56 mg L^{-1}), Ni^{2+} (59 mg L^{-1}) and Pb^{2+} (207 mg L^{-1}) were added to 50 mL medium individually. Besides, to study the metal removal with multicomponent, a mixture of 1 mM Cu^{2+}, Cr^{3+} and Fe^{2+} was introduced. pH value was adjusted to 8.0 in the medium, and incubation temperature was controlled at 30°C. Liquid samples were taken over time for analysis of heavy metal and sulfate. Metal-containing precipitation was collected by further XPS analysis of each metal.

2.5. DNA isolation, PCR and sequencing

DNA was isolated with method described in supplementary material. 16S rRNA gene was amplified with primer a set of 27F and 1492R [25], PCR product of about 1,400 bp could be obtained. Details for PCR amplification were described in supplementary material. 16S rRNA gene sequence of strain BY7 bacteria has been submitted to GenBank (accession numbers MG751338).

2.6. Analytical methods

Protein concentration of biomass was quantified with Biuret method [26]. COD and sulfate were analyzed with standard method [27]. Metal concentration in liquid samples was detected by atomic absorption spectrometer (AAS, Varian, AA240, the Netherlands). See supplementary material for details. Elemental sulfur in mixed liquid sample was extracted as previously reported [28], using HPLC (Thermo Fisher, dionex ultimate 3000) with a Hypersil GOLD aQ column and UV detector (254 nm).

2.7. SEM and XPS

The cellular morphology of strain BY7 was investigated by scanning electron microscopy. In general, strain BY7 bacteria which grew in SRM with and without metal addition were collected, fixation was performed by previously reported in the study by Watsuntorn et al. [29], observation was performed by JEOL microscope (JSM-7001F, Japan).

Composition of collected precipitates were analyzed by XPS (ESCALAB 250XI, Thermo Fisher Scientific, USA), with monochromated Al Kalph anode [30]. Analyzer pass energy was 20 eV for S 2p narrow spectra.

3. Results and discussion

3.1. Heterotrophic growth and sulfate reduction by strain BY7

Strain BY7 was a sulfate-reducing bacterium, able to grow in sulfate-containing mineral medium under anaerobic condition (Fig. S1a). Heterotrophic growth of strain BY7 occurred with t_d of about 15 h and μ_m of 0.045 h^{-1}, when lactate was used as carbon source. Simultaneously removal of COD and sulfate was observed but with different patterns (Fig. S1b), COD concentration was rapidly decreased during the first 21 h (period I) and slowed down afterwards (period II), while sulfate reduction was very slow during the first 21 h and accelerated afterwards. Besides, COD concentration was strongly related to the growth of BY7 with R^2 of 0.99, during the first 21 h, which might suggest that COD consumed during period I was mainly utilized for the growth of strain BY7, and COD removed afterwards might be used as electron donor for sulfate reduction. Stoichiometry ratio of consumed acetate and sulfate was 1:1 during heterotrophic sulfate-reduction process [12,31], indicating the ratio of C:S consumed was 2:1. In this study, the ratio of the consumed carbon and sulfate was 2.1:1 in period II, which was in good accordance with the stoichiometry ratio.

3.2. Phylogeny of strain BY7 based on 16S rRNA gene

According to 16S rRNA gene sequences, the strain BY7 was closely belonged to *Enterococcus avium* 208, with sequence identity of 100% over 1,400 bp sequences [32], as shown in Fig. 1. However, *Enterococcus avium* 208 was an avicin A producer, which was functionally different from strain BY7. Strain BY7 belonged to the class of Bacilli, which was not affiliated to the known SRBs, for instance, *Desulfotomaculum ruminis* strain DSM (82% similarity, [33]), *Desulfovibrio halophilus* (80%, [34]) and *Desulfarculus baarsii* (80%, [35]). Strain BY7 was Gram-positive, non-motile and long rod shaped, with width of 0.3–0.4 μm and length of 3.5–4.5 μm (Fig. S2a), respectively, which was visually very different from other reported ball-like *Enterococcus* sp. [36]. Thus, there might be a misclassification for strain BY7 based on 16S rRNA gene sequence. It has been known that SRB could be both gram-negative and gram-positive, such as gram-negative *Desulfovibrio* sp. [37], gram-positive *Desulfotomaculum* sp. [38] and *Desulfovirgula* sp. [39], respectively. When metals were present in the medium, precipitation was observed. In the present study, when iron was present in the medium, BY7 cells were grown closely onto the surface of precipitations, which might suggest simultaneously sulfate reduction and growth of cells (Figs. S2b and S2c).

3.3. Carbon and sulfur source for growth of strain BY7

As shown in Fig. 2a, strain BY7 could grow under heterotrophic conditions with various carbon source, but could not grow under autotrophic condition. An optimal t_d of about 10 h was achieved with sucrose as carbon source. No growth of strain BY7 was observed when inorganic carbon source was used. Sulfate reduction profiles were in great accordance to the growth of strain BY7, as shown in Fig. 2b. The higher the growth rate of strain BY7, the higher sulfate reduction rate. After 72.5 h, with utilization of sucrose, the maximum sulfate removal efficiency of 91% was obtained, no sulfate removal was observed under autotrophic condition.

Growth of strain BY7 bacteria was observed by utilization of sulfate, sulfite and elemental sulfur (Fig. 3a). Strain BY7 had not grown with thiosulfate, lauryl sulfate, persulfate and SDS. Amongst above sulfur source, strain BY7 bacteria

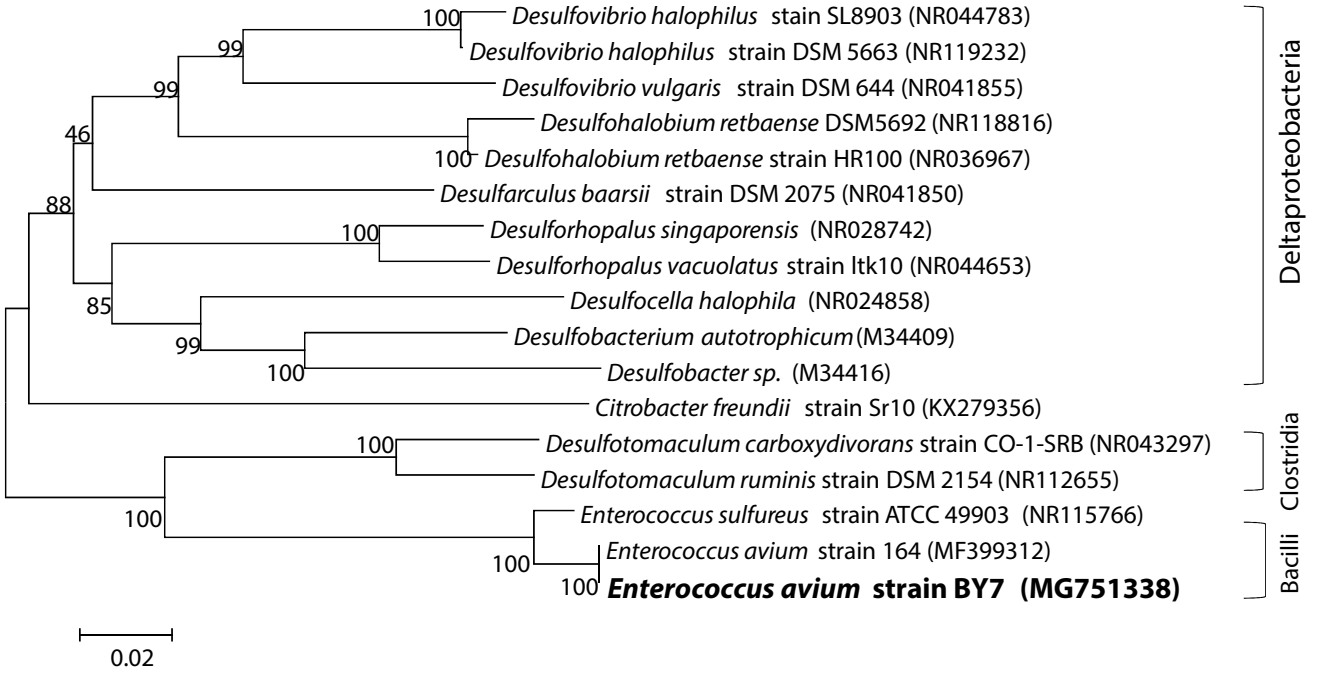

Fig. 1. Phylogenetic tree based on 16S rRNA gene of known SRB and *Enterococcus avium* BY7. The evolutionary history was inferred using the neighbor-joining method. The bar represented 2% estimated sequence divergence. Evolutionary analyses were conducted in MEGA7.

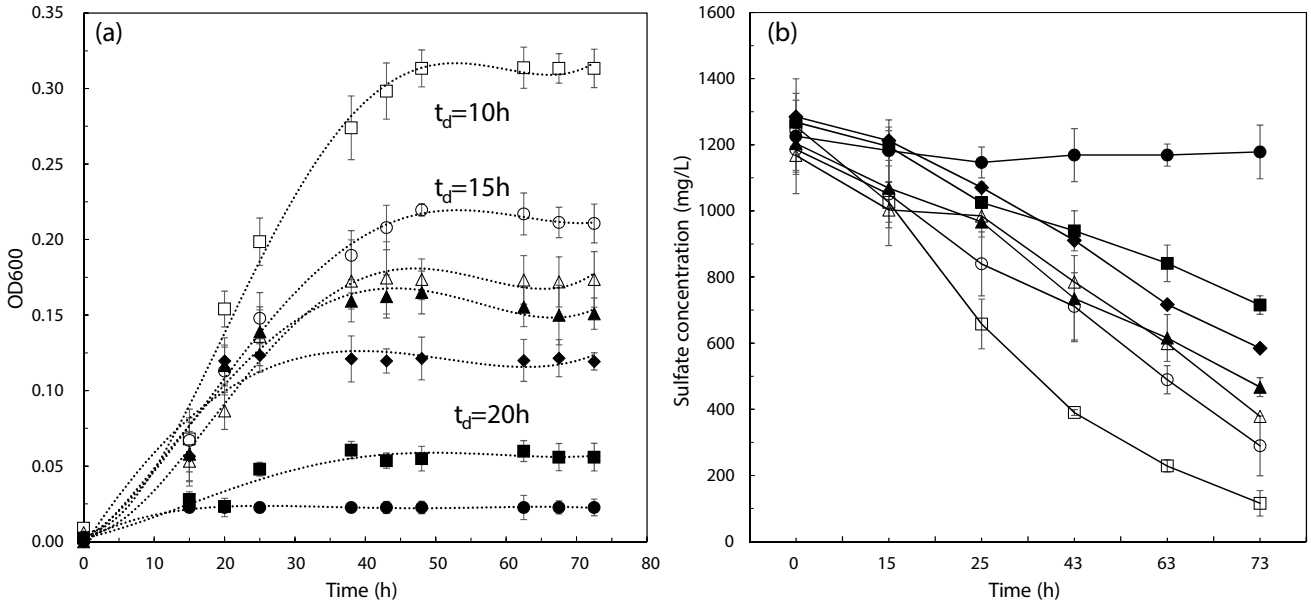

Fig. 2. Growth and sulfate consumption of *Enterococcus avium* BY7 sulfate-reducing bacteria with different carbon source. Bicarbonate (●), sucrose (□), acetate (■), glucose (△), citrate (◆), lactate (○) and ethanol (▲). (a) OD600 and (b) sulfate consumption during growth.

grew remarkably faster when utilizing elemental sulfur as sulfur source (t_d of 8.5 h), while the lowest growth occurred with sulfite (t_d of 23 h). This observation suggested toxicity of sulfite to BY7 bacteria. Sulfite toxicity was commonly known on animals, though sulfite could be used by many sulfate reducers [24,40]. Thus, it might be worthwhile to further investigate the toxicity of sulfite on BY7 bacteria. With addition of iron, ferrous sulfide would be collected as precipitation which was further quantified to evaluate sulfate reduction activity. In Fig. 3b, sulfide production activity of BY7 bacteria was obviously higher by using elemental sulfur. Precipitations were collected from medium with sulfate, sulfite and elemental sulfur at the end of experiment for XPS analysis. In XPS results of S 2p spectra, different

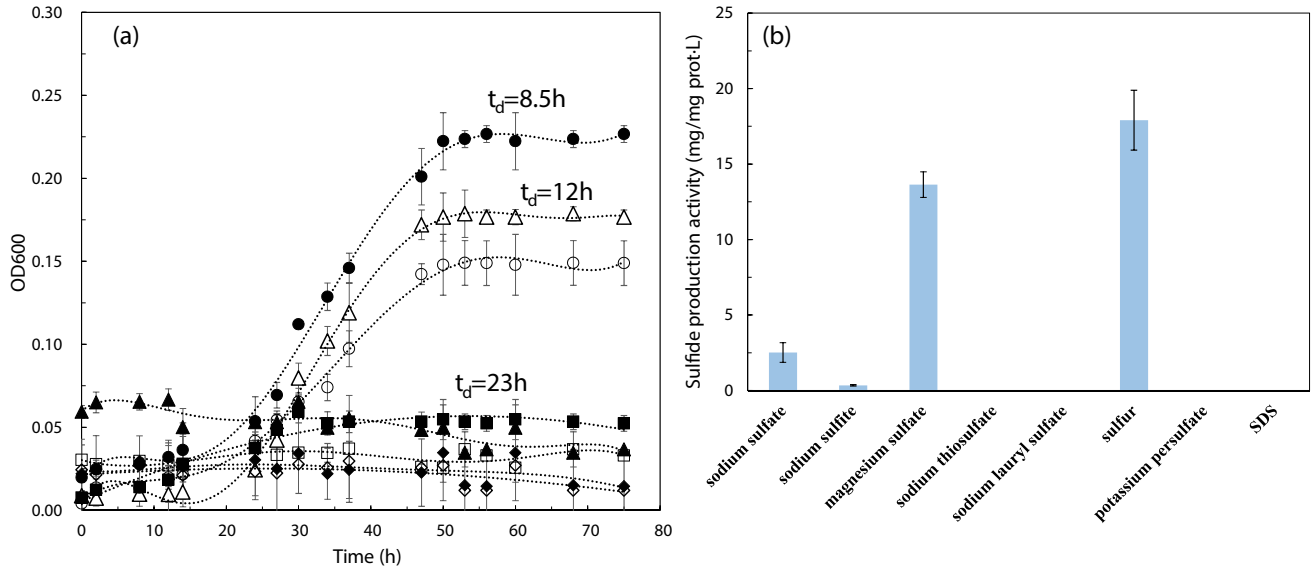

Fig. 3. Growth and sulfite compounds production of *Enterococcus avium* BY7 sulfate-reducing bacteria with different sulfur source. (a) OD600, sodium sulfate (△), sodium sulfite (■), magnesium sulfate (○), sodium thiosulfate (◆), sodium lauryl sulfate (◇), elemental sulfur (●), potassium persulfate (▲) and SDS (□) and (b) sulfite production activity.

peaks represented for SO_4^{2-}, SO_3^{2-}, S^0, S_n^{2-} and S^{2-} were identified (Table 2), which were close to previous reports [41–43]. Formation of S^0, S_n^{2-}, S^{2-} and adsorption of SO_4^{2-} was identified in the precipitation with sulfate as sulfur source, as shown in Fig. 4a. When sulfite was used, production of only S^0, S_n^{2-} and adsorption of SO_3^{2-} was observed, which might be explained by the low sulfide production activity (Fig. 4b). Peak for adsorbed SO_4^{2-} and SO_3^{2-} were not present when elemental sulfur was used in the medium (Fig. 4c). It was known that elemental sulfur with concentration of 20 mM could not be completely dissolved, which led to a moisture at the beginning of experiment. This undissolved elemental sulfur

Table 2
Binding energies and peak full width at half maximum (FWHM) for sulfur and metals peaks in spectra

Element	Binding energy (eV)	FWHM (eV)	Chemical species	Molar ratio of metal and S	Reference
S (2p)	168.8	1.7	SO_4^{2-}		[41]
	169.8	1.9	SO_4^{2-}		
	169.0	1.4	SO_3^{2-}		[41]
	167.8	1.4	SO_3^{2-}		
	163.8–164.8	1.4–1.9	S^0		[43]
	163.0–163.5	1.4–1.5	S_n^{2-}		[43]
	161.5	1.4–1.9	S^{2-}		[62]
Fe (2p)	711.3	3.8	Fe(III)-S	1:1.49	[43]
Cd (3d)	405.1	1.3	Cd(II)-S	1.06:1	[62]
	412.0	1.3			
Pb (4f)	138.7	1.2	Pb(II)-S	1.04:1	[42]
	143.6	1.2			
Ni (2p)	853.3	1.5	Ni(II)-S	1:1.13	[63]
Cu (2p)	932.4	2.1	Cu(II)-S	1:1.02	[57]
	952.3	2.4			
Cr (2p)	577.7	2.9	Cr(III)		[58]
	587.3	3.0			

[#]Binding energy values were accurate to ± 0.1 eV.
[$]Metal and *S* ratio was calculated by the ratio of metal and *S* atomic %, the contribution by sulfate, elemental sulfur peaks were took out from *S* atomic % during calculation.

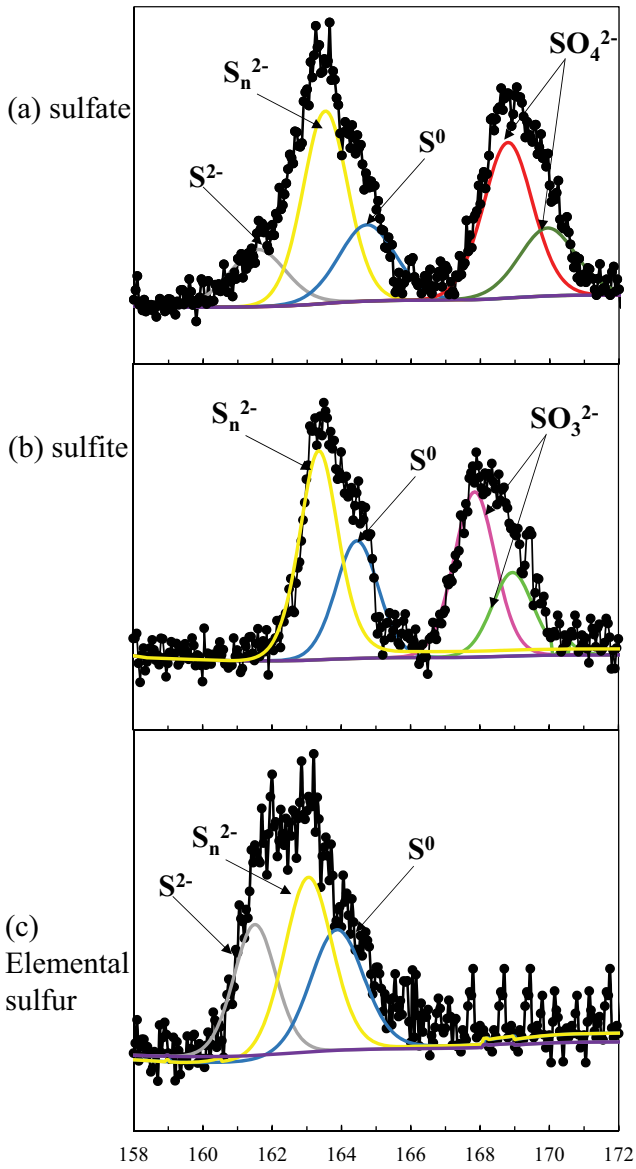

Fig. 4. XPS spectra of S 2p in precipitation collected in the medium with sulfate (a), sulfite (b) and elemental sulfur (c) as sulfur source. Circles were experimental data, purple solid line was the background, grey, yellow, blue, dark green, light green, pink and red solid lines were fit to each spectrum.

would be collected as precipitation at the end of experiment, if they were not converted to dissolvable sulfur compounds. However, XPS results demonstrated that the composition of S^0 in precipitation collected from medium with elemental sulfur was comparable with the precipitation collected from sulfate and sulfite medium. Thus, elemental sulfur, which could not dissolve at the beginning of experiment, was mainly used and converted to S_n^{2-} and S^{2-} during reduction process by strain BY7. Production of elemental sulfur was confirmed by quantification of elemental sulfur with HPLC, up to 45 mg L^{-1} elemental sulfur could be detected after 48 h.

It was very common that SRB could use various organic compounds as electron donors, such as lactate, ethanol, pyruvate, malate, succinate, butanol and propanol, but not acetate [37,44,45]. For most of the SRBs, organic compound was only oxidized incompletely to acetate, but there were still a few SRBs which could further oxidize acetate to CO_2, such as *Desulfobacter postgatei*, *Desulfobacca acetoxidans* [12] and *Desulfovibrio dechloracetivorans* [46]. According to the results, strain BY7 was one of the SRB that could utilize acetate as electron donors. From a chemical viewpoint, sulfate was not a favorable electron acceptor for microorganisms. It has been demonstrated frequently that many SRBs, including species in the genus of *Desulfomicrobium* [9], *Desulfovibrio* [37,45–47], *Desulfotomaculum* [48], could use inorganic sulfur compounds as electron acceptors, including $S_2O_3^{2-}$, SO_3^{2-} and S^0. Eight electrons were required to reduce one molecule of SO_4^{2-} (+VI) to S^{2-} (-II), which was hardly performed by microorganisms. Therefore, the reduction of sulfate was performed stepwise by SRB, first sulfate was activated to adenosine-phosphosulfate (APS), followed by reduction of APS to sulfite, it was finally reduced to sulfide by producing elemental sulfur as intermediate [9,49]. According to the results, with exception of thiosulfate, strain BY7 could use SO_4^{2-}, SO_3^{2-} and S^0 as sulfur source, and produce S^0 as intermediate. Besides, BY7 bacteria grew faster in the presence of elemental sulfur than sulfate, which suggested that BY7 was a better sulfur reducer than a sulfate reducer. Polysulfide (S_n^{2-}) might be formed when sulfur and sulfide co-exist in a system, which accelerated sulfur reduction by improving the solubility and bioavailability of elemental sulfur [40,50].

3.4. Optimization of pH value and temperature

As presented in Fig. S3a, the strain BY7 grew between pH 5.0 and 10.0, with an optimum around pH 9.0. The optimal growth temperature for strain BY7 on lactate and sulfate was 35°C, growth of strain BY7 occurred between 15°C and 45°C, no growth was observed when temperature was below 10°C (Fig. S4a). The optimal sulfate removal efficiency (90%) was achieved under initial pH value of 8.0 at 30°C, as shown in Figs. S3b and S4b. Remarkable sulfate removal (>50%) could be observed from pH value 5.0 to 9.0, and within temperature range of 15°C–40°C, which might indicate tolerance to acidic condition by strain BY7, respectively.

pH value and operation temperature were major factors for the growth and sulfate reduction activity of SRBs. Temperature affected the activity of all enzyme involving reaction. Concentration of H_2S in liquid was strongly correspond to pH value, inhibitory effect of H_2S on SRBs was observed under low pH value. However, SRBs could detoxify H_2S through producing alkalinity during sulfate reduction [51]. In addition, response of SRBs to pH value and operation temperature variation was strain-specific. SRB species in the genus of *Desulfovibrio* were commonly neutrophilic and mesophilic, which grew between pH 6.0 to 8.0, in a temperature range of 15°C–40°C, respectively, such as *Desulfovibrio alaskensis*, *Desulfovibrio bizertensis*, *Desulfovibrio salexigens*, *Desulfovibrio dechloracetivorans* and *Desulfovibrio marinisediminis* [45,47]. Nevertheless, there were still thermophilic or/and alkaliphilic SRBs present, for instance *D. indonesiensis* and *Desulfotomaculum geotbermicum* which were able to grow at temperature over 50°C and under pH 9.0 [52]. It was an unusual physiological feature of strain

BY7 that could growth under such a broad pH range, but a newly isolated novel SRB *Citrobacter freundii* strain SR 10 was able to growth in the pH range of 4.0–9.0 [6]. Therefore, the tolerance to acid condition by strain BY7 might be an important feature in application. Optimized pH value of 8.0 and temperature of 30°C were used for the rest of experiment in this study.

3.5. Metal removal dynamics and resistance

Cd^{2+}, Pb^{2+}, Cu^{2+}, Ni^{2+}, Fe^{3+} and Cr^{3+} removed dynamics by strain BY7 in individual system and mixture system of Cu^{2+}, Fe^{3+} and Cr^{3+}, were presented in Fig. 5a. In the individual systems, the results demonstrated the highest removal efficiency for Ni^{2+} (100%), followed by Pb^{2+} (98.1%), Cu^{2+} (96.2%), Fe^{3+} (93.5%), Cd^{2+} (91.5%) and Cr^{3+} (14.5%). In the mixture system, the removal rate of Cu^{2+} and Fe^{3+} was accelerated (about 30%), and removal of Cr^{3+} was enhanced to 93.4%, which might be due to co-precipitation with CuS/Fe_2S_3 or formation of $Cr(OH)_3$. A synergistic enhancement for metal removal was achieved in this mixture system. It was not normal to achieve such high metal removal efficiency, with initial metal concentration of 1 mM (52–207 mg L^{-1}). Removal of metals with high efficiency was normally reported when metal concentration was lower than 50 mg L^{-1} [6,53]. However, when metal concentration was over 50 mg L^{-1}, metal removal efficiency was sharply decreased, while even complete inhibition could be observed [3,54,55].

Composition of metal-containing precipitations collected in each individual system and mixture system was presented by high resolution XPS, as shown in Fig. S5 and Table 2. The Cd (3d) peak shape and position confirmed the formation of cadmium (II) sulfide (3d5/2 at 405.1 eV and 3d3/2 at 412.0 eV), which was in excellent agreement with literature data on CdS [56]. The Fe (2p) spectrum showed a major peak around 711 eV (Fig. S5b), which corresponded to Fe(III) – S compounds [39]. A strong peak around 932.4 eV might be identified to peak of CuS, which was shifted by +0.2 eV to reported peak [57]. There was a peak located at about 853.6 eV in Fig. S5d, which was contributed to Ni^{2+} in NiS compound [58]. Fig. S5e, the lead peaks represented to Pb(II)S were occurred at 138.7 eV (Pb4f7/2) and 143.6 eV (Pb 4f5/2 peak), which was very similar to previous work on nanoparticle-PbS [42]. The XPS spectrum of Cr, which co-precipitated in the mixture solution with Ni and Fe, was shown in Fig. S5f. The peaks of Cr(III) were appeared at 577 and 587 eV, which were related to Cr 2p3/2 and Cr 2p1/2, respectively. Binding energies of Cr 2p3/2 and Cr 2p1/2 peaks were slightly higher in this study, 0.7 and 0.3 eV respectively, which might be explained by the presence of nickel, iron and chromium hydroxides [58]. SRB was known to reduce metal from higher state to lower state [6,58,59], however, XPS analysis of metal-containing precipitation did not show such a reduction in this study. The molar ratios of metal and S^{2-} (including S_n^{2-}) were very close to the stoichiometry ratio of 1:1 for CuS, NiS, PbS and CdS, and 1:1.5 for Fe_2S_3, respectively, as shown in Table 2. This result also suggested that the metal removal observed in this study was mainly contributed by formation of metal sulfide, but not physical process, such as adsorption.

Sulfate reduction and growth of strain BY7 bacteria were mildly inhibited, with the presence of metal (Fig. 5b). Based on growth and sulfate reduction, the inhibitory effect of metals on strain BY7 followed an order of: Cr > Cu > Pb > Ni > Cd > Fe. However, if we defined metal concentration led to 50% bacterial growth inhibition to be IC_{50}, IC_{50} was only obtained in the test with Cr(III) (1 mM) in the medium. Inhibitory effect for Cu, Fe, Pb, Cd, Ni and mixture (Cu, Fe and Cr) were observed with 34.6%, 17.6%, 30.8%, 21.7%, 26.3% and 36.7%, respectively. The resistance of strain BY7 to metals was obvious. The low toxicity for metal mixture to strain BY7 might contribute to the enhanced metal removal. The inhibition of

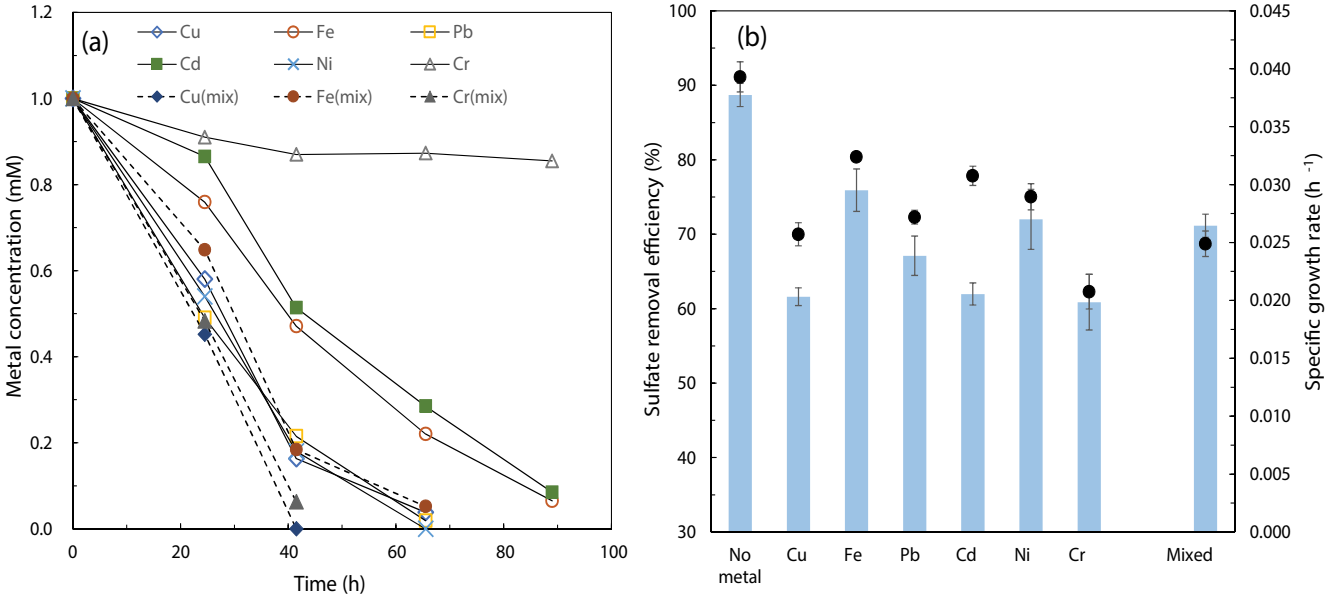

Fig. 5. Heavy metal and sulfate removal efficiency of *Enterococcus avium* BY7 sulfate-reducing bacteria. (a) Heavy metal removal efficiency and (b) sulfate removal efficiency (blue column) and growth rate (black dot).

heavy metal on growth and activity of SRB have been commonly reported [9,10,55,60]. The activity of sulfate reduction was stopped by 0.06 mM Cu^{2+} for *Desulfovibrio vulgaris* SRB, and a *Citrobacter* sp. SRB was completely inhibited with the presence of 0.6 mM Cu^{2+} [61]. Nearly complete inhibition of sulfate reduction was observed in *Desulfovibrio desulfuricans* SRB enrichment, when Cu and Cd concentration was 10 and 15 mg L^{-1}, respectively [54]. A relative order for inhibitory effect of Cu > Cd > Fe > Ni (Cr was not investigated) has been previously reported with *Desulfomicrobium* sp. SRB [9], and the order of Cu > Ni > Mn > Cr > Zn was concluded with *Desulfovibrio* sp. SRB [61], which suggested metals toxicity on SRB was species-specific. In this study, there was sufficient sulfate provided ($M/S^{2-} < 1$), which assured sufficient metal sulfide production for metal removal. The obtained heavy metals removal was mainly attributed to the low solubility of metal sulfides (CuS, PbS, NiS, Fe_2S_3 and CdS). The solubility products constant (K_{sp}) values of CuS, PbS, NiS and CdS were 6×10^{-37}, 3×10^{-28}, 3×10^{-19}, 6×10^{-19} and 8×10^{-28}, respectively [6,55]. Reaction between metal ions and sulfide produced by SRB took place, followed by chemical precipitated of metal sulfide, which consequently reduced the toxicity of the heavy metal to SRBs. Therefore, the highest toxicity of Cr(III) could perfectly link to the lowest removal efficiency to Cr(III) by strain BY7 in the individual system, because Cr_2S_3 was not precipitation. In the mixture system, since co-precipitation of Cr(III), the actual concentration of Cr^{3+} exposed to strain BY7 was reduced significantly, which sharply decreased the toxicity of metal mixture to strain BY7.

4. Conclusion

This is the first report of *Enterococcus avium* strain BY7 as a metal-resistant sulfate-reducing bacteria. Strain BY7 was able to produce sulfide, by using a variety of carbon and sulfur source. Tolerance to acidic condition and resistance to heavy metal were achieved by strain BY7. In this study, it has been demonstrated that removal of heavy metals from both individual and multicomponent system, by strain BY7 with high efficiency. Metals were removed through production of metal sulfide and co-precipitation. Thus, strain BY7 provided great potential in application for simultaneous removal of sulfate and metal in metal-containing soil and wastewater, by an easy-control facultative sulfate-reducing strain.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. Human and animal rights and informed consent. This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary Information

S1. Material and method

S1.1. Genomic DNA isolation, PCR amplification and sequencing analyses

Genomic DNA was isolated from 2 mL medium after centrifugation for 5 min at 10,000 × g, by using TaKaRa Mini BEST Bacteria Genomic DNA Extraction kit Ver.3.0 (TaKaRa, Japan). Quality of extracted DNA was checked on gel (1% agarose). PCR reactions were performed in a PCR apparatus (Whatman-Biometra, Göttingen, Germany) using Premix Taq™ kit (TaKaRa, Japan) according to the instructions of supplier. 16S rRNA gene was amplified with primer a set of 27F and 1492R [22], which obtained a PCR product about 1,400 bp. PCR amplification was performed as follows: Denaturation 2 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, and a final extension for 10 min at 72°C. Size and quality of PCR product was checked on agarose gel. The obtained PCR product was sent to sequencing directly. Phylogenetical analyses were conducted by MEGA 6 software. 16S rRNA gene sequence of stain BY7 bacteria has been submitted to GenBank (accession numbers MG751338).

S1.2. Analytical methods

Protein concentration of biomass was determined by the Biuret method [23]. Bovine serum albumin dilutions (Sigma-Aldrich, USA) were used as standards. OD600 was used to monitor the growth of strain BY7 bacteria, 1 mL liquid sample was taken by syringe from serum bottle, and transferred to a plastic cuvette to detect turbidity at wavelength of 600 nm. To monitor COD and sulfate concentrations, liquid samples of 1 mL were collected and centrifuged, the resulted supernatants were frozen until analyses at –20°C. COD and sulfate analyses were performed according to standard method [24]. For measurement of dry weight of precipitation, precipitation (there were some cells adsorbed onto the precipitation) was collected by filtrating all samples in serum bottle through filter (1–3 μm), which was dried in advance under 105°C for 2 d (W1). Filter and precipitation was dried again under 105°C for 2 d (W2), weight of precipitation (ΔW) was the difference between W2 and W1, which indicated the amount of produced metalsulfide. Metal concentration in liquid samples was detected by using an atomic absorption spectrometer (AAS, Varian, AA240, the Netherlands).

Table S1
Composition of SRM

Chemicals	Concentration (g L^{-1})	Chemicals	Concentration (g L^{-1})
KH_2PO_4	0.5	Sodium lactate	3.5
NH_4Cl	0.1	Na_2SO_4	1.0
Cysteine hydrochloride	0.5	$(NH_4)_2Fe(SO_4)_2$	0.1
Yeast extract	1	Vitamin C	0.1
$CaCl_2$	0.1		

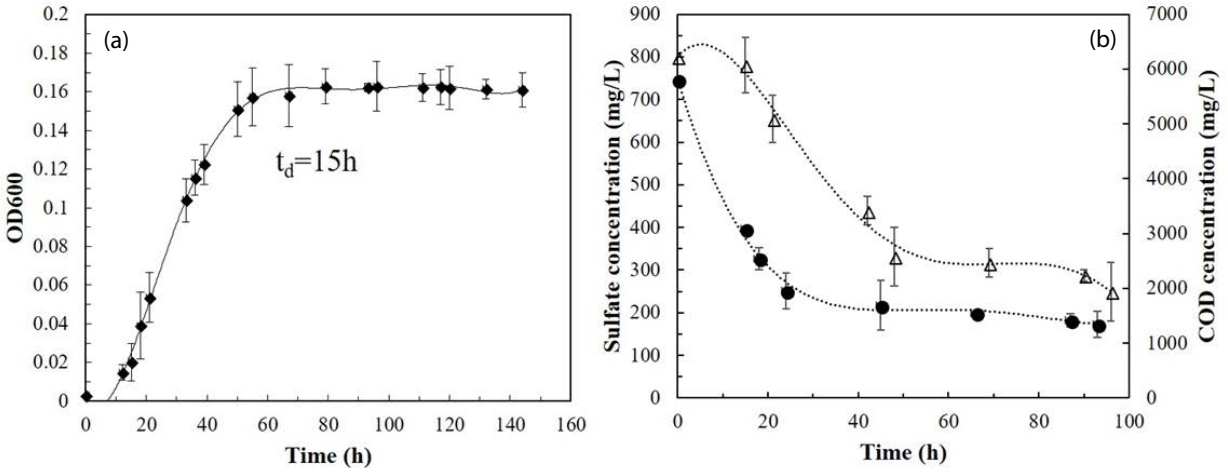

Fig. S1. Growth and substrate consumption of *Enterococcus avium* BY7 sulfate-reducing bacteria. (a) OD600 and (b) sulfate (△) and COD (●) consumption during growth.

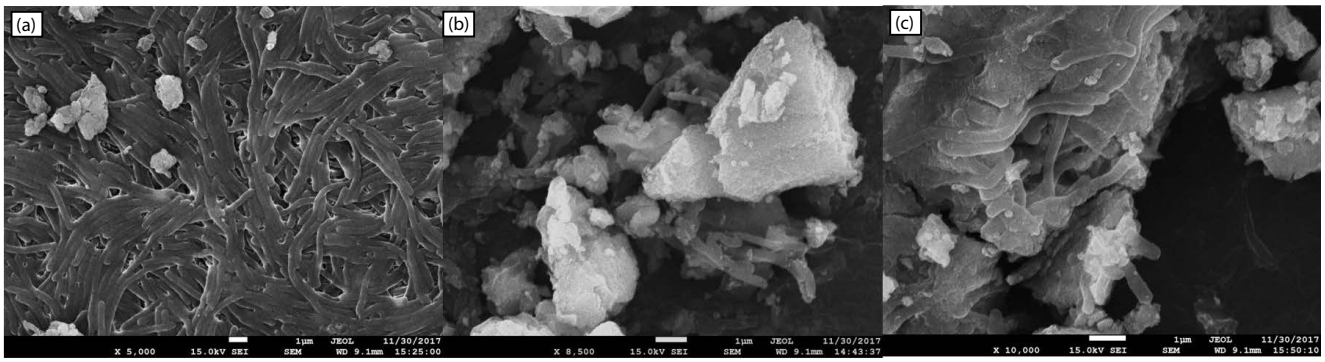

Fig. S2. Scanning electron microscopy of *Enterococcus avium* BY7 sulfate-reducing bacteria in free cell (a) and in precipitation (b,c). Scale bar in the images indicated 1 μm.

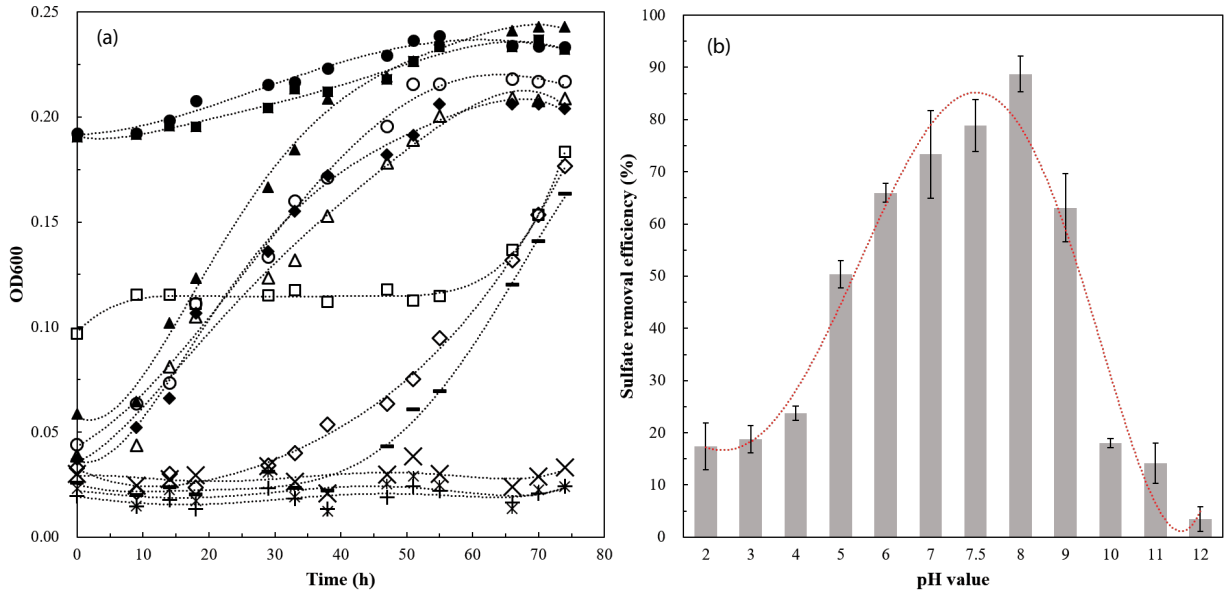

Fig. S3. Growth and sulfate removal efficiency of *Enterococcus avium* BY7 sulfate-reducing bacteria under different initial pH value. (a) OD600, pH 2(*), pH 3 (×), pH 4 (+), pH 5 (_), pH 6 (◇), pH 7 (△), pH 7.5 (◆), pH 8 (○), pH 9 (▲), pH 10 (□), pH 11 (■) and pH 12 (●) and (b) sulfate removal efficiency.

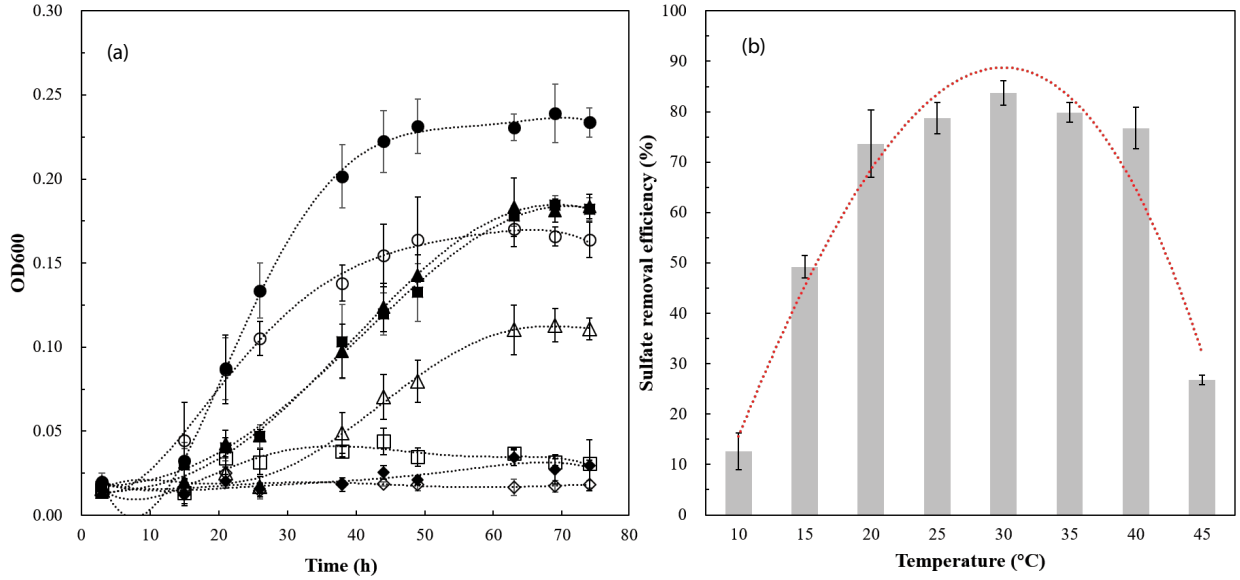

Fig. S4. Growth and sulfate removal efficiency of *Enterococcus avium* BY7 sulfate reducing bacteria under different incubation temperature. (a) OD600, 10°C (◇), 15°C (◆), 20°C (Δ), 25°C (■), 30°C (▲), 35°C (●), 40°C (○) and 45°C (□) and (b) sulfate removal efficiency.

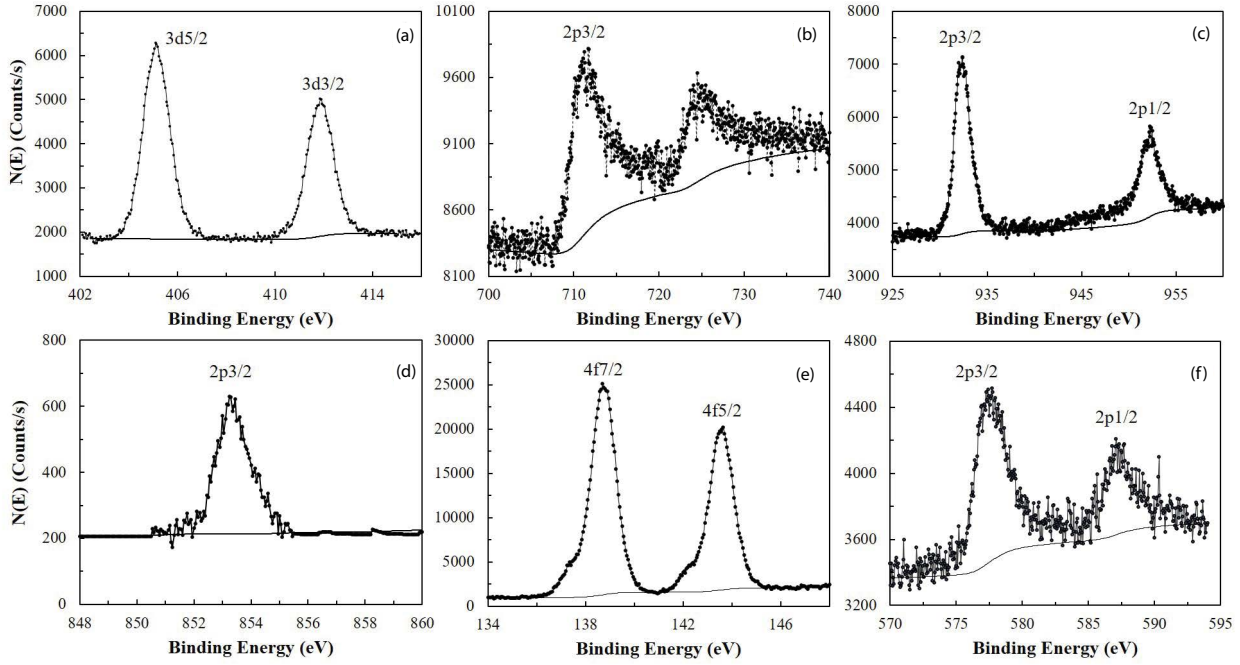

Fig. S5. XPS spectra of Cd(3d), Fe(2p), Cu(2p), Ni(2p), Pb(4f) regions in individual system and Cr(2p) in mixture (a, b, c, d, e and f, respectively). Circles were experimental data, bottom solid line was the background.