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RESEARCH PAPER

Comparative proteomic analysis and characterization of benzo(a)pyrene removal by *Microbacterium* sp. strain M.CSW3 under denitrifying conditions

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Abstract High-molecular-weight polycyclic aromatic hydrocarbons are persistent organic pollutants with great environmental and human health risks and the associated bioremediation activities have always been hampered by the lack of powerful bacterial species under redox conditions. A *Microbacterium* sp. strain capable of using benzo(a)pyrene as sole carbon and energy sources under denitrifying conditions was isolated. The difference in protein expression during BaP removal and removal characterization were investigated. A total of 146 proteins were differentially expressed, 44 proteins were significantly up-regulated and 102 proteins were markedly down-regulated. GO and COG analysis showed that BaP removal inhibited the expression of proteins related to glucose metabolism at different levels and activated other metabolic pathway. The proteins associated with catalytic activity and metabolic process were altered significantly. Furthermore, the BaP removal might be occurred in certain organelle of M.CSW3. The strain removed BaP with a speed of 0.0657–1.0072 mg/L/day over the concentrations range 2.5–100 mg/L. High removal rates (>70%) were obtained over the range of pH 7–11 in 14 days. Carbohydrates and organic acids which could be utilized by the strain, as well as heavy metal ions, reduced BaP removal efficiency. However, phenanthrene or pyrene addition enhanced the removal capability of M.CSW3. The strain was proved to have practical potential for bioremediation of PAHs-contaminated soil and this study provided a powerful platform for further application by improving production of associated proteins.

Keywords Benzo(a)pyrene · Differentially expressed proteins · Bioremoval performance · *Microbacterium* sp. · Denitrifying condition

Introduction

An increasing amount of polycyclic aromatic hydrocarbons (PAHs) are released into the environment due to the incomplete combustion of organic materials and oil spills (including coal tar and lubricants). The PAHs would transport and accumulate in the environment and ultimately deposited in the soil and the aquatic ecosystem [1]. These contaminants may resist the natural removal by indigenous microorganisms because of their high hydrophobicity and low bioavailability properties. Thus, the total amounts of PAHs in the soil and water systems would exceed the environmental risk limits via various accumulation paths [2, 3]. Due to the high bioaccumulation potential and carcinogenic property of PAHs, growing concerns over the rising concentrations and low removal rates of PAHs has spurred researchers to seek ways of treatment [4–6].

Bioremediation is considered as the efficient and cost-effective treatment methods to remove PAHs for the advantages of low inputs and no secondary pollution. But

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when compared with other physicochemical treatments time consuming might be a potential drawback [7, 8]. Among the numerous influential factors, the chemical structure of the PAHs and the environmental conditions (particularly oxygen levels) are relatively difficult to change for large-scale contaminated sites [9]. Thus, changes in population structure and biomass increase of degrading microorganisms would be possible approaches to increase the remediation efficiency [10].

Previous studies have reported that indigenous microorganisms survived in PAH-contaminated sediments and soils were capable of removing PAHs, particularly low-molecular-weight (LMW, 2–3 ring PAHs) PAHs [11]. However, the microorganisms show a limited ability to the removal of PAHs. The removal speed is even lower than the accumulation speed, especially in the sites where oxygen is scarce [12]. Therefore, the isolation of microorganisms capable of removing PAHs effectively has recently been studied intensively. Some studies indicated that the microorganisms isolated from contaminated sediments and soils improved PAHs removal rates in various degrees by changing culturing conditions [13]. These microorganisms were either directly isolated from the site or adapted in the lab under certain conditions. A comparative analysis of the removal behaviors showed that the domesticated strains possessed the ability to remove high-molecular-weight (HMW, 4–7 ring PAHs) PAHs and exhibited higher removal rates of PAHs than strains directly isolated from soil [14–17]. In this study, the *Microbacterium* sp. strain domesticated from PAHs-contaminated soil was used for the bioremoval experiments.

Microbacterium sp. was first reported in association with PAHs removal in 2003 by Gauthier et al. [18]. In their research, the strain was isolated from a two-liquid-phase soil slurry bioreactor, and it could remove pyrene and chrysene only in cultures without naphthalene. Zhang et al. [19] found strain Sphel, which was isolated from Greek soils contaminated with complex waste (e.g., PAHs) and identified as one species of *Microbacterium* sp., capable of removing phenanthrene and n-alkanes. Later studies illustrated that phenanthrene- and pyrene-removing strains isolated from mangrove sediments or oil-polluted soils were assigned to *Microbacterium* sp. [20, 21]. In addition, Li et al. [22] showed that an anaerobic PAHs-removing bacterium (one *Microbacterium* sp.) was harbored in sediment samples collected at a depth of 10–15 cm, and the strain had the potential to remove mixed PAHs (fluorene, phenanthrene, fluoranthene, and pyrene) under anaerobic and anoxic conditions. These studies suggested that *Microbacterium* sp. possessed the ability to remove PAHs with four or fewer fused aromatic rings with or without the presence of oxygen. To date, there have been no reports on the removal of PAHs with five or more fused aromatic rings by the members of the *Microbacterium* sp. However, investigations on the vertical distribution of PAHs in mangrove sediments in Hong Kong, South China, showed that the percentage of PAHs with 4–6 rings in the total PAHs was more than 89% for all sediments at depths between 0 and 20 cm. The highest value for the total PAHs (approximately 5000 ng/g) was found in the deeper anaerobic soil layer (10–15 cm) [22]. To fill the gap of *Microbacterium* sp. on removing HMW-PAHs, our research investigated the anaerobic removal behavior of HMW-PAHs by *Microbacterium* sp.

PAHs has been reported to be associated with human diseases by inhibiting cell differentiation and synthesis [23]. And inhibitory effect against bacteria was also observed in PAHs-contaminated soils [13]. Although survived bacteria have the ability to degrade PAHs and the enzymes mainly involved in PAHs removal in the presence of oxygen have been identified. The enzymes associated with HMW-PAHs removal under anaerobic conditions are rarely reported. Moreover, nothing is still known about the function of related enzymes. Additionally, the effect of BaP on the bacteria has not been thoroughly understood.

As the first attempt to degrade HMW-PAHs by *Microbacterium* sp. under anaerobic conditions, benzo(a)-pyrene (BaP) was selected as the representative and associated removal investigation was carried out in a liquid system under nitrate-reducing conditions. The objective of this study were to (1) identify the differently expressed proteins and asses the effect of BaP on bacteria; (2) determine the BaP removal capability of *Microbacterium* sp. strain when BaP was provided as the sole carbon and energy source; (3) investigate the effect of the experimental factors (e.g., pH, metal ions, and co-substrates) related to bioremediation system on BaP removal behavior.

Materials and methods

Enrichment and isolation of the bacterial strain

The contaminated soil used for enrichments and bacterial isolations was collected from the 20–40 cm soil layer in a coking plant in Beijing. The soil sample (100 g) was suspended in 1000 ml of liquid mineral salt medium (MSM) containing: $Na_2HPO_4 \cdot 10H_2O$ (10.08 g), KH_2PO_4 (1.5 g), NH_4Cl (1 g), $NaNO_3$ (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.03 g), $CaCl_2$ (0.02 g) and 1 mL microelements solution followed the proportions of Dou et al. [24]. The soil-MSM suspension was supplemented with BaP as the enrichment substrate and incubated with shaking at 200 rpm and 35 °C in the dark. After 60 days of continuous shaking without further transfers, the cultures were allowed to stand for 3 h favoring the sedimentation of soil

particles. The supernatant (100 μL) from the culture was transferred and spread on MSM-agar plates which were supplemented with BaP on the surface as the sole carbon source. After incubating for 10 days, the resulting colonies were transferred to MSM medium with high concentration of BaP (50 mg/L), and they were cultured under continuous shaking for 30 days. The diluted supernatants (100 μL) were spread on the MSM-agar plates mentioned above. The steps listed above were repeated, and the colonies forming clear zones on the plates were regarded as BaP-removing strains. It was worthwhile pointing out here the all the steps that exposed to air were carried out in an anaerobic glove box. The isolate M.CSW3 was stored in liquid cultures containing 50% glycerol (v/v) at −80 °C and have been deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number 1.15785.

16S rDNA gene sequence and phylogenetic analysis

The 16S rDNA of the isolate M.CSW3 was amplified by polymerase chain reaction (PCR) with the universal primers 27F and 1492R. The PCR products were tested by Beijing Biomed Co., LTD. The 16S rDNA sequences obtained were aligned with previously published sequences from the GenBank database using the BLAST program.

Culture conditions and inocula preparation

The strains growing on MSM-agar plates were transferred to LB liquid medium and sealed in the anaerobic glove box, then cultured at 200 rpm and 35 °C for 24 h. The cells were then harvested by centrifugation at 6000×*g* for 15 min and washed with MSM three times to remove the residual LB. The inocula were obtained by resuspending the cell pellets with MSM medium.

All the media used in these experiments were previously sterilized at 121 °C for 30 min, and all the operations were performed in an anaerobic glove box filled with pure argon (99.9999%).

BaP bioremoval experiment

The bioremoval experiments were conducted in detest oxygen bottles (100 mL). Single or mixed PAH stocks (1 g/L) were prepared by adding PAHs in acetone, and they were spiked into 20 mL of MSM. These bottles were placed in a fume hood for 2 h to allow for the complete evaporation of acetone, and then $Na_2S \cdot 9H_2O$ (0.2 g) and the inocula were added into the medium. The bottles were capped with Suba-Seal silicone septa and wrapped with aluminum foil after. All the bottles were incubated in a shaker 200 rpm at 35 °C, and samples from individual bottles were collected completely at the designed time to measure the residual BaP. All the experiments were conducted in triplicate, and parallel samples without inocula were employed as blank controls to monitor the abiotic loss of BaP. The automated Bioscreen C system (Finland) was used to evaluate the influence of different additions on the growth of M.CSW3 in liquid culture media. Readings were taken at $\lambda = 600$ nm (OD_{600nm}) at 6-h intervals.

To find out the differentially expressed proteins, M.CSW3 strain was incubated in liquid MSM media containing BaP (10 and 50 mg/L). The bacteria incubated in media with glucose were used as the normal control.

The removal behavior of BaP as the sole carbon and energy source used by M.CSW3 was also investigated. The initial BaP concentrations were set to 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 mg/L by adding different amounts of BaP stock into MSM broth.

To investigate the effect of environmental factors on BaP removal, a serious of batch experiments under different pH, co-substrates and metal ions conditions were carried out. The initial pH of the medium was adjusted to 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 by adding a solution of 0.1 M NaOH or 0.1 M HCl with an initial BaP concentration of 10 mg/L. The impact of the single metal ion on BaP bioremoval by M.CSW3 was conducted by adding inorganic compounds $Co(NO_3)_2$, $FeSO_4$, $MnCl_2$, $CuCl_2$, $PbCl_2$, $Zn(NO_3)_2$, $HgCl_2$, $CdCl_2$, $BaCl_2$ and K_2CrO_4. The initial concentration of metal ion was controlled at 10 mg/L with a BaP concentration of 10 mg/L in control group. The co-substrates carbohydrates (glucose and lactose), organic acids (succinic acid, D-malic acid, L-malic acid, tartaric acid, citric acid, phenol, benzoate, salicylic acid, phthalic acid) and mixed PAHs (phenanthrene, pyrene and BaP) were divided into three groups. Both additive concentrations of glucose and lactose were 0.5, 1.0, 5.0, 10.0 and 50.0 mg/L, and the organic acids were all applied at 10.0 mg/L with a BaP concentration of 10.0 mg/L. The mixed PAHs were split into three subgroups (G1, G2 and G3): G1 contained phenanthrene and BaP, G2 contained pyrene and BaP, G3 contained phenanthrene, pyrene and BaP. The concentrations of phenanthrene, pyrene and BaP in each group were all 10 mg/L. While a group containing BaP at a concentration of 10 mg/L was served as control.

Analytical method for PAHs

To obtain bacteria pellets, samples were collected in triplicates by centrifugation from the media containing BaP (10 and 50 mg/L) and glucose at day 10 and then were centrifuged at 8000×g for 10 min at 4 °C. The total proteins of the bacteria pellets were treated following method of Zhang et al. [25]. Identification and quantification were

conducted by Beijing Genomics Institute (Beijing, china) using the methods of isobaric tags for relative and absolute quantitation (iTRAQ) technology. Spectra were searched against the National Center for Biotechnology Information (NCBI) protein database using Mascot server (version 2.3.02). Identifications of proteins were accepted with an unused ProtScore of >1.2 (>95% CI). And accepted protein identifications were required to have a "Local False Discovery Rate (FDR)" estimation of no higher than 1%. Following identification, differential expression of proteins between glucose and BaP cases was determined by calculating the weighted average log ratios of the peptides for each protein as calculated by "the parsimony principle" after a protein level FDR estimation of no higher than 1%. Only proteins with a p value less than 0.05 was considered statistically significantly. Quantification of proteins was determinated using Iquant method (Beijing Genomics Institute) to screen the differentially expressed proteins. iTRAQ quantification underestimates the amount of "real" fold change between different samples, thus a protein with >1.5-fold difference and a p value <0.05 was regarded as being differentially expressed.

To detect the residual BaP concentration, samples from the bottles were mixed with an equal amount of cyclohexane (CG, JT Baker USA), and the PAHs were extracted by ultrasonic wave treatment (25 °C water bath at 40 Hz). The organic phase was then collected from the two-liquid-phase mixture. The previous step was reprocessed, and the organic solutions obtained from the previous steps were mixed together. The solution was further diluted to the appropriate BaP concentration after removing the water inside with hydrous sodium sulfate. The solution was then filtered through a nylon Millipore membrane filter (0.22 μm) and injected into an Agilent vial for determination. The PAHs were determined by high-performance liquid chromatography (HPLC) using an instrument fitted with a 4.6 mm × 250 mm reverse-phase C18 column with methanol (CG, JT Baker USA)-ultrapure water (90/10, v/v) as the mobile phase and a flow rate of 1.0 mL/min. Phenanthrene, pyrene and BaP were all detected with a UV detector at 254 nm, and chromatography was performed at 25 °C. The injection volume was 20 μL. The entire detection program took 25 min, and the concentration of each PAH was calculated from the calibration curve based on the peak areas of serial concentrations of standards.

The PAH removal rate (%) was calculated as follows:

$$R = \frac{C_0 - C_t}{C_0} \times 100\%,$$

where R represents the removal rate (%), and C_0 and C_t are the initial and final PAH concentrations, respectively. All of the values were the means of three replicates.

Results

Phylogenetic analysis of strain M.CSW3

The 16S rDNA sequences of M.CSW3 were deposited in the GenBank database and the top 100 hits of Blast searches were mostly within the epsilon subdivision of the division *Microbacterium* sp. This includes the best-matching sequences and sequences of representatives of genera within the epsilon subdivision. So the strain was identified as *Microbacterium* sp. and named *Microbacterium* sp. M.CSW3. The genome information of M.CWS3 was deposited in GenBank under the accession number KX496339. A phylogenetic tree (Fig. 1) constructed by the closest relatives of M.CSW3 according to the database of GenBank was obtained with MEGA 5.0. Most of the branches are supported by high bootstrap values. The M.CSW3 was proved to have a strong relationship with the members of *Microbacterium* sp. XT11 (99% identity) and *Microbacterium* sp. PAMC 28756 (99% identity). The *Microbacterium* sp. XT11 was reported to produce a xanthan-degrading enzyme and the enzyme could fragment xanthan to form oligosaccharides [26]. *Microbacterium* sp. PAMC 28756 isolated from an Antarctic lichen is a carotenoid-producing strain and its genome sequence data revealed series of key genes involved in C_{50} carotenoid biosynthesis [27]. So the M.CSW3 might possess the potentiality of producing enzyme of degrading refractory organics.

Effect of BaP on bacteria

From the data of iTRAQ quantification and analysis by comparing the total proteins of bacteria cultured in glucose and BaP, a total of 146 proteins were identified as differently expressed proteins. And 44 proteins were marked up-regulated and 102 proteins significantly down-regulated during BaP removal. To better understand the influence of BaP on strain M.CSW3, the function of the differently expressed proteins were classified by gene ontology (GO) enrichment analysis. 85 proteins were assigned to different functional categories of GO term. As shown in Fig. 2a, main biological process categories represented were metabolic process (36.3%), cellular process (28.08%) and single-organism process (23.97%). According to the molecular function properties, the proteins were mainly involved in catalytic activity (42.47%) and binding (29.45%). This illustrated that large numbers of proteins involved in the regulation of cell metabolism and physiological processes during BaP removal. Besides, the top two proteins in cellular component were cell (16.44%) and cell part (12.33%), indicating that the changed proteins were

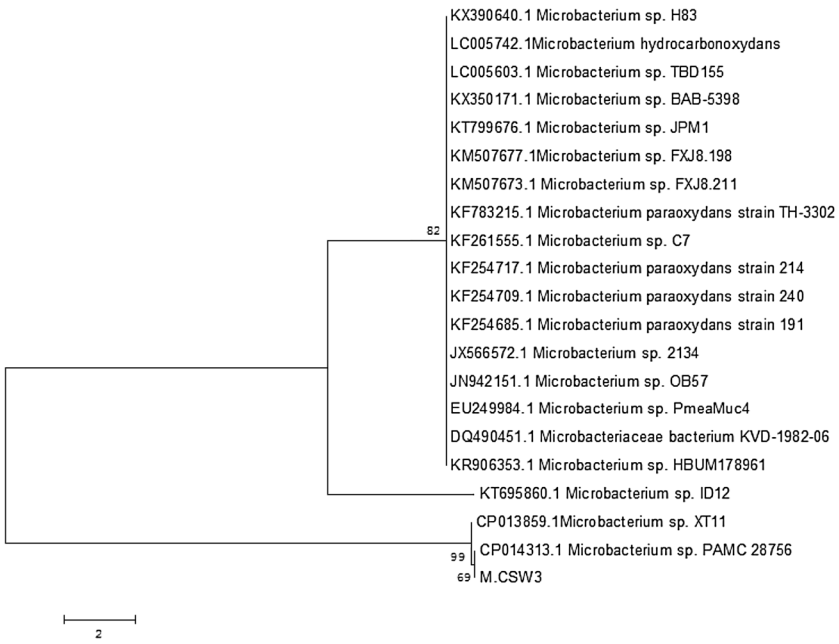

Fig. 1 Phylogenetic tree inferred from 16S rDNA sequence of the strain M.CSW3. The phylogenetic tree was constructed with MEGA 5.0 software based on the homologous 16S rDNA sequences. Bootstrap values were calculated based on 1000 replications shown at nodes of the tree

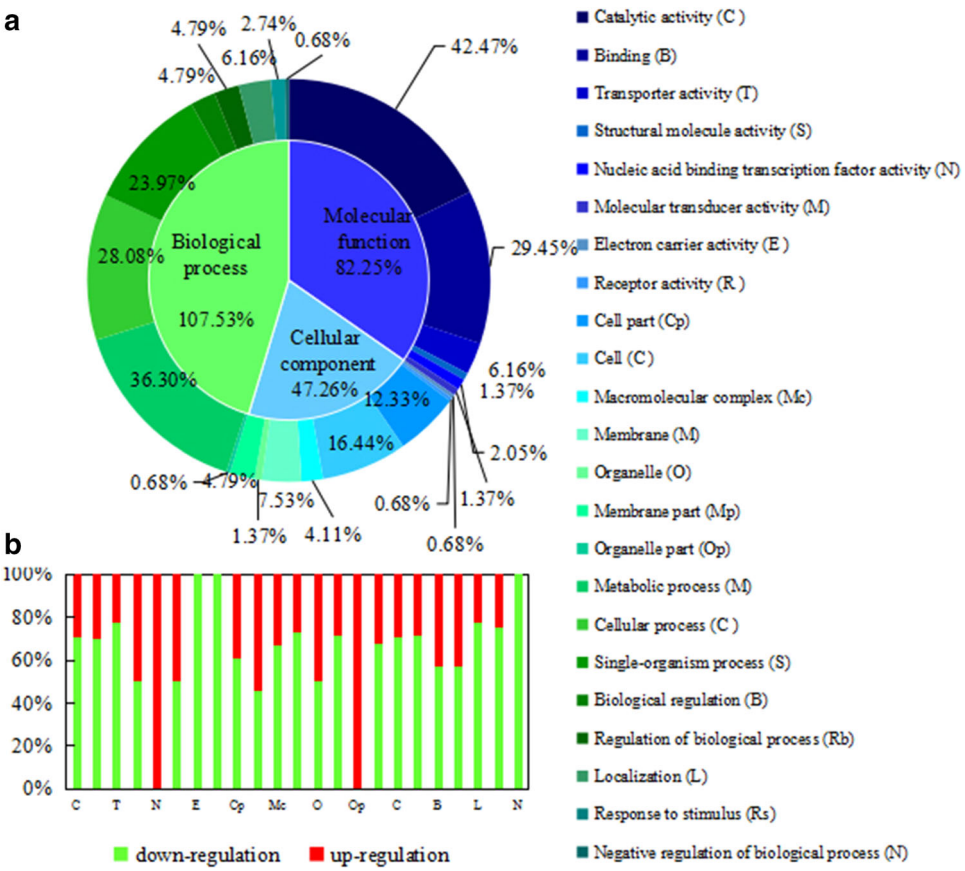

Fig. 2 Classification by GO. To facilitate the global analysis of protein expression, all the differentially expressed proteins were assigned to different functional categories. However, only 85 proteins were successfully matched to GO term. **a** 85 proteins were divided into three types and 23 categories based on their predicted GO function. A given gene product might exhibit one or more molecular functions. **b** The proportion of up-regulated and down-regulated proteins in each category

mainly involved in cellular structure and function. In addition, the proportion of up-regulated and down-regulated proteins in each group is described in Fig. 2b. The proteins in groups of nucleic acid binding transcription factor activity and organelle part were all up-regulated, while all the proteins in electron carrier activity, receptor activity and negative regulation of biological process groups were down-regulated. Thus, it was concluded that

the increase of certain protein level could facilitate the removal of BaP, but the decreased proteins involved in electron transfer suggested a limited biological process. Further database analysis demonstrated that these proteins were associated with 18 groups based on cluster of orthologous groups (COG) of protein function classification as shown in Fig. 3a . As the biggest category of all the differential proteins in *Microbacterium* sp. cells induced by BaP, metabolic proteins (45.21%) were classified into seven categories and these proteins were known to be involved in degradation and metabolism of benzene rings, TCA cycle, amino acid, and lipids. The proteins percentage of functional categories of amino acid transport and metabolism and carbohydrate transport and metabolism was larger than 10% of the total protein, indicating that there is a difference between the proteins involved in BaP degradation and glucose degradation. Meanwhile, transcription classified as one of the group of information storage and processing accounted for 12.33%, and these proteins play an important role in transmitting genetic information. While the function of proteins associated with translation, ribosomal structure and biogenesis is to protect the cellular growth and metabolism, and proteins associated with replication, recombination and repair would function in prevention of proteins damage under changing environments. Most of the proteins classified as cellular processes and signaling group involved in cell wall/membrane/envelope biogenesis, signal transduction mechanisms and posttranslation modification, protein turnover, chaperones. And the proteins ranked as posttranslation modification, protein turnover, chaperones is a stress response protein, particularly the chaperones. It occurred in protein folding under both optimal and stressful conditions as well as in cell division, inhibition of cell clustering and renaturation of protein. The M.CSW3 strain was easier to survive from the media with high concentration of BaP in the presence of chaperones. Though the function of proteins associated with the poorly characterized group (including general function prediction only and function unknown) have to be further studied, they were essential to catalytic activity of enzymes during BaP removal. It can be seen from Fig. 3b that the differentially expressed proteins involved in energy production and conversion, coenzyme transport and metabolism, secondary metabolites biosynthesis, transport and catabolism, cell wall/membrane/envelope biogenesis, defense mechanisms, intracellular trafficking, secretion and vesicular transport and cell motility were found to be significantly down-regulated, which demonstrated that the expression of these proteins were inhibited by BaP.

BaP removal behavior

The removal tests were conducted with BaP concentrations of 0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100 mg/L over the pH range of a natural MSM system. The results are described in Fig. 4a. It was clear that no significant abiotic losses were exhibited by BaP under all conditions, that is, no photodegradation and adsorption of BaP occurred during the bioremoval. Thus, abiotic loss of BaP was negligible

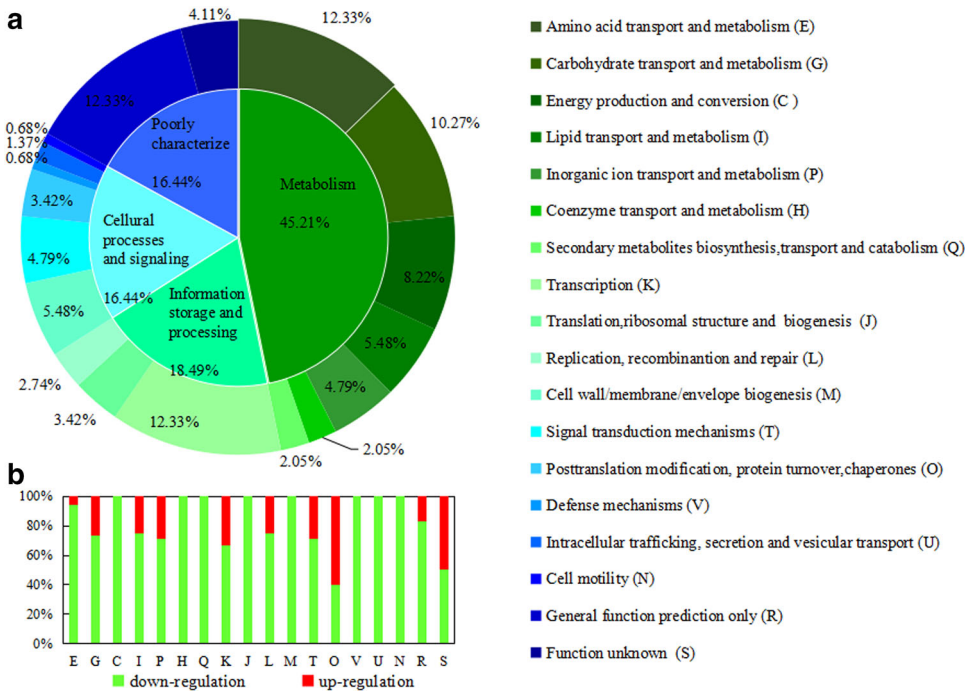

Fig. 3 Classification by COG. **a** 133 differentially expressed proteins were divided into 4 types and 18 categories based on their predicted COG functions. **b** The proportion of up-regulated and down-regulated proteins in each category

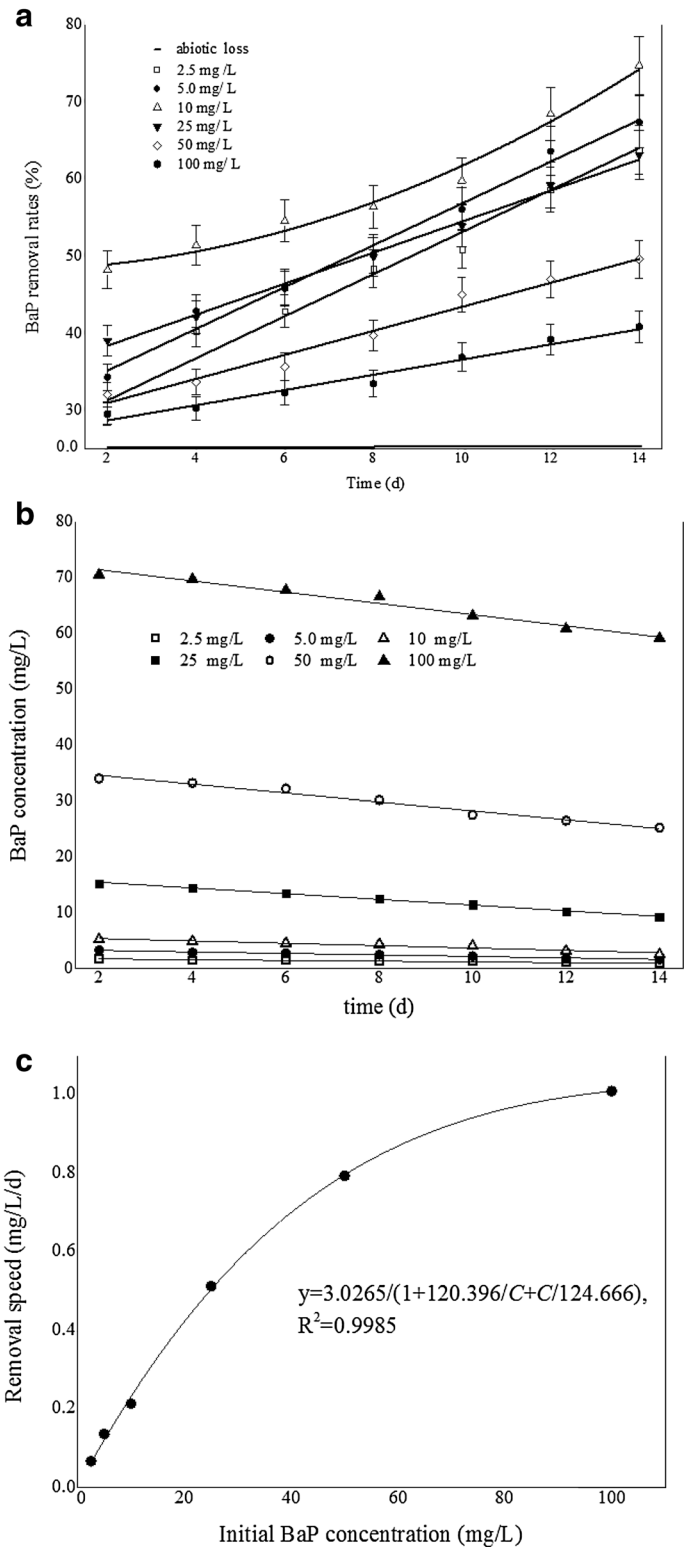

Fig. 4 Effects of initial concentrations on BaP removal by strain M.CSW3. **a** BaP removal rates, **b** BaP concentration changes with removal time and **c** removal dynamics under various concentrations; All the testings were conducted at pH 7.0 and BaP concentrations were measured every other day. The abiotic loss showed in the figure represents the percentage of abiotic loss under all conditions. The error bars represent the standard deviations of triplicate sample measurements

when removal rates were calculated. There were significant differences in the BaP removal performance among the six graded concentrations, and the order of the final removal rates was 10.0 mg/L > 5.0 mg/L > 2.5 mg/L ≈ 25.0 mg/L > 50.0 mg/L > 100 mg/L. The removal speed at 10 mg/L was higher than that of the other concentrations over the whole removal process. It could be seen from the curves of BaP concentration changes versus removal time of Fig. 4b

that the BaP bioremoval speed was constant at various initial concentration. And these curves were expressed as $y = -0.0657t + 1.8315$, $R^2 = 0.9756$ (2.5 mg/L), $y = -0.1348t + 3.5051$, $R^2 = 0.9881$ (5.0 mg/L), $y = -0.2119t + 5.7895$, $R^2 = 0.9381$ (10 mg/L), $y = -0.5111t + 16.471$, $R^2 = 0.9958$ (25 mg/L), $y = -0.7919t + 36.151$, $R^2 = 0.9765$ (50 mg/L), $y = -1.0072t + 73.421$, $R^2 = 0.9761$ (100 mg/L) (y was the BaP concentration at time t, mg/L; t was the removal time, day). Thus, the removal kinetics could be described based on these data of removal speed as the Monod model, and this model was put forward by Andrews [28] to describe cell growth of bacteria under substrate-inhibited conditions. The kinetics model was as follows:

$$V = V_{\max}/(1 + K_s/C + C/K_1),$$

V was the removal speed (mg/L/day); $V_{\max}$ was the maximum removal speed (mg/L/day); C was the initial concentration of BaP; K_s was the saturation coefficient (mg/L); K_1 was inhibition constant (mg/L).

Figure 4c shows that the maximum removal speed of strain M.CSW3 was 3.0265 mg/L/day when BaP concentration was among 2.5–100 mg/L. The saturation concentration was 120.396 mg/L under the fixed amount of cell addition, and the inhibition concentration was 124.666 mg/L.

Thus, the initial BaP concentration was the major fact affecting the efficiency of removal of strain M.CSW3. The highest removal rate (74.7%) was obtained in 14 days when the initial BaP concentration was 10 mg/L. So the following experiments were performed at a BaP concentration of 10 mg/L.

Effect of environmental factors on BaP removal

The solution pH was an important factor influencing removal process. As shown in Fig. 5a, the strain showed a high BaP removal efficiency under neutral and alkaline conditions. Higher BaP bioremoval efficiency was observed when the solution pH ranged from 7 to 9, and the highest removal rate (80%) was recorded at pH 8. The BaP removal rate reached 67.5% even the pH of the medium was as high as 12, and the BaP removal declined to 44.1% when the broth pH decreased to 3. The OD_{600nm} values of the strain indicated that the growth characteristics of the strain were influenced by the medium pH. The cells cultured in the medium of pH 3 did not show obvious changes indicating that the extreme acid prolonged the lag phase of strain M.CSW3. While the population of the bacteria derived from the solutions of pH 4 and 5 continually decreased through the 10-day period. The strain under the solution pH of 6–12 increased first and then decreased with the increased time. However, a comparison between the initial and final OD_{600nm} values suggested solution pH 6 and 7 was conducive to the growth of the strain. Though the strain M.CSW3 showed a high tolerance to alkalinity, it would suppress their growth when exposed in an alkaline environment for long period. Therefore, the medium with pH 7 was used to conduct the subsequent tests.

Metallic ions not only affect the growth of the organisms, but also activities of certain enzymes associated with the metabolism. An investigation was carried out to evaluate the impact of metallic ions on BaP removal by strain M.CSW3 for the reason that they always co-exist with PAHs in the environment [29]. As can be seen from Fig. 5b, the removal rates of BaP that co-existed with Fe(II), Zn(II), Mn(II) and Hg(II) in the broth were higher than that in control group. And the presence of Cu(II), Co(II), Ba(II) and Cr(VI) in medium resulted in a slight reduction of BaP removal. The phenomenon that BaP was removed by strain M.CSW3 at a similar rate with or without Pb(II) indicated that Pb(II) did not involve in BaP metabolic process. However, the removal was severely suppressed by Cd(II) and the removal rate was decreased to 39.8%. According to the OD_{600nm} values, the strain cultured in the medium containing Fe(II) or Hg(II) exhibit different levels of growth compared with that of control. The phenomenon biomass increased in the presence of Fe while it decreased significantly in the presence of Hg suggested that Fe ions promoted bacterial growth and Hg ions inhibited bacterial growth.

To promote bacterial growth and BaP removal, different amount of glucose and lactose was added to the media containing BaP respectively. As illustrated in Fig. 6a, neither additive enhanced the BaP removal. Glucose addition significantly inhibited the removal of BaP, and the lowest removal efficiency of BaP (25.3%) was obtained from the glucose addition at a concentration of 10 mg/L. The extent of inhibition of BaP removal by lactose addition was less than that by glucose addition, and the BaP removal rates with lactose additive were reduced about 1% (5 mg/L) to 6.6% (0.5 and 1 mg/L) compared with that of the control group (59.9%). The OD_{600nm} values also showed the bacterial growth under all conditions were different. The strain co-cultured with glucose showed cell proliferation during BaP removal, the strain co-cultured with lactose showed cell death, and the strain of the control group kept a stable stage.

Organic acids, as metabolic intermediates of various carbon sources, are also used by organisms as carbon sources. In addition, the utilization of corresponding intermediates would enhance removal of the original carbon source [30]. Figure 6b shows that benzoate accelerated the bioremoval of BaP significantly, and the removal rate reached to 83.4% in 10 days, 24.31% higher than that of the control. The succinic acid addition had no effect on the

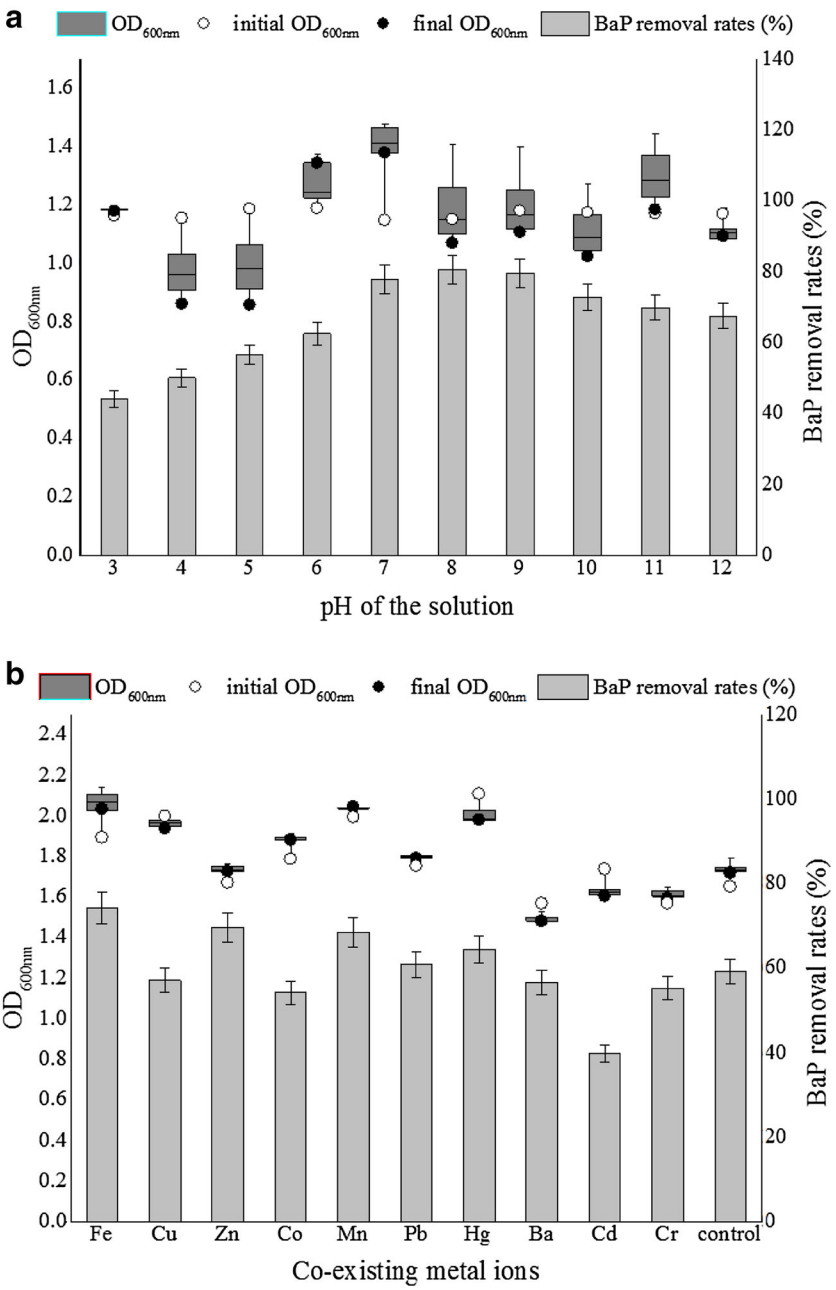

Fig. 5 Effects of environmental factors on BaP removal by M.CSW3 under denitrifying conditions. **a** Effects of solution pH on BaP removal, **b** effects of co-existing metal ions on BaP removal; both the initial BaP concentration and metal ion in each treatment were 10 mg/L, and BaP concentration was measured at day 10. The error bars represent the standard deviations of triplicate sample measurements

removal of strain M.CSW3. The others of the suspected intermediates inhibited the bioremoval activities to different degrees. Citric acid significantly reduced BaP removal of the strain with the lowest removal rate of 39.1%. The OD_{600nm} values showed that the cells cultured with D-malic acid, L-malic acid, and tartaric acid grow rapidly during the first 6 h. The quantity of bacteria growing in medium with phenol, benzoate, salicylic acid, phthalic acid addition showed a descending trend after a short period of growth. The growth of bacteria cultured in succinic acid showed a similar decreasing tendency with the control. Only the strain M.CSW3 stably grew in the medium with citric acid. These data illustrated that strain M.CSW3 could utilize malic acid, tartaric acid, and citric acid as carbon source. The organic acids with benzene rings were different to use as a carbon source by strain M.CSW3. However, different degrees of inhibition of the co-substrate on BaP removal were observed.

Typically, BaP together with other aromatic compounds co-exists as complex mixtures in contaminated sites. And these co-existing PAHs would have an impact on BaP removal. As shown in Fig. 6c, the addition of phenanthrene to the medium led to an obvious increase of BaP removal, and pyrene had no appreciable effect on BaP bioremoval

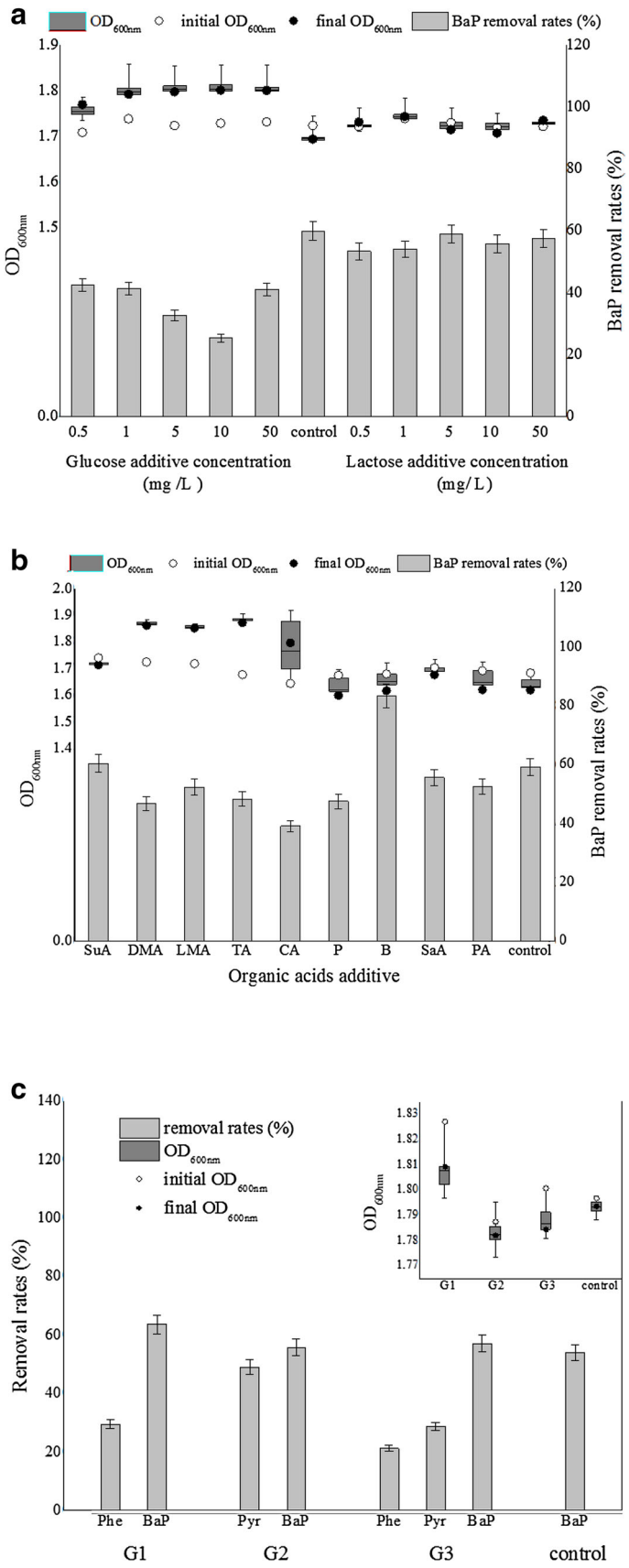

Fig. 6 Effects of co-substrates on BaP removal by M.CSW3 under denitrifying conditions. **a** glucose and lactose at various concentrations as co-substrates. **b** succinic acid (SuA), D-malic acid (DMA), L-malic acid (LMA), tartaric acid (TA), citric acid (CA), phenol (P), benzoate (BA), salicylic acid (SaA), phthalic acid (PA) at a concentration of 10 mg/L as co-substrates. **c** Phenanthrene (Phe) and pyrene (Pyr) as co-substrates. The co-substrate of group 1–3 were phenanthrene (G1), pyrene (G2) and phenanthrene and pyrene (G3), respectively, and each concentration was 10 mg/L. The initial BaP concentration for all the tests was 10 mg/L and the BaP concentration was measured at day 10. The error bars represent the standard deviations of triplicate sample

when added individually. Meanwhile, 29.5% of phenanthrene and 48.9% of pyrene were observed to be removed, respectively. However, removal of phenanthrene and pyrene by M.CSW3 from the phenanthrene, pyrene and BaP combined pollution system decreased with a slight increase in BaP removal. Thus, it could be concluded that strain M.CSW3 could remove a wide range of PAHs and the BaP removal rates were not influenced by removal of phenanthrene and pyrene. The OD_{600nm} readings of these groups showed tiny fluctuations of biomass among them. However, it was obvious that the addition of phenanthrene and pyrene had a side-effect on bacterial growth.

Discussion

Effect of BaP on bacteria

Among the 146 differentially expressed proteins, the expression of 102 proteins was relatively down-regulated when M.CSW3 utilized BaP as sole carbon and energy source, compared to healthy control. And this indicated that BaP removal inhibited the expression of proteins associated with glucose metabolism and activated the expression of proteins involved in other metabolic pathway [31]. Over 35% of the total differentially expressed proteins were involved in catalytic activity and metabolic process during BaP removal, suggesting that BaP was used as carbon source by strain M.CSW3 and the removal depended mainly on enzymatic catalysis. However, the expression of proteins menaquinol-cytochrome C reductase cytochrome C subunit involved in electron carrier activity and ATPase involved in receptor activity were down-regulated because of the absence of oxygen. The proteins associated with nucleic acid binding transcription factor activity and organelle part showed up-regulation. And it was hypothesized that BaP might be degraded in certain organelle of strain M.CSW3 and the organelle was controlled by regulation of gene expression. The phenomenon of inhibition of protein expression by BaP removal was observed obviously based on COG protein function classification. The proteins with the metabolism function of energy production and conversion, coenzyme transport and metabolism, secondary metabolites biosynthesis, the information storage and processing function of transport and catabolism, translation, ribosomal structure and the cellular processes and signaling function of biogenesis, defense mechanisms, intracellular trafficking, secretion and vesicular transport and cell motility were all down-regulated. While the functional groups related to metabolisms involved in removal and metabolism. The proteins associated with information storage and processing mainly involved in the ensuring precise gene expression. The cellular activity and signal transduction were controlled by the group of cellular processes and signaling. The loss of function of these proteins illustrated that M.CSW3 strain changed its metabolic pathway, in other words, M.CSW3 strain had activate other metabolic pathway in response to environmental stimuli. While metabolic pathways are always controlled at different levels for different carbon sources, and this control is achieved at the transcriptional level of gene expression or by a set of proteins that modulate the gene expression [32]. And the changes occurred in the proteins related to amino acid transport and metabolism, transcription and carbohydrate transport and metabolism during BaP removal confirmed a different metabolic pathway, compared to that of healthy control. This also showed that strain M.CSW utilized BaP as sole carbon and energy sources.

BaP removal behavior

Anaerobic bioremoval of PAHs is a very broad field and involves a wide range of biologically related factors, such as environmental conditions, bacterial population and the bioavailability of PAHs. BaP with five benzene rings is removal-resistant for its highly hydrophobic and non-volatile character [3, 33]. The isolated strain M.CSW3 had the ability to degrade BaP in aqueous solution under denitrifying conditions. However, Fig. 4 shows that the removal capability of BaP in the six graded concentrations was different, and the highest removal rate was achieved at BaP concentration of 10 mg/L. Verrhiest et al. [34] arrived at the same conclusion in an investigation of interactions between a PAH mixture and the microbial communities in a natural freshwater sediment. PAHs at a concentration of 30 mg/kg had no effect on bacteria for all the bacterial parameters, but the quantity of total bacteria and the proportion of viable bacteria markedly decreased when PAHs was at 300 mg/kg. Besides, the removal speed of strain M.CSW3 increased with increasing initial BaP concentration. However, they had no obvious correlation when initial concentration was over 10 mg/L, and the speed increases in removal decreased with multiplied initial concentration. So it was predicted that high concentration of BaP would inhibit the removal process of M.CSW3, and the Monod model depicted it well. Generally, the smaller the K_1 value is, the stranger the inhibition of microorganism. In this study, the value was higher than the values of benzene, *p*-xylene and *o*-xylene in the study made by Dou et al. [35]., indicating that the M.CSW3 had a good adaptability to BaP. Thus, the M.CSW3 strain possessed a strong BaP removal capability.

Effect of environmental factors on BaP removal

The complete removal of organics requires the cooperative action of a variety of enzymes, especially refractory organic pollutants [36]. The enzymes associated with removal show activities under different pH regimes, and certain enzymes display optimum activity under specific conditions [37]. It could be seen from Fig. 5a that the strain could grow within a pH range of 6–11, while high removal rates (>70%) were obtained within the pH range of 7–10. The phenomenon that the removal rates at pH 11 and 12 were higher than that at pH 6 suggested the activities of enzymes related to BaP removal were improved in an alkaline condition. Though the bacteria grow well at pH 6, the proteins with the function of catalytic activity during BaP removal were inhibited. And the survived bacteria under extreme alkaline conditions still had the ability for removal due to the regulation of proteins involved in response to stimulus. The expression of proteins associated with metabolism and removal were inhibited simultaneously under acidic environment. The inhibition effect was significantly enhanced by the increase of total acidic groups, and finally, inactivation, protein damage might be induced at pH 3.

The refractory organic decomposition primarily depends on the activities of a series of degrading enzymes. The activities of enzymes were easily influenced by metal ions and were always inhibited by heavy metal ions. Thus, bacterial resistance to metal ions in the environment was important as starter strains for further bioremediation applications. The strain M.CSW3 showed excellent heavy metal resistance except for Cd(II). The ions of Fe(II), Zn(II), Mn(II) and Hg(II) improved the activities enzymes related to BaP removal followed by increased BaP removal rates [38, 39]. Pb(II) seemed to be unresponsiveness to involve in bioremoval of BaP. The heavy metal ions Cu(II), Co(II), Ba(II) and Cr(VI) slightly inhibited the proteins expression or enzyme activity. According to the result, Cd(II) had high toxicity to the M.CSW3 strain by directly and severely suppressing the function of certain proteins involved in bacterial metabolism and removal. The results from Chen et al. [40] indicated that the activities of manganese peroxidase, lignin peroxidase were generally inhibited by Cd(II). The Cd(II) would cause cytotoxicity to cells and directly and severely impact the function of enzyme activity in bacterial metabolism and a decreased BaP removal rate of 30% was reported by Bhabananda et al. [41].

Co metabolic bioremoval of refractory organic pollutants was always used to improve metabolic capability. The carbon sources that served as co-substrates could be utilizable carbon sources, metabolic intermediates or compounds with similar structures. Glucose is easily utilized by microbes and growth-promoting, so it is widely used as co-substrates for bioremoval of PAHs with or without oxygen [42]. Indeed, the removal capability did not increase, but decreased with the increased biomass of strain M.CSW3 when concentrations of glucose additive were 0.5–50 mg/L. As discussed above, the metabolic pathway of BaP by M.CSW3 strain was different from that of glucose, and the expression of proteins associated with glucose removal were inhibited. Therefore, the strain could not degrade BaP when it used glucose as carbon source. And the more bacteria used glucose as carbon source, the lower BaP removal efficiency were obtained. The BaP removal rates were decreased only by 7% due to small numbers of bacteria using lactose as the carbon source. Thus, it was also concluded that the strain M.CSW3 used BaP as sole carbon and energy source and it would show a decreased BaP consumption when the strain utilized additional substrate as the carbon source.

Organic acids were common intermediate products in anaerobic digestion of organics, and different strains are associated with different intermediate [43, 44]. Previous studies have demonstrated that the intermediate compounds formed during PAHs removal contained carboxyl and hydroxyl groups in the presence and absence of oxygen. The compounds phenol, benzoate, salicylic acid and phthalic acid are carboxyl- and hydroxyl-substituted aromatic group, while the malic acid, citric acid, succinic acid, and tartaric acid were organic acids containing 4 or 6 carbon atoms. The order of the pH values for these solutions is phthalic acid < tartaric acid < salicylic acid < citric acid < malic acid < benzoate < succinic acid < phenol. As discussed above, the removal rates decreased with decreased pH. However, the removal rates did not follow this order because of the utilization of certain substrate. The result that benzoate significantly promoted the BaP removal illustrated that benzoate was one of metabolites produced by M.CSW3 strain, and the transient accumulation of benzoate as a putative intermediate was observed when benzene was degraded by sulfate-reducing bacteria. The research also showed that benzoate was intermediate in many anaerobic metabolic pathways [45]. Though bacterial growth was not observed, the degrading enzymes were stimulated by benzoate and improved activities.

Since organisms always have the ability of utilization of similar compounds, it was deduced that the strain M.CSW3 could remove other PAHs as well under nitrate-reducing conditions. The results proved that strain M.CSW3 was capable of removing a wide range of PAHs, particularly LMW PAHs such as phenanthrene and pyrene. And the presence of phenanthrene or pyrene induced the expression of the removal-related protein and accelerated the metabolism of BaP. A hypothesis was generated that

phenanthrene, pyrene and BaP shared the same removal pathway, and phenanthrene and pyrene were the intermediate during BaP bioremoval. When phenanthrene, pyrene and BaP were degraded by the strain simultaneously, the proteins involved in the metabolites of phenanthrene or pyrene was affected by the exogenous phenanthrene and pyrene. In addition, the detection of metabolites over the BaP removal process by M.CSW3 under denitrifying conditions successfully supported the existence of phenanthrene and pyrene (Fig. S1–Fig. S4). And another work about BaP degradation mechanism was also proved that phenanthrene and pyrene were the metabolism products during BaP degradation process [46]. Liang et al. [15] arrived at the same conclusion in the study on BaP anaerobic removal process by *Pseudomonas* sp. JP1.

It could be concluded that strain M.CSW3 used BaP as the sole carbon and energy source with or without additional carbon sources. The bacteria would lack the ability to remove BaP when other substrates were utilized as a carbon source. Besides, the strain M.CSW3 was capable of removing a wide range of PAHs including phenanthrene and pyrene.

Conclusion

It is clear that the M.CSW3 strain can utilize BaP as sole sources of carbon and energy under nitrate-reducing conditions. 44 up-regulated and 102 down-regulated proteins were identified as differentially expressed proteins. GO and COG analysis showed that BaP removal inhibited the expression of proteins associated with energy production and conversion, coenzyme transport and metabolism, secondary metabolites biosynthesis, transport and catabolism, translation, ribosomal structure and biogenesis, cell wall/membrane/envelope biogenesis, defense mechanisms, intracellular trafficking, secretion and vesicular transport and cell motility at different levels. And another metabolic pathway was induced by BaP in which BaP removal mainly determined by enzymatic catalysis. Furthermore, the BaP removal might be occurred in certain organelle. The BaP removal speed were 0.0657–1.0072 mg/L/day at BaP concentrations of 2.5–100 mg/L. Utilization of additional carbon sources and heavy metal ions would decrease the BaP removal efficiency, and the M.CSW3 strain possessed the capacity of removing other PAHs without reduction of BaP removal rates. Benzoate, phenanthrene and pyrene were considered to be the metabolic products. Thus, the M.CSW3 strain was a practical potential strain for bioremediation of PAHs-contaminated sites and this study provided a powerful platform for further application by improving production of associated proteins.

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Compliance with ethical standards

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

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