



Pathway for biodegrading coumarin by a newly isolated *Pseudomonas* sp. USTB-Z

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Abstract

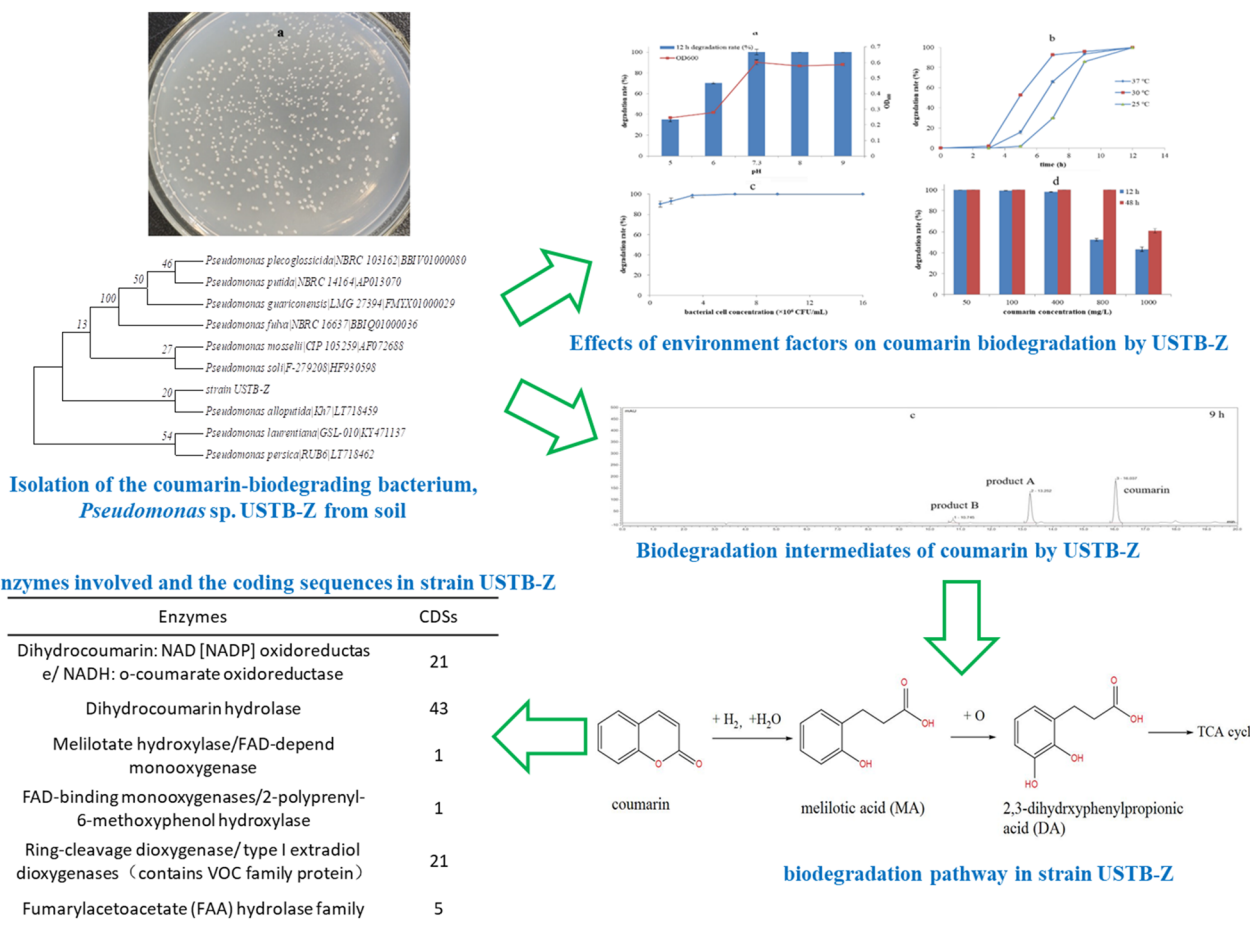
Coumarin is widely used in personal care products and pharmaceutical industry, which leads to the release of this compound into environment as an emerging contaminant. Here, a promising strain USTB-Z for biodegrading coumarin was successfully isolated from botanical soil and characterized as a potential novel *Pseudomonas* sp. based on 16S rDNA sequence analysis and orthologous average nucleotide identity tool. Initial coumarin up to 800 mg/L could be completely removed by USTB-Z within 48 h at the optimal culture conditions of pH 7.3 and 30 °C, which indicates that USTB-Z has a strong capacity in coumarin biodegradation. The biodegradation products of coumarin were further investigated using HPLC and Q-TOF LC/MS, and melilotic acid and 2,3-dihydroxyphenylpropionic acid were identified. The draft genome of strain USTB-Z was sequenced by Illumina NovaSeq, and 21 CDSs for NAD (P)-dependent oxidoreductase, 43 CDSs for hydrolase, 1 CDS for FAD-depend monooxygenase, 1 CDS for 3-hydroxycinnamic acid hydroxylase, 21 CDSs for dioxygenase, and 5 CDSs for fumarylacetoacetate (FAA) hydrolase were annotated and correlated to coumarin biodegradation. The present study provides a theoretical basis and microbial resource for further research on the coumarin biodegradation.

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Graphic abstract



Keywords Coumarin · Biodegradation · Pathway · *Pseudomonas* sp. · Enzyme

Introduction

Coumarins (coumarin and its derivatives), consisting of an aromatic ring fused to a condensed lactone ring, are widely distributed in higher plants and microorganisms (Stringlis et al. 2019), and have been ubiquitously used in food, tobacco products, alcoholic beverages, pharmaceutical and personal care products as a flavoring and fragrance enhancer after chemical synthesis in 1868 (Egan et al. 1990). Based on containing structure of π - π conjugate system, coumarins are easily bound to active sites of enzymes in organisms and have a wide range of biological activities, such as anticancer (Venugopala et al. 2013), antioxidant (Joubert et al. 2017), antibacterial (Kapp et al. 2017), anti-parasite (Mandlik et al. 2016). In addition, coumarins are used as fluorescence sensors (Helal et al. 2011), dye sensitizers (Margar and Sekar 2016), fluorescent whitening agent (Kothavale et al. 2017). It was reported by International Fragrance Association

that the worldwide use volume of coumarin as a fragrance was more than 1000 metric tons per year (Api et al. 2019). These applications can lead to the release of coumarin into the environment through various sources. It was reported by British Geological Survey that coumarin as one of the emerging contaminants in groundwater was detected in samples from 20 monitored sites (Stuart et al. 2011; Montanaro et al. 2017). The effects of coumarin on the environment and human health are still uncertain. However, some researches have shown that coumarin can cause hepatotoxicity and tumor developments in rodents (Born et al. 2003) and that there is a percentage of the human population much more susceptible to its toxicity (Abraham et al. 2010). Moreover, coumarin metabolism can lead to the formation of toxic metabolites such as coumarin 3,4-epoxide and *o*-hydroxyphenylacetaldehyde (Born et al. 1997). Finally, coumarin has been reported to elicit estrogenic responses in mammals, which makes it a potential endocrine disrupting compound

(McLachlan et al. 2012). For these reasons, there is a need to find appropriate technologies for the efficient removal of coumarin from the environment.

At present, only few studies have addressed this issue by physical and chemical methods in removing coumarin (Montanaro et al. 2017; Tan et al. 2019), which were limited in application because of high cost. Microbial biodegradation is considered to be a green and economically competitive technology, because microorganisms are well known as the efficient and selective catalysts from the environment. Several coumarin-degrading bacteria have been isolated successfully, such as *Arthrobacter* (Levy and Weinstein 1964), *Pseudomonas* (Nakayama et al. 1973), *Fusarium* (Shieh and Blackwood 1968), *Saccharomyces* (Häser et al. 2006), *Aspergillus* (Aguirre-Pranzoni et al. 2011), *Glomera* (Marumoto and Miyazawa 2011) and *Cunninghamella* (Ghadai et al. 2016). However, microbial resources of high efficient strains were limited, and no genome sequences of active strains have been reported and analyzed previously, and there is too limited genetic information about coumarin biodegradation by *Pseudomonas* genus.

In this study, we aimed to isolate microorganisms capable of biodegrading coumarin, explore the mechanism of biodegradation, characterize the environmental factors influencing the degradation process, and analyze the main functional genes at the genomic level.

Materials and methods

Chemicals, soil sample and culture medium

Coumarin with 99.98% purity was purchased from Sigma Aldrich Co., Ltd (Shanghai, China). All organic solvents were of chromatographic grade (Macklin Biochemical, Shanghai, China), and chemicals were of analytical grade (Nanjing Chemical Reagent Co., Ltd).

A soil sample collected from Beijing Botanical Garden in the western suburbs of Beijing, P. R. China in 2018 was passed through a 10-mesh sieve to remove stones and plant debris and allowed to air-dry overnight before experiments.

Mineral salt medium (MSM) consisted of 0.5 g NH_4Cl , 0.3 g Na_2HPO_4 , 0.05 g KH_2PO_4 , 0.1 g MgSO_4 , 0.01 g CaCl_2 , 0.01 g ammonium ferric citrate, 0.5 mL trace elements per litre water at pH 7.3 (Karn et al. 2010).

Nutrient broth medium (NB) used in this study contains (g/L) peptone 10 g, Yeast extract 5 g, and sodium chloride 10 g, glucose 10 g and the final pH is 7.2–7.6.

Enrichment of coumarin biodegrading organism

Ten grams of soil samples and 100 mL MSM were placed in a 250 mL Erlenmeyer flask, cultured for seven days in the

concentration of 1000 mg/L coumarin as the sole carbon source. The suspension was incubated on a rotary shaker (in dark) operated at 150 rpm and 30 °C. An aliquot of 1 mL was subcultured to 99 mL fresh medium every five days, for three times. The final enrichment cultures were plated onto the coumarin-containing agar plates. Isolates were screened for the ability to biodegrade coumarin, and a promising bacterial strain USTB-Z was isolated and used.

Inoculum preparation

To prepare the inoculum, 10 mL cultures were centrifuged (5000 rpm, 5 min) and washed three times with sterile physiological saline water. The washed bacterium was then resuspended in 10 mL sterile physiological saline water to obtain the bacterial suspension used as inoculum, which was approximately 3.2×10^8 CFU/mL tested by plate counting method.

Biodegradation of coumarin by bacterial strain USTB-Z

The biodegradation experiments were performed in MSM supplementing with coumarin on a rotary shaker operated at 150 rpm and 30 °C. When the coumarin concentration was lower than 400 mg/L, all the coumarin and its products could be biodegraded completely within 10 h by strain USTB-Y, which was too rapidly to detect. Therefore, coumarin concentration at 400 mg/L was chosen as initial concentration. The growth curve and biodegradation curve were obtained with sampling at 0, 3, 5, 7, 9 and 12 h. The following single-factor experiments were tested to investigate the biodegradation ability and the effect of environment factors on coumarin biodegradation by strain USTB-Z (cultured for 12 h): coumarin concentrations (50–1000 mg/L); temperature (25 °C, 30 °C, and 37 °C); initial pH (5, 6, 7.3, 8, and 9); inoculum size (initial cell concentration was 0.8×10^6 , 1.6×10^6 , 3.2×10^6 , 6.4×10^6 , 9.6×10^6 CFU/mL, respectively). The blank control was set in the same conditions without inoculating bacterium USTB-Z.

Half a milliliter of culture broth was sampled and mixed with 0.5 mL of methanol by shaking for 1 min on a vortex. After centrifugation (6000 rpm, 5 min), the supernatant was filtered with 0.22 μm organic nylon micropore membrane for HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific Inc., USA) analysis. Ten microliter of aliquot was injected into the HPLC system, which were equipped with a Dikema Diamonsil C18 reverse phase column ($250 \times 4.6 \text{ mm}^2$, 5 μm particle size, Dikema Technology Co. Ltd., China), and a UV-detector at 310 nm. Methanol and water (65:35, v/v) were used as the mobile phase with the flow rate of 1.0 mL/min. The temperature of column oven was 30 °C. For the quantification of coumarin, an external standard method

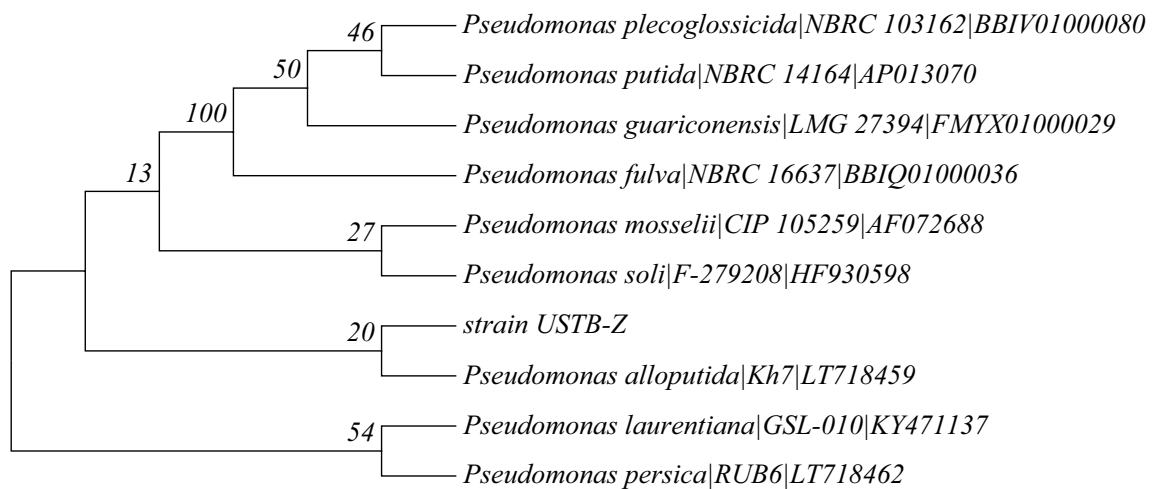


Fig. 1 Phylogenetic tree of strain USTB-Z based on 16S rDNA sequences by the neighbor-joining approach

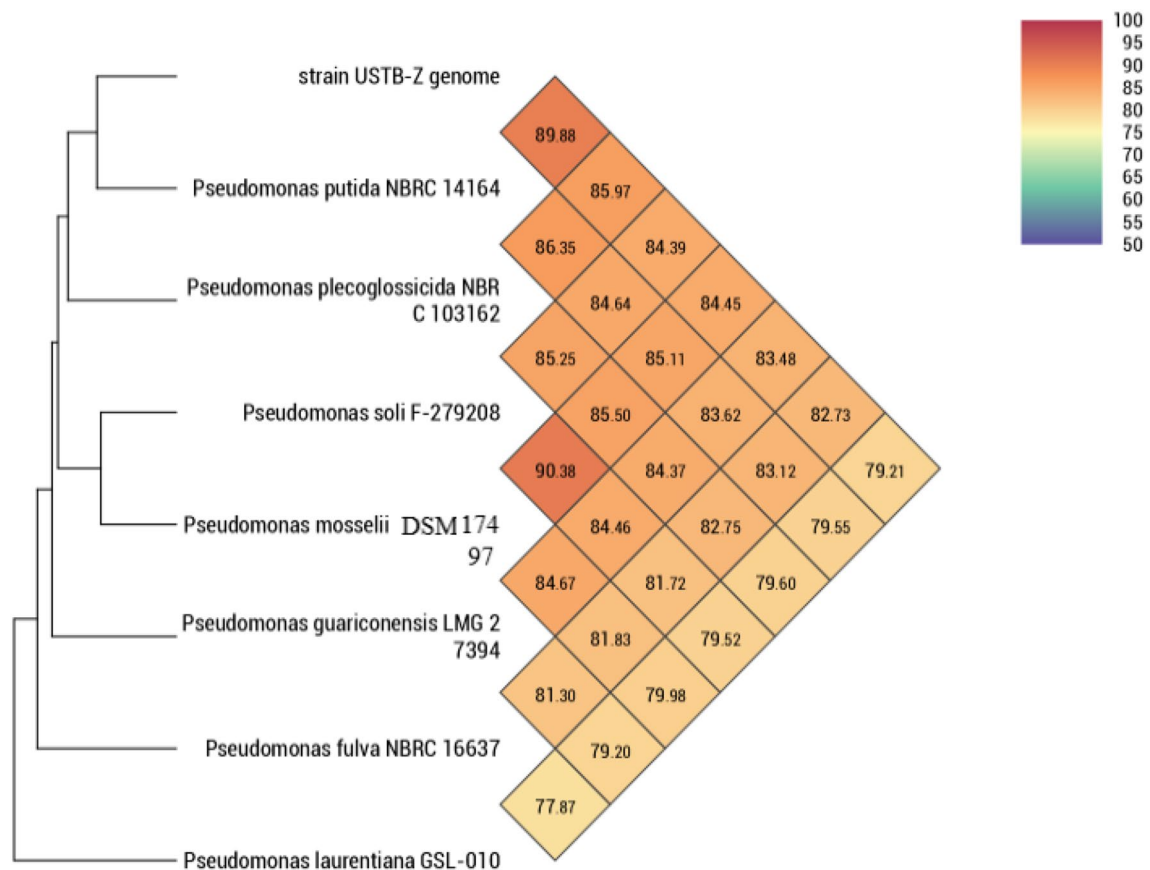


Fig. 2 Phylogenetic analyses of eight genomes of *Pseudomonas* sp. strain USTB-Z and representative related *Pseudomonas* species by OAT. *Pseudomonas putida* NBRC 14164 (GCA_000412675.1), *Pseudomonas plecoglossicida* NBRC 103162 (GCA_000730665.1), *Pseudomonas soli* F279208 (GCA_900110655.1), *Pseudomonas*

mosselii DSM 17497 (GCA_000621225.1), *Pseudomonas guariconensis* LMG 27394 (GCA_900102675.1), *Pseudomonas fulva* NBRC 16637 (GCA_000621265.1), *Pseudomonas laurentiana* (GCA_010671685.1) were downloaded from NCBI for phylogenetic analysis

was applied and the standard curve was built following a six-point calibration curve (ranged from 100 to 800 mg/L, and the R^2 was above 0.99, Online Resource 1, Fig. S1-2). All experiments were conducted in triplicates and were subjected to statistical analysis.

Identification of coumarin biodegradation products

Two milliliters of inoculum and 198 mL MSM were added into a 500 mL Erlenmeyer flask with 400 mg/L coumarin, with sampling at 0, 3, 5, 7, 9 and 12 h to analyze the products. Two controls were carried out, which contained either bacterial cells grown on glucose inorganic medium or coumarin with inorganic medium.

Extraction and cleanup procedures were performed as follows. Two hundred milliliters of the liquid cultures was extracted twice with 200 mL ethyl acetate, respectively, and all the organic phase was combined and concentrated by a rotary evaporator (65 °C). The residue was resuspended in 4 mL acetonitrile and filtrated for HPLC (Dionex Ultimate 3000) analysis. A gradient program having two mobile phases, ultra-pure water and acetonitrile, was used. The gradient composition process was started with 95% water for 1 min, then decreased to 5% water gradually in 20 min, kept for 10 min, then again was increased to 95% water in 1 min, and kept for 10 min. The biodegradation products were further analyzed based on Q-TOF LC/MS (6545, Agilent

Technologies Inc., USA) equipped with an Agilent Eclipse plus C18 column (50×3.0 mm², 1.8 μm particle size). The solvent composition consisted of 5 mM ammonium formate aqueous solution (A) and Methanol (B) in a flow rate of 0.3 mL/min. The gradient composition process was started with 95% A and decreased to 35% A gradually in 13 min, then decreased to 0% A gradually in 3 min, kept for 4 min, then again was increased to 95% A in 2 min, kept for 2 min. Five microliters of aliquot was injected into the HPLC system at 40 °C of column temperature. The MS detection conditions were: capillary voltage of 4500 V, nebulizer gas (N₂) pressure of 40 psi, desolvation gas flow rate of 11 L/min and desolvation gas temperature of 350 °C. MS analysis was full scan mass spectra (m/z 50–1700) using both positive and negative modes electrospray ionization (ESI +/ESI -).

Identification of strain USTB-Z

Strain USTB-Z was identified using 16S rDNA sequence analysis. Total DNA was extracted from the purified bacterium using the Bacterial Genome Extraction kit (Tiangen, Beijing) following the manufacturer's instructions. Following centrifugation, the supernatant was used as template for polymerase chain reaction (PCR) with the primer pair of 27F (5'-AGAGTTTGGATCCTGGCTCAG-3') and 1492R (5'-GGT TACCTTGTACGACTT-3') (Sharma, 2020). The PCR products were sequenced by Shengggong Biotechnology Co.,

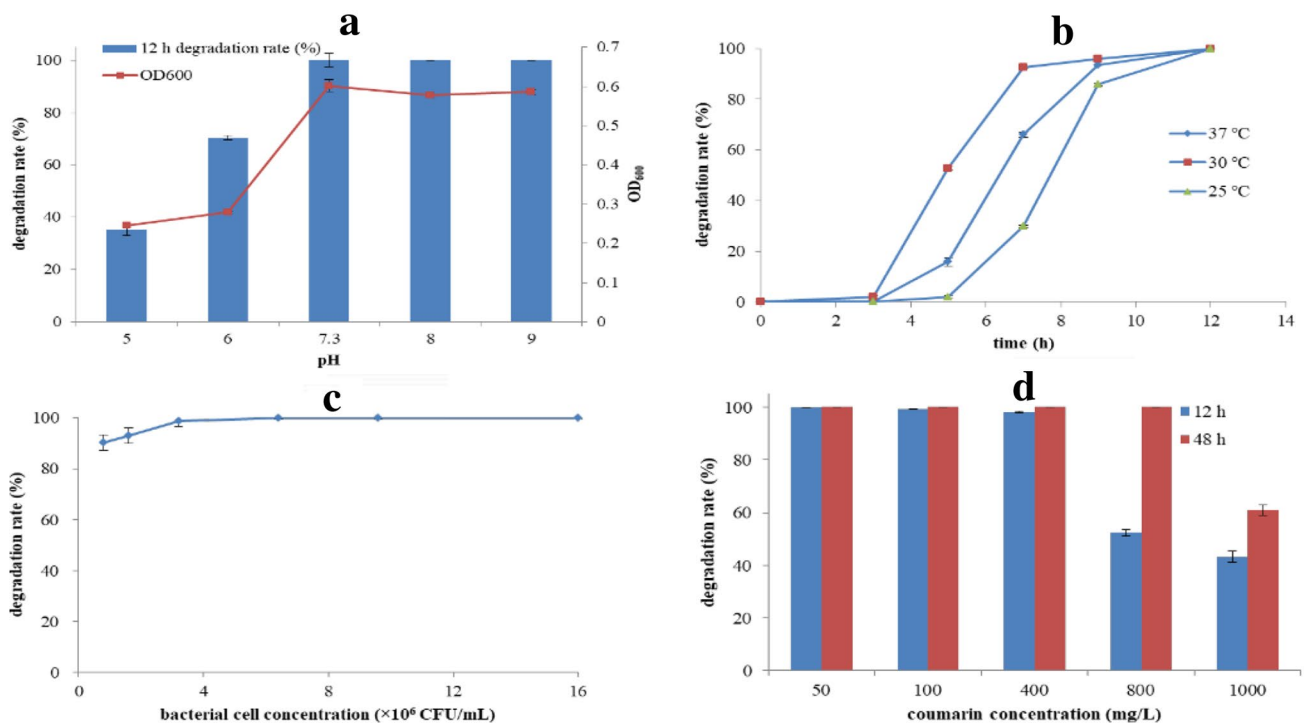


Fig. 3 Effects of environment factors on coumarin biodegradation by bacterium USTB-Z. **a** Temperatures optimum; **b** pH profile; **c** profile of inoculum size; **d** profile of different initial coumarin concentrations. Error bars indicate the standard error of three replicates

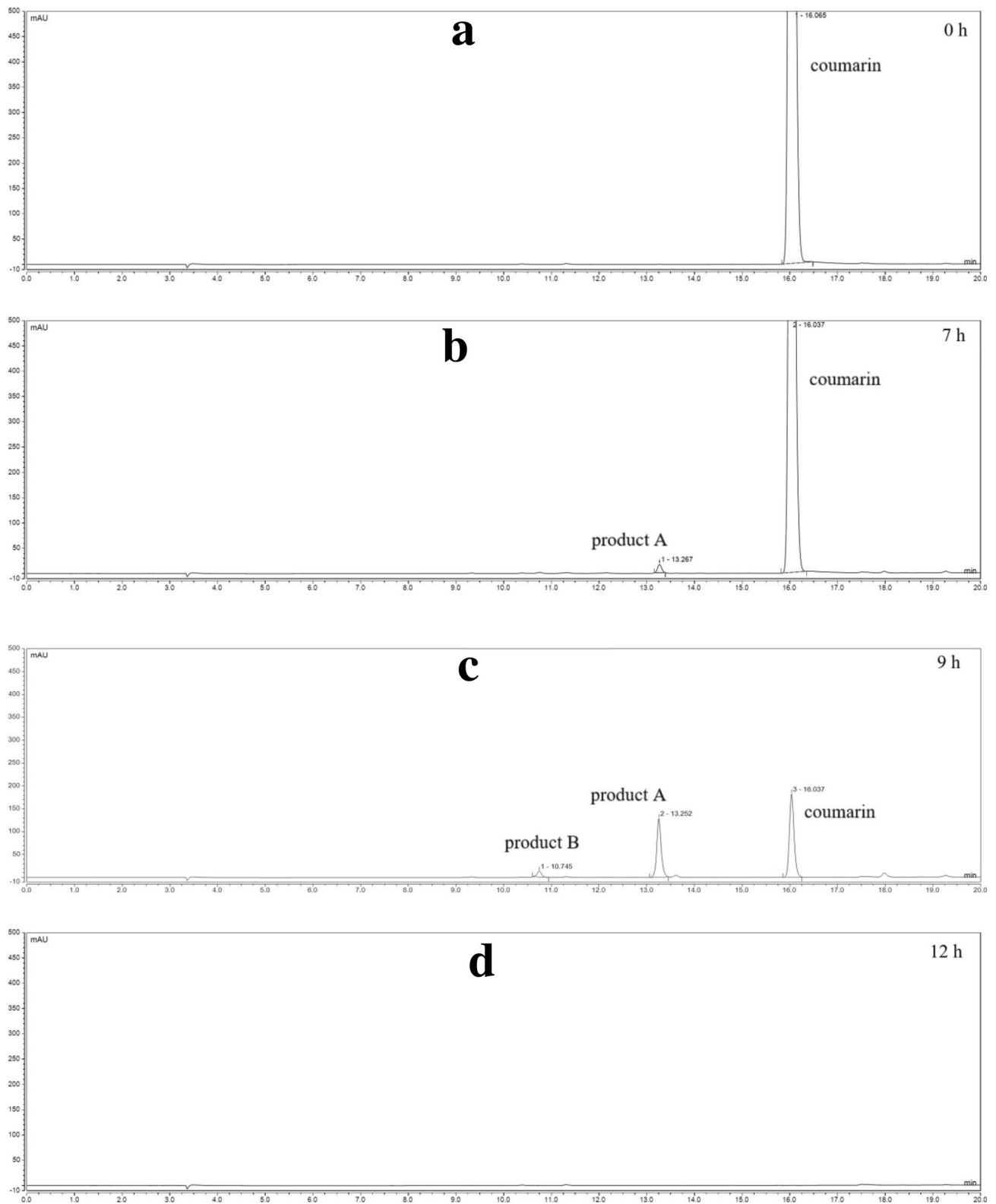


Fig. 4 HPLC chromatograms for coumarin biodegradation by USTB-Z after the following times: **a** 0 h; **b** 7 h; **c** 9 h; **d** 12 h

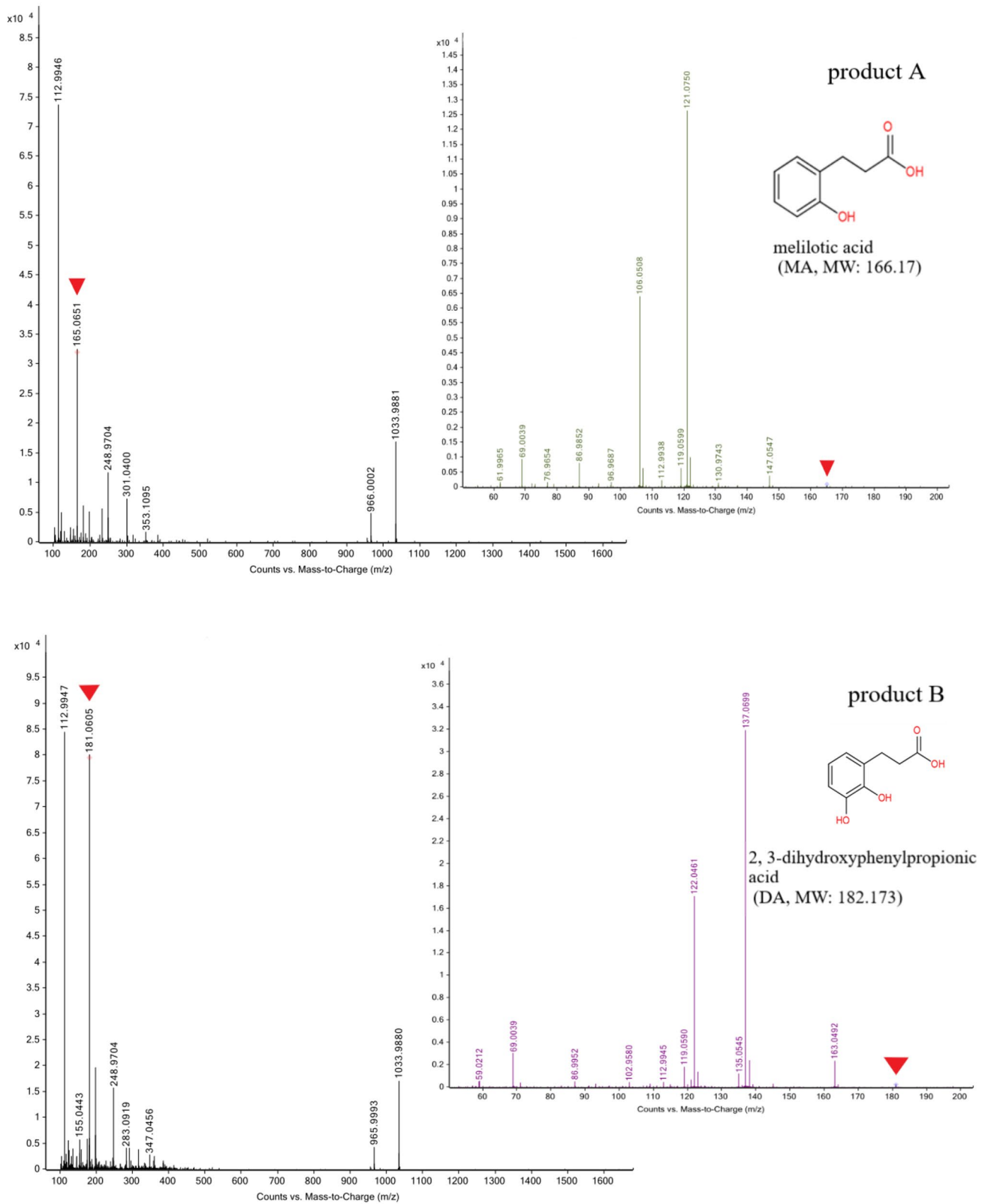


Fig. 5 Mass spectrograms of coumarin biodegradation intermediates using negative modes electrospray ionization (ESI⁻). Product a: Melilotic acid; product b: 2, 3-dihydroxyphenylpropionic acid

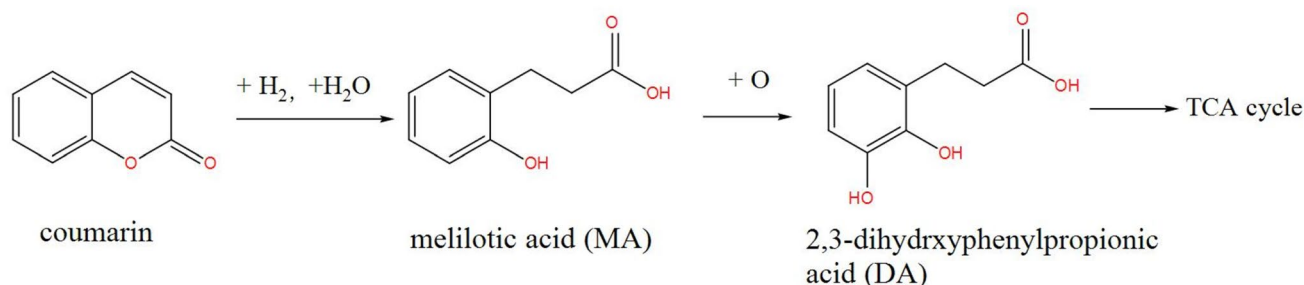


Fig. 6 Proposed pathway of coumarin biodegradation in strain USTB-Z

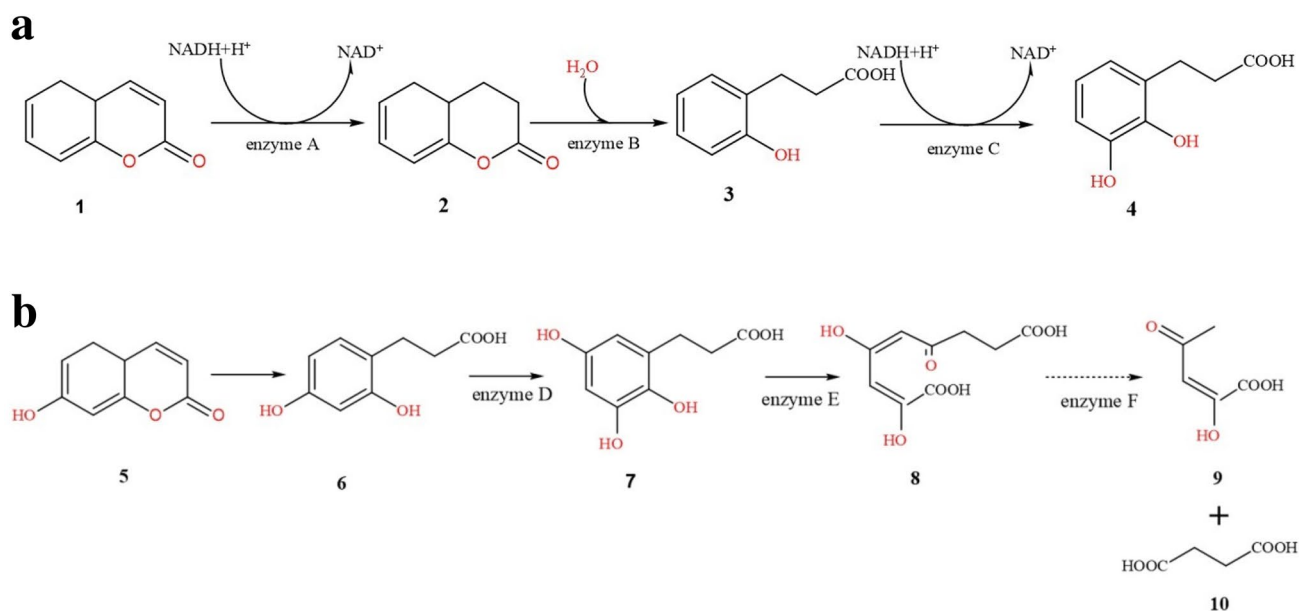


Fig. 7 a Coumarin metabolic routes in *Pseudomonas* sp. 301 (Nakayama et al., 1973). **b** Proposed metabolic pathway of 7-hydroxycoumarin in *Pseudomonas* sp. 7HK4 (Krikštaponis and Meškys 2018). **1**—coumarin, **2**—dihydrocoumarin, **3**—melilotic acid, **4**—2,3-dihydroxyphenylpropionic acid, **5**—7-hydroxycoumarin, **6**—3-(2,3-dihydroxyphenyl)-propionic acid, **7**—3-(2,3,5-trihydroxyphenyl)-propionic acid, **8**—(2E,4E)-2,4-dihydroxy-6-oxonona-2,4-dienedioic acid, **9**—(E)-2-hydroxy-4-oxopent-

2-enoic acid, **10**—succinic acid. Enzyme A—NAD(P)-dependent oxidoreductase, enzyme B—hydrolase, enzyme C—FAD-depend monooxygenase, enzyme D—FAD-binding monooxygenases/2-poluprenyl-6-methoxyphenol hydroxylase, enzyme E—ring-cleavage dioxygenase/extradiol dioxygenases, enzyme F—fumarylacetoacetate (FAA) hydrolase family. The dash arrow indicates a hypothetical reaction

Ltd. (Shanghai, China) and deposited in the GenBank database under the Accession Number MT889639. The resulting sequence of strain USTB-Z were aligned and compared with the known gene sequences in EzBioCloud database. The nearest neighbor sequences were aligned using Clustal W, and a phylogenetic tree based on the 16S rDNA sequence data was constructed by the neighbor-joining method with MEGA 7 software. To define the phylogenetic relationship, the genome sequences of several nearby neighbor species were downloaded from NCBI and the phylogenetic analysis with strain USTB-Z was performed using an orthologous average nucleotide identity tool (OAT) (Lee et al. 2016).

Whole genome sequencing (WGS) of strain USTB-Z

Preparation of genomic DNA was carried out according to the same method above. The draft genome of strain USTB-Z was sequenced by Illumina NovaSeq platform of Beijing Fixgene Co., Ltd. Low reads were trimmed by fastp software. High quality reads were then assembled by Spades software. The genome sequence was annotated by the PGAP on NCBI (October, 2020). The sequence data were submitted to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) with Accession Number JAECZP000000000 and SRA Number SRR13221997.

Results

Isolation and identification of the coumarin-biodegrading bacterium

A bacterium capable of utilizing coumarin as the sole source of carbon and energy was isolated from botanical garden soil. We designated it as USTB-Z. The colony was creamy, round, opaque, smooth in surface and neat in edge on a coumarin-containing agar plate, morphological examination under the light microscope revealed short rods (Online Resource 1). The cell was aerobic and gram-negative.

Basic Local Alignment Search Tool (BLAST) search against the EzBioCloud database showed that the 16S rDNA gene sequence of strain USTB-Z was > 98% identical to eight sequences of members of genus *Pseudomonas* such as *P. allopitida* Kh7 (98.65%), *P. plecoglossicida* NBRC 103162 (98.50%), and > 97% identical to more than 20 sequences of members of genus *Pseudomonas* such as *P. putida* NBRC 14164 (97.97%), *P. soli* F279208 (97.60%). The blast analysis was also supported by Phylogenetic tree based on 16S rDNA sequences as shown in Fig. 1. Furthermore, several nearby neighbor species to strain USTB-Z were chosen to phylogenetic tree analysis on the basis of the whole genome sequences by using OAT. There was no genome information of *P. allopitida* Kh7 and *P. persica* RUB6 in EzBioCloud database. The result revealed that strain USTB-Z shared highest identities (89.88%) to *P. putida* NBRC 14164 (Fig. 2). According to current bacterial taxonomy, strain USTB-Z is a potential novel species within *Pseudomonas* genus when their average nucleotide identity (ANI) < 95% (Kim et al. 2014).

Environment factors influencing biodegradation process

As shown in Fig. 3a, coumarin was biodegraded completely at pH 7.3–9.5 after 12 h, which exhibited a high biodegradation of coumarin and a high growth of USTB-Z in alkaline condition. The optimal pH value for USTB-Z to degrade coumarin was 7.3. The relationship between the biodegradation rate and temperatures for USTB-Z is shown in Fig. 3b. The optimum culture temperature was 30 °C, and the biodegradation rate reached 92.62% after 7 h. Coumarin biodegradation rate was weakly influenced by the inoculum size (Fig. 3c). It improved from 90.12 to 100% when the initial bacterial cell concentration increased from 0.8×10^6 to 9.6×10^6 CFU/mL. When the initial bacterial cell concentration was $\geq 3.2 \times 10^6$ CFU/mL, the biodegradation efficiency was above 98%. Hence the initial bacterial concentration of 3.2×10^6 CFU/mL was used in the following experiments. To further investigate

biodegradation ability for coumarin, different initial concentrations (50–1000 mg/L) of coumarin were used as the sole carbon and energy source in MSM. When coumarin concentration was less than 400 mg/L, biodegradation rate could reach almost 100% in 12 h in the mean while increasing concentration of coumarin to 800 mg/L and 1000 mg/L would cause biodegradation rate to decrease to 52.4% and 43.2% respectively (Fig. 3d). Initial coumarin up to 800 mg/L could be completely removed by USTB-Z within 48 h.

Identification of biodegradation products

Biodegradation products were analyzed by HPLC and Q-TOF LC/MS to elucidate the probable biodegradation pathway of coumarin by USTB-Z. HPLC chromatograms (Fig. 4) showed that during the first 9 h of incubation, the peak area of coumarin (retention time at 16.065 min) decreased and two new peaks appeared at 13.267 min (product A) and 10.745 min (product B), respectively, implying the formation of two new compounds. As the biodegradation proceeded, the areas of all the three peaks decreased rapidly and no accumulative metabolites were observed at the end of experiment (12 h). In the control group without strain USTB-Z, the concentration of coumarin was shown to be constant. (Figs. 5 and 6).

It has been reported that melilotic acid and 2, 3-dihydroxyphenylpropionic acid, as shown in Fig. 7, are the intermediates in a known coumarin metabolic pathway in several microorganisms (Levy and Weinstein 1964; Levy 1967; Shieh and Blackwood 1968; Nakayama et al. 1973; Aguirre-Pranzoni et al. 2011). Proposed molecular formulas of coumarin and products based on Q-TOF LC/MS analyses were supplemented in Online Resource 2 and 3. Figure 5 showed that *Pseudomonas* sp. USTB-Z cells cultivated in the presence of coumarin produced melilotic acid (MA, $C_9H_{10}O_3$, MW: 166.17) and 2, 3-dihydroxyphenylpropionic acid (DA, $C_9H_{10}O_4$, MW: 182.173) as biodegradation products. The deprotonated molecular ion of product A was m/z 165.0651 which was m/z 20 more than coumarin (MW: 146.15), indicating that product A was hydrogenated and hydrolyzed coumarin $[M + H_2 + H_2O - H]^-$. The deprotonated product B was detected at m/z 181.0605 which was m/z 16 more than product A, coinciding with the oxygenation of product A. These products were not found in the coumarin inorganic medium without USTB-Z or bacterial culture with glucose inorganic medium. Therefore, coumarin was reduced at the double bond in the α -pyrone ring and hydrolyzed between the oxygen and carbonyl carbon atoms of the ring to produce MA as the first intermediate product, and then MA was oxidized to DA as the second intermediate product. And no accumulative metabolites were observed at the

end of experiment. Based on the above results, a tentative metabolic pathway for the degradation of coumarin by *Pseudomonas* sp. strain USTB-Z may be proposed (Fig. 6).

Overview of the *Pseudomonas* sp. strain USTB-Z genome

The draft genome of strain USTB-Z has a single chromosome of 5,906,080 bp with a G+C content of 61.7%. The genome contains 5325 predicted protein coding genes (CDS) with an average size of 990 bp, giving a coding intensity of 88.373%. Analysis revealed 53 pseudogenes, 73 tRNA genes, 4 noncoding RNA (ncRNA), and 4 rRNA operons in the genome. Of 5325 protein coding sequences (CDSs), 5264 could be assigned to 21 different categories of clusters of orthologous groups (COGs). These results clearly suggested the organism's efficient amino acid transport and metabolism, energy production and conversion, inorganic ion transport and metabolism, etc. (Online Resource 1, Fig. S1-3).

In the present study, *Pseudomonas* sp. strain USTB-Z was found to be able to biodegrade coumarin. According to genome sequence analysis and annotation (Online Resource 4), there were 21, 43 and 1 coding sequences (CDSs) encoding for NAD (P)-dependent oxidoreductase, hydrolase and FAD-depend monooxygenase, respectively, and 1, 21 and 5 CDSs encoding for 3-hydroxycinnamic acid hydroxylase, dioxygenase, and fumarylacetoacetate (FAA) hydroxylase family, respectively (Online Resource 5). Importantly, based on a BLAST search, it was found that there were three continual genes which were annotated as bifunctional 3-(3-hydroxy-phenyl) propionate/3-hydroxycinnamic acid hydroxylase (gene_001906, 1560 bp genes encoded 519 amino acid proteins), vicinal oxygen chelate (VOC) family protein (gene_001907, 558 bp genes encoded 185 amino acid proteins), and fumarylacetoacetate hydrolase family protein (gene_001908, 852 bp genes encoded 283 amino acid proteins), and had the function of biodegradation aromatic compounds. Protein sequence alignment revealed that products of the three genes exhibited relatively high identity with the induced proteins encoded by a gene cluster *hcdABC* from 7-hydroxycoumarin-degrading *Pseudomonas mandelii* 7HK4 (DSM 107615) (Krikštaponis and Meškys 2018), such as 77.6% sequence identity to the flavin-binding hydroxylase (HcdA), 72.08% to the extradiol dioxygenase (HcdB), and 62.91% to the putative hydroxymuconic semialdehyde hydrolase (HcdC) (Online Resource 6 and 7).

Table 1 Enzymes involved in the biodegradation of coumarin or hydroxycoumarin and the coding sequences (CDSs) in strain USTB-Z

| Enzymes | Enzyme-catalyzed reactions in bacteria | CDSs in strain USTB-Z | References |
|--|--|-----------------------|--|
| Dihydrocoumarin: NAD [NADP] oxidoreductase/ NADH: <i>o</i> -coumarate oxidoreductase | Coumarin + NADH + H ⁺ + O ₂ = dihydrocoumarin + NAD ⁺ + H ₂ O Catalyze the reduction of <i>o</i> -coumaric acid to melilotic acid | 21 | Levy and Weinstein 1964; Nakayama et al. 1973 |
| Dihydrocoumarin hydrolase | Dihydrocoumarin + H ₂ O = melilotate | 43 | Nakayama et al. 1973 |
| Melilotate hydroxylase/FAD-depend monooxygenase | Catalyze the hydroxylation of melilotic acid (<i>o</i> -hydroxyphenylpropionic acid) to 2, 3-dihydroxyphenylpropionic acid | 1 | Levy 1967 |
| FAD-binding monooxygenases/2-polypropenyl-6-methoxyphenol hydroxylase | Hydroxylation of 3-(2,4-dihydroxyphenyl)-propionic acid to 3-(2,3,5-trihydroxyphenyl)-propionic acid | 1 | Krikštaponis and Meškys 2018 |
| Ring-cleavage dioxygenase/ type I extradiol dioxygenases (contains VOC family protein) | Oxidative cleavage of benzene rings of 3-(2,3,5-trihydroxyphenyl)-propionic acid to (2E,4E)-2,4-dihydroxy-6-oxonona-2,4-dienedioic acid | 21 | Krikštaponis and Meškys 2018 |
| Fumarylacetoacetate (FAA) hydrolase family | Biodegradation of (2E,4E)-2,4-dihydroxy-6-oxonona-2,4-dienedioic acid to (E)-2-hydroxy-4-oxopent-2-enoic acid and succinic acid | 5 | Krikštaponis and Meškys 2018 |

Discussions

Coumarin as a typical material of pharmaceutical and personal care products is produced and released into environment at a growing rate. As its potential toxicological properties, great concerns heightened the need for effective removal of this emerging contaminant from environment. In the present study, a promising bacterial strain USTB-Z for biodegrading coumarin was successfully isolated and identified as a potential novel *Pseudomonas* sp. strain by 16S rDNA sequence analysis and OAT. *Pseudomonas* sp. isolated from environment (soils, intertidal sediment, sewage sludge and so on) have been confirmed to be capable of degrading various organic contaminants such as furfuryl alcohol (Kumar and Rashmi 2017), PAHs (Obayori et al. 2008), nicotine (Chen et al. 2008), pyridine, indole, quinoline (Fetzner 1998) and so on. Strain USTB-Z could efficiently biodegrade coumarin, with 100% of removal percentages for 400 mg/L coumarin within 12 h and 800 mg/L coumarin within 48 h, higher than that by *Saccharomyces cerevisiae* DSMZ 2155 and *Bacillus cereus* Delaporte and *Pseudomonas orientalis* (100% within 120 h, 50% within 150 h and 40% within 150 h, respectively, at initial 400 mg/L coumarin) isolated from strawberry leaves (Häser et al. 2006). It indicated that *Pseudomonas* sp. USTB-Z is a high efficient candidate for biodegrading coumarin.

Environmental factors influence the biodegradation process. The rate of coumarin biodegradation increased from 70 to 100% when pH value was increased from 6.0 to 9.0. The alkaline condition at pH 7.3 was favorable for the degradation of coumarin, and it was also the optimum pH for the growth of strain USTB-Z. When the temperature sustained between 25 °C and 37 °C, 400 mg/L of coumarin was biodegraded completely within 12 h, and the optimal temperature was 30 °C. These results indicated that *Pseudomonas* sp. USTB-Z has a great potential in removing coumarin in mild environment conditions. The laboratory conditions are far different from those found in nature. Based on these findings, future work will be attempted to study the performance of biodegrading coumarin by strain USTB-Z in nature.

The key biodegradation intermediates during coumarin catabolism in bacteria is MA. The bioconversion of coumarin to MA by microorganisms can be achieved in two different pathways: (a) Coumarin was hydrolyzed at the lactone moiety to give *o*-coumaric acid [3-(2-hydroxyphenyl)-2-propenoic acid], and then reduced at the double bond to yield MA, which was the metabolic pathway in *Arthrobacter* genus (Levy and Weinstein 1964). (b) Coumarin was reduced to dihydrocoumarin and only then hydrolyzed to MA, which was the biodegradation pathway in *Pseudomonas* sp. 30-1

(Nakayama et al. 1973), *Aspergillus niger* ATCC 11394 (Aguirre-Pranzoni et al. 2011), *Saccharomyces cerevisiae* DSMZ 2155 and *Bacillus cereus* (Häser et al. 2006). MA was oxidized to DA by *Arthrobacter* and *Pseudomonas* species, and no data are available on further conversions of DA before into tricarboxylic acid cycle in these bacteria. In the present study, MA and DA were detected as the main biodegradation products by *Pseudomonas* sp. USTB-Z. However, dihydrocoumarin wasn't detected at any sampling time. DA was further biodegraded by *Pseudomonas* sp. USTB-Z into CO₂ and H₂O after the tricarboxylic acid cycle, but its products were not detected in the study.

The above biodegradation mechanism of coumarin by *Pseudomonas* sp. USTB-Z was further confirmed by its draft genome annotation and analysis. Biodegradation of coumarin or 7-hydroxycoumarin can be achieved in two stages, the bioconversion of coumarin to DA (Stage A), and the metabolism of DA (Stage B) as shown in Fig. 7 (Levy 1967; Nakayama et al. 1973; Krikštaponis and Meškys 2018). Several kinds of enzymes are involved in the two stages (Table 1), including NAD (P)-dependent oxidoreductase, hydrolase and FAD-depend monooxygenase in stage A (biodegradation of coumarin), and FAD-binding monooxygenases/2-polyprenyl-6-methoxyphenol hydroxylase, ring-cleavage dioxygenase/dioxygenases and fumarylacetoacetate (FAA) hydrolase family in Stage B (biodegradation of 7-hydroxycoumarin). Based on the results of genomic analysis of strain USTB-Z, 21 CDSs encoding for NAD (P)-dependent oxidoreductase, 43 CDSs encoding for hydrolase, and 1 CDS encoding for and FAD-depend monooxygenase, respectively, were annotated, which catalyzed the coumarin conversion to DA, and 1 CDS encoding for 3-hydroxycinnamic acid hydroxylase, 21 CDSs encoding for dioxygenase, and 5 CDSs encoding for fumarylacetoacetate (FAA) hydrolase family, respectively, were found and annotated, which were reported and speculated to participate in biodegradation of DA (Krikštaponis and Meškys 2018). Especially, the FAA family proteins are usually involved in the last stages of bacterial metabolism of aromatic compounds (Roper and Cooper 1993; Díaz and Timmis 1995; Lim et al. 2000). It was suggested that there are some kinds of enzymes in *Pseudomonas* sp. USTB-Z that participates in the final steps of coumarin metabolism after oxidative cleavage of the aromatic ring.

The operon *hcdABC*, which encodes a flavin-binding hydroxylase (HcdA), an extradiol dioxygenase (HcdB), and a putative hydroxyumuconic semialdehyde hydroxylase (HcdC), were found for the biodegradation of 3-(2,4-dihydroxyphenyl)-propionic acid (hydroxylation of DA) in *Pseudomonas mandelii* 7HK4 (DSM 107615) (Krikštaponis and Meškys 2018). It was confirmed that

there was three highly similar (with respect to gene organization, size, and homology) corresponding genes in USTB-Z. Based on comparison of protein sequences, it exhibited relatively high identity between *Pseudomonas* sp. USTB-Z and *Pseudomonas mandelii* 7HK4 (62.91–77.6%). These findings provided biological information base for coumarin biodegradation by *Pseudomonas* sp. USTB-Z, which is helpful in bioremediation of the emerging contaminant coumarin.

Conclusions

Pseudomonas sp. USTB-Z is a high efficient bacterium for biodegrading coumarin. It could biodegrade almost 100% of coumarin of 800 mg/L in MSM in 48 h. Optimal conditions for the strain were 30 °C and pH 7.3 with supplementation of coumarin at 400 mg/L. The biodegradation products of coumarin were further investigated using Q-TOF LC/MS and melilotic acid and 2,3-dihydroxyphenylpropionic acid were identified. Importantly, the draft genome analysis revealed that many genes in USTB-Z encoded related enzymes involved in coumarin (and its analogues) biodegradation. This study is helpful to the development of microbe resources for bioremediation of coumarin contaminated water and soil.

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Author contributions ZZ and HY conceived and designed the experiments. ZZ performed the experiments. CL was involved in sample preparation. QX, YL, XL, CY and HZ were involved in identification of biodegradation products and genome analysis. Data was statistically analyzed by ZZ. The paper was written by ZZ, reviewed and edited by HY.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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