Biodegradation of 2-methylisoborneol by enzyme separated from *Pseudomonas mandelii*

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ABSTRACT

As a kind of odorous substance, 2-methylisoborneol (2-MIB) is difficult to be degraded naturally. Some isolated strains of bacteria can degrade 2-MIB effectively. In this study, a strain of bacteria which can remove 2-MIB from drinking water efficiently was obtained from activated carbon in a filter, and was identified to be *Pseudomonas mandelii* based on 16S rRNA gene sequence analysis. *Pseudomonas mandelii* was not sensitive to the initial concentration of 2-MIB, and could tolerate a rather high concentration of 2-MIB. The best growth conditions for this degrader were 25–35 °C and initial pH of 7. The concentration of 2-MIB in mineral salt medium was reduced from 2 mg/L to 471.9 μg/L by *Pseudomonas mandelii* in 20 d after incubation. Nineteen bands of degrading enzyme were isolated from *Pseudomonas mandelii*, one of which was identified as a NAD-dependent dehydratase. It was found that 2-methyl-2-bornene was the metabolite in the presence of both the *Pseudomonas mandelii* and the isolated enzymes, indicating that NAD-dependent dehydratase might be involved in the biodegradation process or cooperate with other enzymes in the metabolic process to complete the dehydration process of 2-MIB.

Key words | 2-MIB, biodegradation, dehydration product, enzyme, *Pseudomonas mandelii*

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HIGHLIGHTS

- *Pseudomonas mandelii* was reported to be a 2-MIB degrader.
- Degrading enzymes were isolated from *Pseudomonas mandelii*.
- NAD-dependent dehydratase might help to complete the 2-MIB dehydration process.
- 2-Methyl-2-bornene was found to be the metabolite in the presence of *Pseudomonas mandelii* or the isolated enzymes.

INTRODUCTION

As one of the metabolites of *cyanobacteria* and *actinomycetes* (Klausen *et al.* 2005; Guttman & van Rijn 2009; Li *et al.* 2018), 2-methylisoborneol (2-MIB, $C_{11}H_{20}O$, of which the boiling point and the solubility are 196.7 °C and 150.2 mg/L, respectively) has been considered to be the main substance which can cause musty odor in water, resulting in most consumer complaints in waterworks (Yuan *et al.* 2012). The 2-MIB concentrations in the raw water of waterworks are in the range of 0–200 ng/L, and the values in backwash water may be higher than 1,000 ng/L. The standard limit of 2-MIB for drinking water plants in China has been set to be 10 ng/L, and the value was determined according to the threshold odor concentration of 2-MIB (Xie *et al.* 2015). It is difficult to remove 2-MIB through conventional water treatment processes. Therefore, advanced treatment processes such as activated carbon adsorption and ozonation are often used to remove 2-MIB (Kim *et al.* 2014; Bu *et al.* 2017; Li *et al.* 2018). Activated carbon can easily adsorb 2-MIB (Yu *et al.* 2016); however, large amounts of activated carbon are needed

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because of the competition from natural organic matter (NOM) in water. Ozonation is efficient for 2-MIB degradation, but hazardous oxidation by-products such as BrO_3^- may be produced during ozonation as Br^- is usually contained in water. Therefore, a more cost-effective method for the treatment of 2-MIB is required (Yuan *et al.* 2012).

Previous studies have shown that adsorbed 2-MIB can be biodegraded (Izaguirre et al. 1988b; Azaria *et al.* 2017), and the by-products produced in the process of water treatment are reduced because no chemical is added in the system (Kim *et al.* 2014). Several strains of bacteria, including *Pseudomonas* sp. (Izaguirre *et al.* 1988a; Ho *et al.* 2007; Yuan *et al.* 2012), *Flavobacterium* sp. (Yuan *et al.* 2012), *Micrococcus* sp. (Yuan *et al.* 2012), *Sphingomonas* sp., *Alphaproteobacterium*, *Acidobacteriacea* (Ho *et al.* 2007), *Enterobacter* sp. (Tanaka *et al.* 1996), *Candida* sp. (Sumitomo 1988), *Brevibacterium* sp., *Bacillus fusiformis*, *Bacillus sphaericus* (Lauderdale *et al.* 2004) and *Bacillus subtilis* (Yagi *et al.* 1988), have been isolated and identified to be 2-MIB degraders.

It was reported that 2-methylcamphene and 2-methylenebornane are formed as 2-MIB degradation products by additional bacteria (Tanaka *et al.* 1996; Eaton 2012), and 2-MIB was degraded after pre-activating the enzymatic expression using inducers such as camphor and limonene (Azaria & van Rijn 2018). Three gene copies of cytochrome P-450cam, a gene encoding for an enzyme involved in the degradation of camphor, were detected in *Rhodococcus* sp., which was capable of 2-MIB degradation (Azaria *et al.* 2017). Furthermore, Oikawa *et al.* (1995) confirmed this by excising the entire cam operon from a camphor degrading *Pseudomonas putida*, where its subsequent transformation into *Escherichia coli* demonstrated the acquired ability of 2-MIB degradation. The previous studies have focused on the degradation of 2-MIB by the strains of bacteria, but little attention has been given to the degradation of 2-MIB by the separated enzyme thus far (Azaria & van Rijn 2018).

The objective of this research is to separate the 2-MIB degrading enzyme to promote the removal of 2-MIB in water. In this study, a single strain of bacteria which can degrade 2-MIB was isolated, and the 2-MIB degradation performance and affecting factors of the degrader were examined. In addition, the removal rate of 2-MIB in the presence of the enzyme was investigated.

MATERIALS AND METHODS

Chemicals

The solid 2-MIB (98%) used in this study was purchased from Wako Pure Chemicals, Ltd (Osaka, Japan). Sterile 2-MIB solution (20 mg/L) was prepared by dissolving 2-MIB in deionized (DI) water treated by a Milli-Q system (Millipore Pty Ltd, USA). The solution was then filtered through a sterile 0.22 μm-pore-size micro-porous membrane (MPM) and stored at 4 °C prior to use.

Enrichment procedures

The enrichment of 2-MIB degraders was conducted in a mineral salt medium (MSM, with NH_4NO_3 0.1%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05% and KCl 0.02%, pH 7) (Tanaka *et al.* 1996). The 250 mL flask containing 100 mL MSM was sealed with absorbent gauze and sterilized at 121 °C for 30 min. Five grams of activated carbon, which was obtained from a carbon filter of a water plant in Beijing, was added into 10 mL sterile PBS buffer (0.1 mol/L). The buffer was shaken vigorously for 15 min to obtain the bacteria.

Ten millilitres of the PBS buffer which had been inoculated were poured into the flask containing 100 mL MSM. Then, 2-MIB was added into the MSM, and the initial concentration of 2-MIB was set to be 100 ng/L. The flasks were covered with sealing membrane (Solarbio, Beijing, China), and shaken for 5 days at 30 °C and 120 r/min. The incubation process was repeated to select the 2-MIB degraders by increasing the initial 2-MIB concentration from 200 ng/L to 20 μg/L. All these experiments were carried out in an aseptic processing station.

Isolation of 2-MIB degraders

Luria–Bertani (LB) agar medium (peptone 1%, yeast extract powder 0.5%, NaCl 0.5%, agar 1.5%, pH 7) was used to isolate the 2-MIB degraders. The sterilized agar medium was poured into 90 mm vitreous plates, and cooled to about 40 °C. The cultured MSM (1 mL) was applied on LB agar medium in triplicate, and incubated for two days at 30 °C.

The isolated cultures were streaked onto the LB agar medium at least twice for purification. The pure cultures were dissolved in 1 mL sterile PBS buffer (0.02 mol/L), and centrifuged at 8,496 × *g* for 3 min. The cells in vials were washed twice with 1 mL sterile PBS buffer and stored at 4 °C. The bacteria were identified by a commercial laboratory, TaKaRa Biological Company (China).

Degradation performance of bacteria

During these experiments, all the glassware and solutions were sterilized at 121 °C for 30 min before use. Ten millilitres of the prepared bacterial suspension with an optical density at a wavelength of 600 nm (OD_{600}) of 1.0 was added into 150 mL MSM containing 20 μg/L 2-MIB, and the degradation temperature (5, 10, 15, 20, 25, 30 and 35 °C) was optimized by rotating the solutions in an incubator shaker at 120 r/min, followed by measuring the OD_{600} of the samples at 0, 3 and 5 d. Similar experiments were conducted to investigate the effects of temperature, initial pH, 2-MIB concentration and bacteria dosage on the growth of degraders and 2-MIB removal rates, except that 1,000 mL of MSM with 2 mg/L of 2-MIB was used as the initial solution in the experiments for detecting the removal rate of 2-MIB. In addition, the degradation rate of 2-MIB in the effluent of the sand filter in the water plant was also considered.

Separation of enzyme

The total enzymes were extracted as follows. Fifty millilitres of cell suspensions with the same OD_{600} were centrifuged at 25 °C and 2,124 × *g* for 10 min to obtain the bacteria, and the bacteria were washed by Tris-HCl buffer (pH 7) three times. The prepared cells were dispersed in 10 mL Tris-HCl buffer, and ultrasonicated in an ice bath for 40 min (work for 2 s and rest for 2 s alternately). Three millilitres of the Tris-HCl buffer containing degrader cells were taken into 20 mL 20 μg/L 2-MIB solution at 30 °C for 1 h, while a control experiment was carried out at 80 °C. In order to determine the enzyme activity, the samples were filtered through sterile 0.22 μm-pore-size filters, and transferred into crimp cap vials to measure the concentration of 2-MIB. Then, the degrading enzyme was separated through column separation. The appropriate column was selected to separate the enzyme, and the separation protocol was obtained according to the activity of the gradually separated enzyme.

Analytical method

The 20 mL samples were filtered through sterile 0.22 μm-pore-size MPM filters, and supplemented with 0.3 g/mL NaCl. The samples were transferred into crimp cap vials, and then incubated in a heated magnetic stirrer at 1,000 r/min and 60 °C for 50 min. The solid-phase microextraction (SPME) fiber was immersed into the headspace of the liquid sample vial for 5 min, and agitated to allow the analyte to be absorbed onto the SPME fibers. The concentration of 2-MIB was determined by gas chromatograph (GC, Agilent 6890N)–mass spectrometer (MS, Agilent 5975C) (Kang *et al.* 2017) which was equipped with a capillary column (HP-5MS, 30 m × 0.25 mm × 0.25 μm, Hewlett-Packard). The oven temperature was held at 40 °C for 2 min from injection, increased to 240 °C at 8 °C/min, and held at 240 °C for 5 min. Helium gas with a purity of 99.999% was used as the carrier gas, and the column flow rate was 1.0 mL/min in the constant flow mode.

The biomass of living bacteria was also measured by the plate-counting method (GB/T 5750.12-2006, China), which had a similar process as the separation of the 2-MIB degraders, and only the plates with 30–300 colony-forming units (CFU) were utilized.

RESULTS AND DISCUSSION

Isolation and identification of 2-MIB degraders

One kind of bacteria, which was observed from the agar containing 2-MIB as the sole carbon source after 72 hours inoculation, was detected to be gram-negative bacilli. The colony-forming time of this bacterium was about 48 h, and a flat colony morphology with diameter of 3 mm was found at 6 d. The micromorphology of the bacteria was observed by 100 times oil immersion lens. The 16S rRNA phylogeny result is shown in Figure 1, indicating that the isolated bacteria shared 99% 16S rRNA gene sequence identity with their nearest relative of *Pseudomonas mandelii*. This is

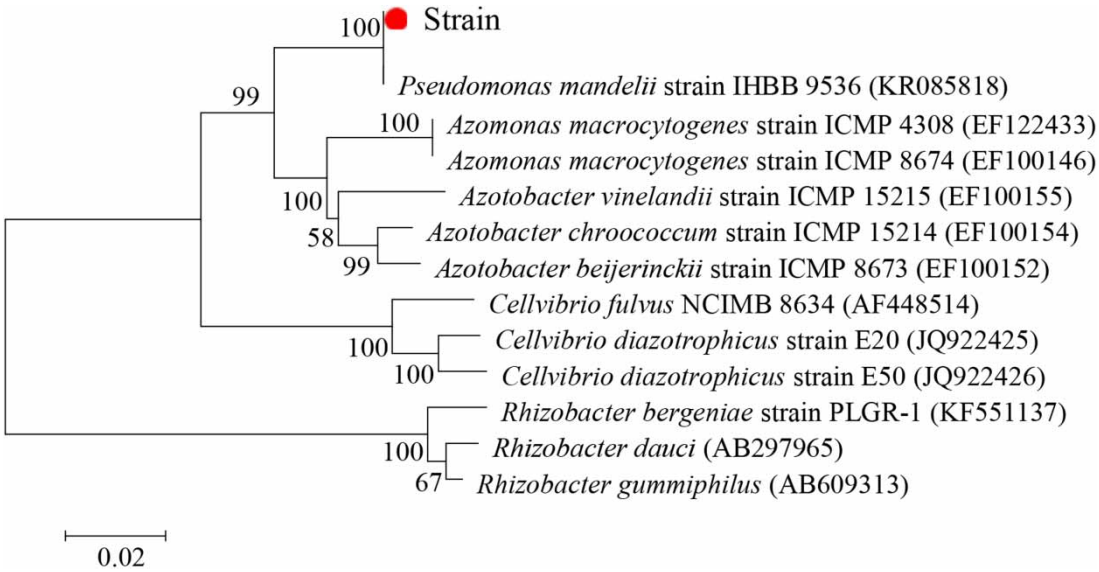

Figure 1 | Neighbor-joining trees showing the phylogenetic relationships of the predominant strains in the isolated culture with the closest-matching species.

the first report of this kind of bacteria being capable of degrading 2-MIB since the samples used for degrader isolation were different from those in the previous studies.

Growth of 2-MIB degraders

The effect of ambient conditions including the environmental temperature, the initial pH and the initial 2-MIB concentration on the specific growth rate of *Pseudomonas mandelii*, which was calculated by (OD_{600} at 3 d)/(OD_{600} at 0 d), were investigated in this study, and the results are shown in Figure 2.

Effect of temperature

The same amounts of *Pseudomonas mandelii* were added to MSM containing 20 μg/L 2-MIB and placed in various temperatures between 5 and 35 °C to investigate the effect of environmental temperature on the growth of the degraders. The OD_{600} values of the medium were measured on the third day (Figure 2(a)). When the temperature was below 10 °C, there was very slow bacterial growth. The growth of the bacteria accelerated along with the increasing temperature, and reached the maximum rate when the temperature was between 25 and 35 °C, which agreed with the general conditions of most microorganisms, indicating that the bacteria could grow and propagate rapidly by utilizing the energy generated from 2-MIB decomposition at 25–35 °C. Thus, the cultivation temperature was selected to be 30 °C in the following studies.

Effect of initial pH

The optimum pH for bacterial growth is generally between 6 and 9, and most microorganisms grow in neutral or alkaline environments. In this experiment, HCl or NaOH was added to MSM containing 20 μg/L 2-MIB to adjust the value of pH, and the results for the effect of initial pH on 2-MIB degrader growth are shown in Figure 2(b). It can be seen that the bacteria hardly grew at pH 5, and began to grow slowly at pH 6, indicating that an acidic environment was not suitable for *Pseudomonas mandelii*. The highest specific growth rate was obtained at pH 7–8, showing that the bacteria grew better in neutral or weak alkaline environments since the enzymes necessary for bacterial metabolism have strong biological activities in this pH range. In this experiment, pH 7 was chosen as the most suitable growing condition. Another experiment showed that the pH of the culture medium on the third day was slightly increased. A final pH value of 7.16 was detected compared with the initial neutral condition (pH 7), probably due to constant lysis of the thallus cells during their growing process. The hydrolysis of the cytoplasmic components and the enzymes would cause the increase of pH value.

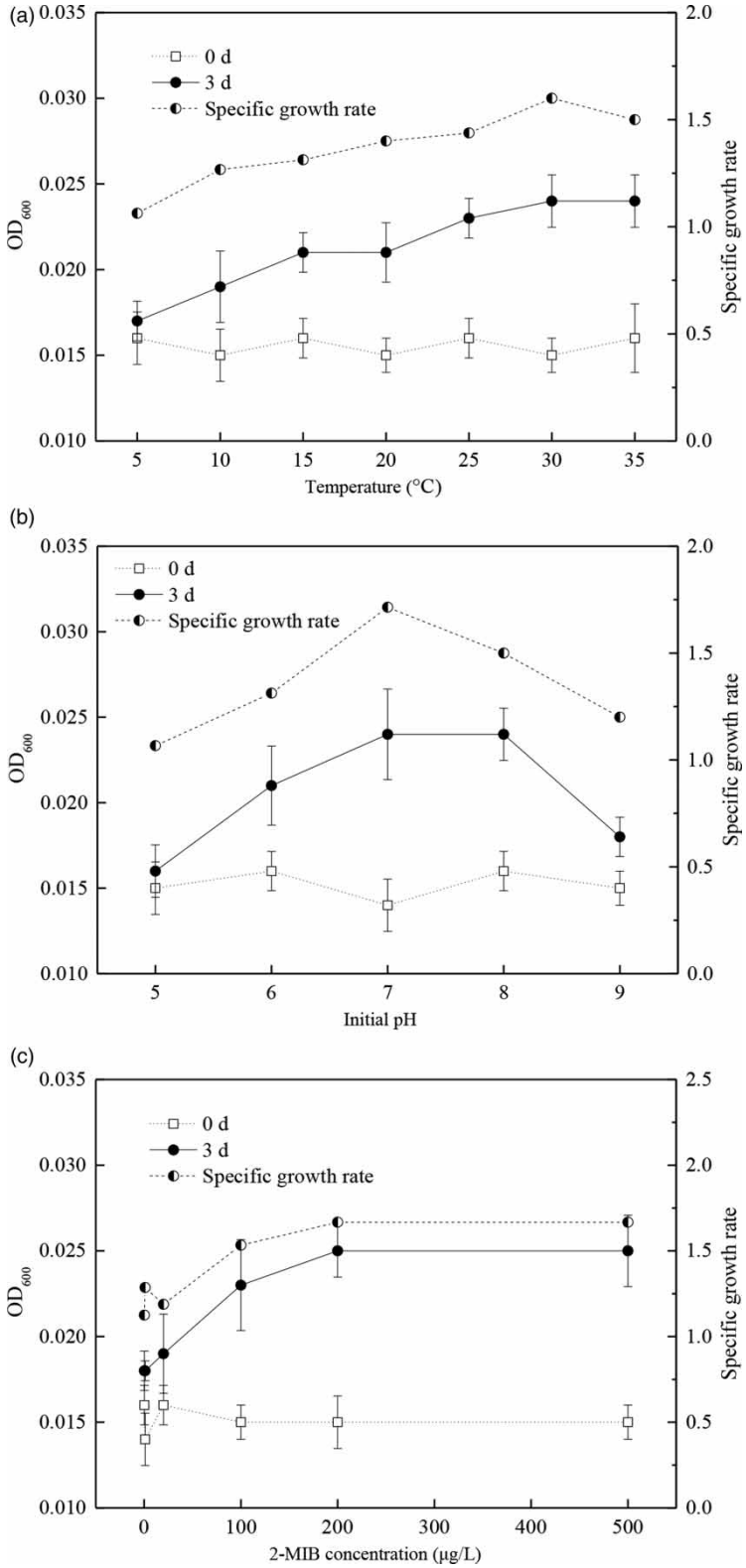

Figure 2 | Effect of ambient conditions on the growth of *Pseudomonas mandelii*: (a) temperature; (b) initial pH; (c) initial 2-MIB concentration.

Effect of 2-MIB initial concentration

As the carbon source, 2-MIB offers the energy necessary for the growth and reproduction of *Pseudomonas mandelii*. It is necessary to consider the utilizability of low concentrations of 2-MIB, as well as the strain and the tolerance of the degraders to high concentrations of 2-MIB. In this study, the effect of 2-MIB concentration on the growth of the bacteria was investigated (Figure 2(c)). The results indicate that with the increase of the initial 2-MIB concentration, the degradation and utilization of the degraders to 2-MIB were also increased accordingly, especially in the concentrations of 20–200 μg/L. When the concentration of 2-MIB was up to 500 μg/L, the growth rate of the strain was a bit higher compared with that at 200 μg/L, indicating that the strain had good tolerance to 2-MIB at this concentration, and could use high concentrations of 2-MIB as the energy substance for its growth and reproduction. In addition, the growth rate of *Pseudomonas mandelii* fits with the 'Monod equation' (Equation (1)), with a calculated maximum specific growth rate of 0.56 /d and half-saturation coefficient of 3.85 μg/L, respectively:

$$\mu = (\mu_{\max} \times S)/(K_s + S) \quad (1)$$

where μ is the specific growth rate of the bacteria (/t), $\mu_{\max}$ is the maximum specific growth rate (/t), K_s is the half-saturation coefficient, and S is the substrate concentration.

Degradation of 2-MIB by *Pseudomonas mandelii*

The experiments for the removal of 2-MIB with initial concentration of 2 mg/L were carried out in a 2,000 mL flask containing 1,000 mL MSM, and the effect of initial bacteria dosage was investigated (Figure 3(a)). The growth curve of the degraders during the degradation process was also detected.

Effect of initial dosage of bacteria on 2-MIB degradation rate

A larger amount of microorganisms often have higher metabolic capacity. However, the concentration range most suitable for strain growth should be determined. It can be seen from Figure 3(a) that when the initial OD_{600} was 2, the biodegradable removal rate on the third day was 25.8%, which was much higher than the rate for the lower OD_{600}.

Growth curve of *Pseudomonas mandelii*

Figure 3(b) shows that after *Pseudomonas mandelii* was added to MSM with a high concentration of 2-MIB, the bacterial growth was slow in the first 1–2 d. Then the number of viable bacteria increased sharply in the logarithmic growth phase from 3 to 5 d. The stationary phase of the bacteria was from 6 to 15 d, a long time which was helpful for bacterial degradation of 2-MIB, and the decline phase was from 15 to 20 d. The cardinal number of viable cells was large, indicating that *Pseudomonas mandelii* had strong viability and metabolic ability in MSM containing 2 mg/L of 2-MIB. *Pseudomonas mandelii* could degrade 2 mg/L 2-MIB to 471.9 μg/L in a degradation period of 20 d, which indicated that the bacteria had strong tolerance and degradation ability for super-high concentration odorous substances.

Isolation of degrading enzyme

According to the determination of enzyme activity after gradual separation, the separation protocol of *Pseudomonas mandelii* was finally identified. The protein was purified by a high-throughput HiPrep 16/10 Q FF anion exchange column at first, and then by a SOURCE 30S (10 mL) cation exchange column; finally, the protein was purified by a Mono Q (1 mL) anion exchange column.

The degrading enzyme was isolated from *Pseudomonas mandelii*, and 19 bands were obtained after three steps of chromatographic separations. The bands were numbered as 1–19, and the proteins were identified by MALDI-TOF-MS. The identification map is shown in Figure 4. Based on the total protein sequence encoded by *Pseudomonas mandelii* which was uploaded to the Mascot software, the peptide digestion database was obtained. By comparing with the total protein sequence of *Pseudomonas mandelii* which had been sequenced and reported before, 19 functional enzymes were identified (Table 1), among which the No. 17 band was identified as a nicotinamide adenine dinucleotide (NAD)-dependent dehydratase, and the others were identified as catalytic proteins such as histidine kinase, DNA

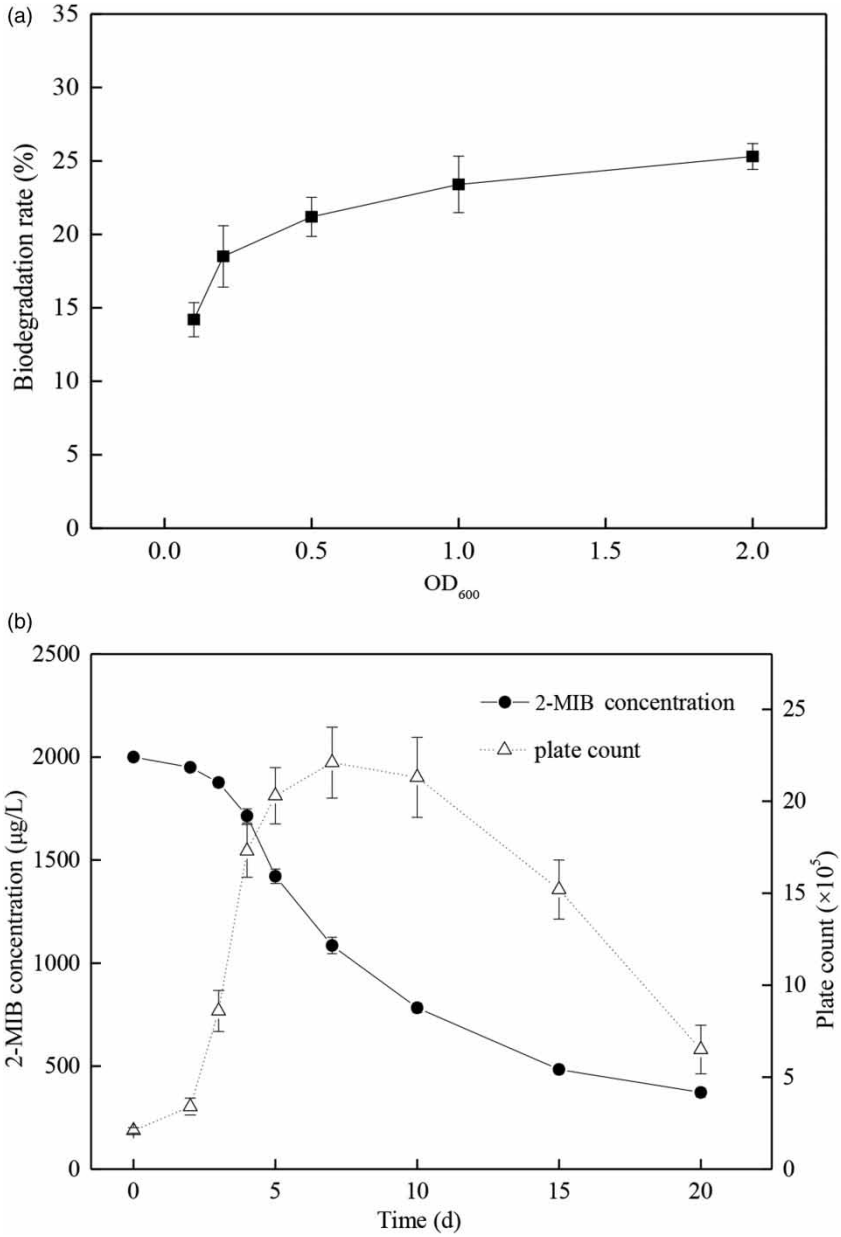

Figure 3 | Effect of degrader dosage on the degradation of 2-MIB: (a) effect of degrader dosage on 2-MIB (20 μg/L) degradation in the third day; (b) growth curve of degraders during 2-MIB (2 mg/L in MSM) degradation.

polymerase I, translation initiation factor and some unknown proteins.

Degradation mechanism of 2-MIB

The metabolites of 2-MIB degraded by *Pseudomonas mandelii*, as well as those by the isolated enzymes, were analyzed using GC-MS (Figure 5); 2-methyl-2-bornene, which had been reported to be one of the dehydration products of 2-MIB by *Pseudomonas* sp. and *Enterobacter* sp. according to ^{1}H-NMR analysis (Sumitomo 1988; Tanaka *et al.* 1996), was identified at a retention time of 8.235 min. The dehydration product 2-methyl-2-bornene was also detected by GC-MS when the enzymes were added in the

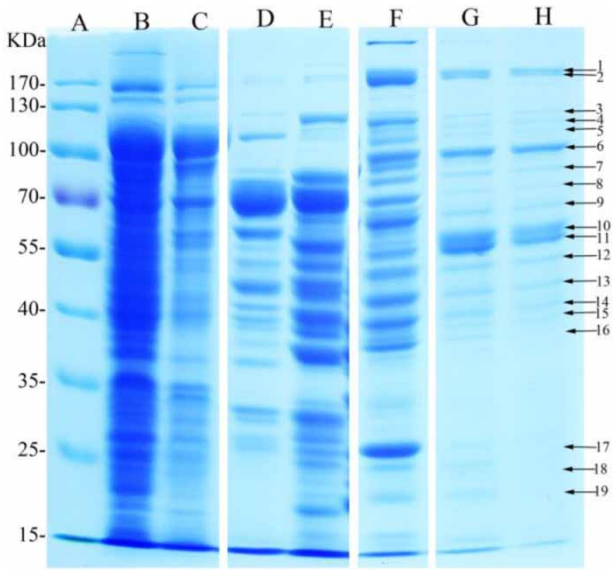

Figure 4 | Purification and identification map of *Pseudomonas mandelii* protein: (A) standard molecular weight of protein; (B) total protein extract; (C) HiPrep 16/10 Q FF anion exchange column penetration component; (D) and (E) HiPrep 16/10 Q FF anion exchange column elution collection component; (F) SOURCE 30S (10 mL) cation exchange column elution component; (G) and (H) Mono Q (1 mL) anion exchange column elution component.

solution (Figure 5(c)), showing that NAD-dependent dehydratase might be involved in the biodegradation process or cooperate with other enzymes in the metabolic process to complete the dehydration process of 2-MIB.

Degradation of 2-MIB in effluent of sand filter

In order to investigate the removal of 2-MIB in the effluent of sand by biodegradation, both the degrader and the isolated enzyme were used, and the results are shown in Figure 6. It can be seen that in both of the experiments, 2-MIB in real water could be degraded efficiently. The degradation rate of 2-MIB in real water by *Pseudomonas mandelii* was a little lower than that in MSM, indicating that the presence of organic matter decreased the removal rates of 2-MIB because NOM was proposed as a competitor for utilization by the bacteria. In the first 5 d, the removal rate in the presence of the enzymes was higher than that in the presence of the bacteria because the enzymes should be used immediately. However, the removal rate for degradation of 2-MIB by the degraders increased rapidly because of the propagation of the bacteria, which could be proved by the increase of the OD_{600} value.

Table 1 | Annotation of 19 functional enzymes

Sample	Locus tag	MW (kDa)	Annotation
1	WP_010459347.1	223.05	hemolysin-type calcium-binding region
2	WP_010466867.1	217.17	sensor histidine kinase
3	WP_010456331.1	128.57	transcription-repair coupling factor
4	WP_010457195.1	122.05	hypothetical protein
5	WP_010456066.1	111.88	multidrug transporter
6	WP_010465624.1	100.98	DNA polymerase I
7	WP_010463483.1	90.84	translation initiation factor IF – 2
8	WP_010461877.1	83.86	acylaldehyde oxidase
9	WP_049870757.1	67.15	FAD-dependent oxidoreductase
10	WP_010465559.1	60.04	membrane protein
11	WP_010464417.1	55.48	cell division protein
12	WP_010467398.1	53.98	aldehyde dehydrogenase
13	WP_010462550.1	47.43	4-hydroxybenzoate transporter
14	WP_010461925.1	47.91	23S rRNA pseudouridylate synthase B
15	WP_010459363.1	39.9	dehydrogenase
16	WP_010463461.1	36.41	transcriptional regulator
17	WP_010467311.1	33.36	NAD-dependent dehydratase
18	WP_003186059.1	22.6	MULTISPECIES: 50S ribosomal protein L3
19	WP_010461373.1	19.18	transcription elongation factor

CONCLUSIONS

One kind of bacteria capable of removing 2-MIB from drinking water was isolated from a carbon filter of a water plant in Beijing, and was identified to be *Pseudomonas mandelii* based on 16S rRNA gene sequence analysis. The suitable conditions for the growth of *Pseudomonas mandelii* were 25–35 °C and pH 7. The concentration of 2-MIB could be reduced from 2 mg/L to 471.9 μg/L by *Pseudomonas mandelii* in 20 d in these conditions. The degrading enzyme was isolated from *Pseudomonas mandelii*, and 19 bands were obtained after three steps of chromatographic separations, in which the No. 17 band was identified as a NAD-dependent dehydratase by MALDI-TOF-MS. It was found that 2-methyl-2-bornene was the degradation product

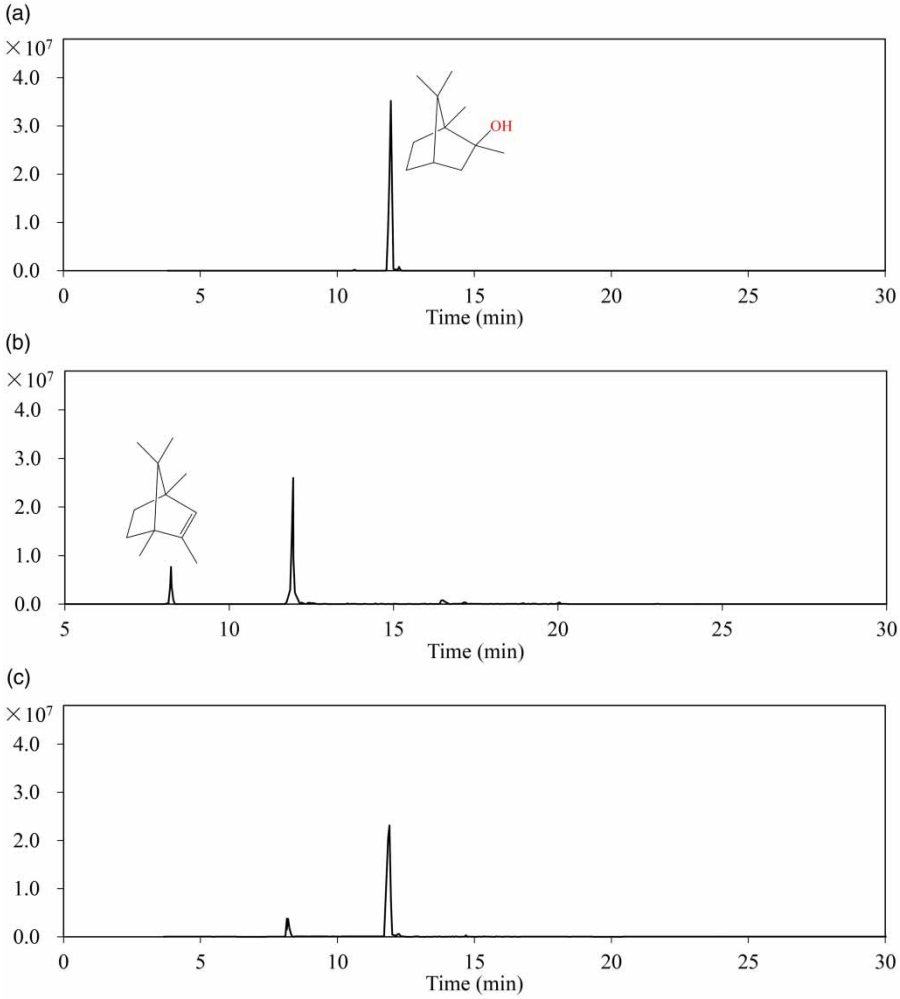

Figure 5 | Chromatograms of 2-MIB metabolites: (a) control; (b) degraded by *Pseudomonas mandelii*; (c) degraded by the isolated enzymes.

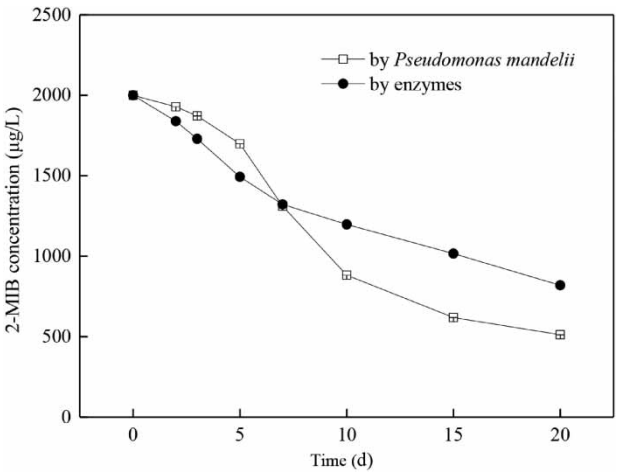

Figure 6 | Degradation of 2-MIB in the effluent of the sand filter.

both in the presence of the *Pseudomonas mandelii* and when isolated enzymes were added, indicating that NAD-dependent dehydratase might be involved in the biodegradation process or cooperate with other enzymes in the metabolic process to complete the dehydration process of 2-MIB.

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