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Novel strains with superior degrading efficiency for lincomycin manufacturing biowaste

Yonghong Li [a], Luping Fu [a], Xuan Li [a], Yun Wang [a], Yongjun Wei [a,*], Jinfeng Tang [c,d,**], Hongmin Liu [a,b,***]

[a] Key Laboratory of Advanced Drug Preparation Technologies, Ministry of Education, School of Pharmaceutical Sciences, Zhengzhou University, Henan, Zhengzhou 450001, China
[b] Henan Province Collaborative Innovation Center of New Drug Research and Safety Evaluation, Henan, Zhengzhou 450001, China
[c] Key Laboratory for Water Quality and Conservation of Pearl River Delta, Ministry of Education, School of Environmental Science and Engineering, Guangdong Provincial Key Laboratory of Radionuclides Pollution Control and Resources, Linköping University – Guangzhou University Research Center on Urban Sustainable Development, Guangzhou University, Guangzhou 510006, China
[d] Nuclear Chemistry and Industrial Material Recycling, Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Gothenburg 412 96, Sweden

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ABSTRACT

As the antibiotic pollution source in the environment, a large amount of biowastes generated from antibiotic fermentation manufacture needs proper disposal. Recycling the biowaste as resources and nutrients is of great interest. Besides, degradation or removal of antibiotics is indispensable for the reclamation of antibiotic manufacturing biowaste. To establish environmentally friendly disposal strategies for lincomycin manufacturing biowaste (LMB), we screened the microbial strains that could efficiently degrade lincomycin from the antibiotic wastewater treatment plant. Among them, three novel strains were identified as *Bacillus subtilis* (strain LMB-A), *Rhodotorula mucilaginosa* (strain LMB-D) and *Penicillium oxalicum* (strain LMB-E), respectively. LMB-A and LMB-D could degrade 92.69% and 74.05% of lincomycin with an initial concentration of 1117.55 mg/L in 144 h, respectively. The lincomycin degradation products were formed by the breakage of amide bond or losing N-demethyl/thiomethyl group from the pyrrolidine/pyranose ringcata cata catalyzed by the strains. Moreover, LMB-A could decontaminate LMB, and the decontaminated LMB could be used as a nitrogen source to culture salt-resistant bacteria and other useful microorganisms. LMB-A and LMB-D have the potential to be used for the bioremediation of water and soil polluted by lincomycin and its analogs. LMB-E could degrade 88.20% LMB after 144-h cultivation. In summary, this study gives an insight into the green disposal of LMB, and the established strategy has potential application for biotreatment of other antibiotic fermentation manufacturing biowastes.

1. Introduction

Solid wastes and wastewater generated during antibiotic manufacturing processes are among the primary antibiotic pollutant sources (Phillips et al., 2010). These antibiotic manufacturing biowastes (AMBs) are mainly composed of cell biomass and fermentation waste. The AMBs contain rich proteins and other nutritional materials that could be used as valuable resource. However, residual antibiotics in AMBs may affect environmental microbiota by inducing the generation of antibiotic-resistant bacteria (ARB) and leading to the spread of antibiotic resistance genes (ARGs). It would disturb natural elemental cycles and alter the structural, genetic and functional diversity of natural microbial communities (Cycoń et al., 2019). Moreover, the ARGs might be transferred among environmental microbiota and human microbiota,

* Corresponding author.
** Corresponding author at: Key Laboratory for Water Quality and Conservation of Pearl River Delta, Ministry of Education, School of Environmental Science and Engineering, Guangdong Provincial Key Laboratory of Radionuclides Pollution Control and Resources, Linköping University – Guangzhou University Research Center on Urban Sustainable Development, Guangzhou University, Guangzhou 510006, PR China.
*** Corresponding author at: Key Laboratory of Advanced Drug Preparation Technologies, Ministry of Education & School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou 450001, China.
E-mail addresses: yongjunwei@zzu.edu.cn (Y. Wei), jinfeng@gzhu.edu.cn (J. Tang), liuhm@zzu.edu.cn (H. Liu).

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and harm human health (Zhou et al., 2018). Therefore, AMBs without proper disposal are listed as 'hazardous wastes', and are prohibited from being used as animal feedstuff and land fertilizer.

Currently, landfill and incineration are the widely used AMBs treatment methods, but these methods are not eco-friendly. The landfill has the potential risk of antibiotic leakage and migration, while incineration requires expensive combustion equipment and suffers a high energy consumption for drying (Chen et al., 2017a, 2017b). Physical and chemical methods require high cost and cannot damage ARGs completely (Cai et al., 2018; Wang et al., 2018a; Zhang et al., 2020). Biotreatment of AMBs is the most promising eco-friendly strategy. More than 99% lincomycin is degraded by co-compost of lincomycin mycelia dregs (LMDs) and slag. However, the total abundance of ARGs increased 180 times, and the relative abundance of ARGs increased five times. Besides, the horizontal gene transfer level increased after the treatment (Ren et al., 2019). Yang et al. established a comprehensive treatment strategy consisting of 'high temperature and oxidant pretreatment' before compost and 'thermal treatment' after compost to treat tylosin fermentation dreg. Tylosin concentration in the final product was below the detection limit (< 1 mg/kg) and the ARGs disappeared (Yang et al., 2020). However, the pretreatment process needs more than 15 days with high temperature and oxidant, and the cost is high. Lincomycin manufacturing biowaste (LMB) treatment by the mesophilic bio-liquefaction strategy produced volatile fatty acids and ammonium nutrient, but little lincomycin was degraded (Wu et al., 2011). Other recycling strategies for AMB treatment were rarely reported and of great interest.

China has been the world's largest antibiotic bulk drug producer since 2007 (Zhang et al., 2015), and generates large amounts of AMBs every year. Therefore, it is necessary to develop alternative reclamation strategies for treating AMBs. For reclamation, AMBs should be pretreated to degrade or remove the residual antibiotics. Microbiological degradation is believed to be the most environmentally-friendly and cost-effective (Hurtado et al., 2016). For example, the *Klebsiella pneumoniae* Z1 strain isolated from the penicillin fermentation residue could remove 99% penicillin with the initial concentration of 300 mg/L in 24 h (Wang et al., 2015). When the initial penicillin concentrations are less than 2.0 g/L, one immobilized *Paracoccus* sp. strain could degrade all the penicillin (Wang et al., 2020). Bio-augmentation with the culture of an *Aspergillus* strain significantly increased the removal of persistent benzanthracene and benzopyrene in the soil (Silva et al., 2009). Microbiota in a constructed antibiotic treatment wetland could effectively degrade enrofloxacin and ceftiofur (Alexandrino et al., 2017). One fungal strain isolated from solid waste and gentamicin production sewage was reported to remove more than 95% gentamicin after 7-day treatment at the optimized culture conditions (Liu et al., 2016). Moreover, AMQD4, a gentamicin-degrade bacterial consortia enriched from gentamicin production biosolids could remove about 50% gentamicin in gentamicin production sewage (Liu et al., 2017a). Bioaugmented with *A. terreus* FZC3 strain, the co-composting system of gentamicin and lovastatin fermentation residues showed a rapid gentamicin degradation speed ($t_{1/2}$ = 4.4 days) and high gentamicin degradation ratio (96.7%) (Liu et al., 2017b).

Lincomycin has a stable structure and steady physiochemical properties, so it is resistant to degradation (Bertelkamp et al., 2014; Zhang et al., 2014). Physical or chemical degradation strategies, such as acidic hydrothermal treatment, photo-degradation, the coupling of solar photolysis and membrane, and photo-Fenton process (Andreozzi et al., 2006; Augugliaro et al., 2005; Bautitz and Nogueira, 2010; Wang et al., 2018b), have been reported for LMB treatment; however, microbial degradation is the most eco-friendly and cost-effective method (Hurtado et al., 2016). In this study, we isolated several lincomycin degradation strains and established the green LMB biotreatment strategy based on three isolated microbial strains. The potential application of these strains in AMB treatment was discussed.

2. Materials and methods

2.1. Chemicals and inoculum

Lincomycin hydrochloride monohydrate standard substrate was purchased from Sigma Aldrich. LMB was supplied by Tianfang pharmaceutical Co, Ltd in Henan province, China. According to the company's test results, the fermentation biowaste (dry weight) consists of 48.25% crude proteins, 11.85% crude fats, 9.82% crude fibers, 6.43% calcium, 1.22% phosphorus, 22.51% ash content, and 1117.55 mg/kg residual lincomycin. LMB was stored at 4 °C before use. The inoculation culture was sampled from the aerobic tank of lincomycin wastewater treatment plant.

Halotolerant bacterial strains of ZZU-N1 (*Bacillus subtilis*) and ZZU-N2 (*Bacillus amyloliquefaciens*) were isolated from the Qarhan Salt Lake and preserved in our Lab. These two strains cannot grow at lincomycin concentration of more than 125 mg/L. Their 16S rRNA gene sequences have been deposited in GenBank with the accession numbers of MW082827 and MT722033.

2.2. Analysis method

Lincomycin concentration in the culture broth was analyzed by high performance liquid chromatography (HPLC) with UV detector (DAD, Agilent 1100). The following steps pretreated the samples for HPLC analysis. Firstly, the samples were centrifuged at 8000 r/min for 10 min, and then filtered by 0.45-μm filter membrane to remove macromolecular particles. The collected supernatant was then acidified with borate and further diluted with methanol before denaturation and precipitation of protein via keeping it at 60 °C for 30 min. Finally, the samples were centrifuged at 8000 r/min for 10 min and filtered by 0.45-μm filter membranes. The obtained supernatant was used for HPLC analysis (Wang et al., 2018a). HPLC separation was performed with an ODS RP-C18 column (5 μm,200 mm × 4.6 mm) at 30 °C. The mobile phase running at a flow rate of 1 mL/min comprises a 40:60 mixture of methanol and borax solution (0.05 mol/L, pH 6.10). The injection volume was 20 μL, and the eluate was continuously monitored by a UV detector at 214 nm (Wang et al., 2018a). Lincomycin would be eluted at 12.0 min. The lincomycin concentration was linear with the HPLC diagram's peak area in the range from 0 to 8 mg/L, with the linear regression equation as $y = 1.1013x - 0.0203 (R^2 = 0.9997)$. Lincomycin concentration could be calculated from the peak area of the HPLC chromatogram according to the above equation.

To understand the lincomycin biodegradation mechanism, we treated lincomycin (3500 mg L^{-1} in mineral media) with LMB-A and LMB-D for three days. Then the degradation intermediates were analyzed by HPLC- (+) ESI-MS. The following steps pretreated the samples for HPLC-MS analysis. Firstly, the samples were extracted by dichloromethane to remove inorganic salts and other water-soluble impurities. The obtained organic phase was dried by air-blowing, and then the acquired powder was dissolved by methane and filtered with 0.45 μm filter membrane to remove proteins. This process was repeated three times (Wang et al., 2015). Finally, the solution was dissolved in methane for HPLC- MS analysis.

HPLC (SHIMADZU LC-30A) coupled to an ion trap mass spectrometer (ABSCIEX6600) with a Kinetex RP C18 column (2.6 μm, 50 mm × 2.1 mm) was used to determine the biodegradation products of lincomycin. The electrospray ionization (ESI) source on the positive mode was chosen for MS measurements with a capillary voltage of 5.5 kV and nebulizer temperature of 450 °C. Conditions were optimized in tuning sections to achieve the highest sensitivity. Lincomycin and its degradation products were separated with gradient elution with a flow rate of 0.3 mL/min at 40 °C. The mobile phase was a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient elution procedure was as followed: 0–3 min, 100% of A; 3–6 min, 90% of A and 10% of B; 6–9 min, 70% of A and 30% of B;

9–12 min 50% of A and 50% of B; 12–13 min, 80% of A and 20% of B; 13–15 min, 90% of A and 10% of B. Mass accuracy of recorded ions was ± 5 ppm. Mass spectra were collected in a range of 100–1000 *m/z* using the full-scan mode. Lincomycin biodegradation products were identified with the total ions chromatograph (TIC) by extracting *m/z* of potential lincomycin degradation metabolites (Wang et al., 2018a).

2.3. Enrichment, screening and evaluation of lincomycin-degradation strains

Wastewater sampled from the aerobic tank of a lincomycin wastewater treatment plant was served as inoculation culture. Sterile modified base mineral medium (BMM) with 1 g/L lincomycin was inoculated with wastewater at a 10% inoculation rate. The inoculated flasks were kept on the orbital shaker at 200 rpm and 30 °C for 24 h. Then, 10 mL enrichment broth, as mentioned above, was transferred to another 100 mL fresh BMM containing lincomycin and cultured under the same conditions. The enrichment procedure, as mentioned above, was repeated three times with lincomycin concentration increased from 1 g/L to 10 g/L gradually in the process. Afterwards, the enrichment suspension was spread on solid nutrient agar plates containing 10 g/L lincomycin, and then incubated at 30 °C for 12 h (Wang et al., 2015). The morphologically distinct isolates were selected and streaked on solid nutrient agar plates for further purification. The pure colonies were preserved on nutrient agar slants at 4 °C for further biodegradation study. One liter of BMM contained 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.025 g/L $CaCl_2 \cdot 2H_2O$, 0.0023 g/L $FeCl_3 \cdot 6H_2O$, and 0.5 g/L NH_4NO_3, with a pH value of 7.0. One liter of solid nutrient agar media contained 10 g peptone, 3 g beef extract, 5 g NaCl, and 17 g agar, with a pH value of 7.2. All the media were sterilized at 121 °C for 20 min.

Cells of studied strains were harvested by centrifugation (8000 rpm, 4 °C, 10 min) and washed twice with 0.85% (w/w) sodium chloride solution. Then the harvested cells were inoculated into BMM containing 3.5 g/L lincomycin. The inoculated media were cultured at 200 r/min and 30 °C for 48 h to evaluate their lincomycin degradation ability. All the tests were repeated three times. Lincomycin degradation ratio was calculated by the following formula,

$$Lincomycin\ degradation\ ratio(\%) = (3.5 - Cx) \div 3.5 \times 100$$

where 3.5 is the initial lincomycin concentration, and C_X represents the lincomycin concentration in BMM after being treated for 48 h.

2.4. Screening of effective LMB degradation strains

The cells of screened strains stored in nutrient agar slants were inoculated into their respective medium and cultured at 200 r/min and 30 °C, and the incubation time for bacteria, yeast, and fungi were 24 h, 48 h and 72 h, respectively. The obtained culture suspension was used as an inoculum to evaluate the strains' LMB degradation ability. The broth medium containing 10 g/L peptone, 1 g/L beef extract, 5 g/L glucose and 5 g/L NaCl, with a pH value of 7.40 ± 0.1, was used for culturing bacterial and yeast strains. The medium consisting of 20 g/L glucose and 20% (w/v) potato extract was used to culture fungal strains. All mediums were sterilized at 121 °C for 20 min.

Sterilized LMB samples were inoculated with the culture broth acquired by the procedure mentioned above and cultured steadily at 30 °C. The LMB sample prepared using the same method without inoculation were used as controls. LMB samples were weighed every 24 h within a culture period of 144 h. LMB biodegradation ratio was calculated by the following formula

$$LMB\ biodegradation\ ratio(\%) = (M_0 - M_x) \div M_0 \times 100$$

where M_0 and M_X represent the weight of the control samples and that of the biotreated samples, respectively.

2.5. Strain identification

The screened strains were identified by morphological characterization combined with phylogenetic analysis. Microbial phenotypic characteristics were determined by observing colonial and mycelia morphology by naked eyes and an optical microscope, respectively. Phylogenetic analysis was based on their 16S/18S rRNA sequences. For bacteria, the 16S rRNA gene sequence fragments were amplified with the primer pair of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Wei et al., 2015). For yeast and fungi, the 18S rRNA gene sequence fragments were amplified with the primer pair of NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS2 (5′-GGCTGCTGGCACCAGACTTGC-3′) (Liang et al., 2020; Wei et al., 2020). The PCR protocol consisted of the following steps: 5 min at 94 °C for the first denaturation step, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, and ended with a final extension step at 72 °C for 7 min. The PCR reaction mixture (20 μL) consisted of an appropriate amount of DNA template (10–100 ng), 1.0 μL Taq DNA polymerase (Beijing Com Win Biotech Co. Ltd. China), 0.5 μL of 10.0 μM each primer, and 8.5 μL of ddH_2O. The reaction mixture without template DNA was used as a negative control. The PCR products were verified using agarose gel electrophoresis and purified using the QIA Quick purification kit (Qiagen) (Zhang et al., 2014). Pure PCR products from bacterial and fungal isolates were sequenced by TsingKe Biological Technology Co. Ltd. Zhengzhou, China. Afterwards, the 16S rRNA gene sequences of strain LMB-A, and the 18S rRNA gene sequence of strain LMB-D and LMB-E were deposited in GenBank with the accession numbers of MH644857, MH644858 and MK850210. Among the three tested strains, LMB-A and LMB-E showed an excellent lincomycin or LMB biodegradation performance and potential commercial value. They have been stored in China General Microbiological Culture Collection Center with the preservation numbers of CGMCC No.15560 and CGMCC No.15400, respectively.

Multiple sequence alignments were performed with ClustalW in MEGA 6, and phylogenetic trees were constructed from the evolutionary distance data calculated from Kimura's two-parameter model using the neighbor-joining method by MEGA 6. Bootstrap analyses were performed based on 1000 random resampling. Reference sequences were retrieved from GenBank with the accession numbers indicated in the trees (Zhang et al., 2014).

2.6. Degradation of the residual lincomycin in LMB

LMB-A and LMB-D with higher lincomycin degradation efficiency were used to treat LMB. To effectively degrade the residual lincomycin in LMB, we optimized their medium components and culture conditions such as carbon source, nitrogen source, initial pH, culture temperature, liquid loading ratio, rotation speed and culture time in the trial test. The optimal medium for LMB-A was the modified YEPD 2 containing 20 g/L peptone, and 20 g/L maltose, with a pH value of 8.0. The optimal medium for LMB-D was the modified YEPD2 containing 20 g/L yeast extract and 20 g/L sucrose, with a pH value of 5.0. Their oxygen demand was similar, with an optimum liquid loading ratio of 10% and an optimum rotation speed of 200 r/min. Their optimum culture temperatures were 35 °C and 25 °C, respectively. Their optimum culture times were 24 h and 36 h, respectively.

LMB-A and LMB-D cells stored in nutrient agar slants were inoculated into their optimal media and cultured at 30 °C and 200 r/min for 24 h. The broth was then inoculated into the fresh medium with a 10% loading ratio and cultured under the optimum conditions. Microbial cells were harvested by centrifugation at 8000 rpm for 10 min and used for subsequent LMB biotreatment. Sterilized LMB inoculated with LMB-A and LMB-D cells were cultured steadily at 30 °C for 144 h. Lincomycin concentration in the samples was analyzed every 24 h by HPLC. The total nitrogen content was analyzed with the Kjeldahl method after the biodegradation process was finished.

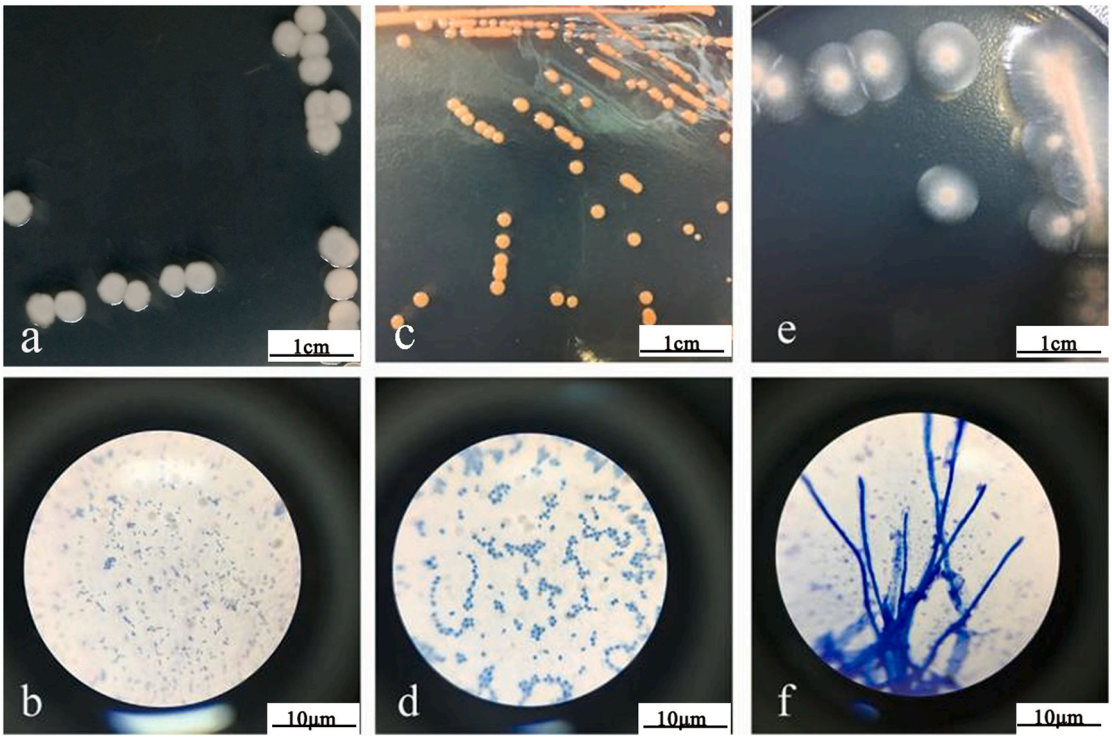

Fig. 1. Colonial morphology and microscopic structure of the strain LMB-A, LMB-D and LMB-E. (a), (c), (e) colonial morphology of LMB-A, LMB-D and LMB-E. (b), (d), (f) microscopic structure of LMB-A, LMB-D and LMB-E observed by microscope with 1000 folds amplification.

2.7. Reclamation of decontaminated LMB

LMB pretreated by the cells of LMB-A strain had a lower residual lincomycin concentration and a higher organic nitrogen content and was used for further recycling study. Decontaminated LMB was used to replace 20–100% peptone in the nutrient broth medium for culturing salt-tolerant bacteria strains at 37 °C and 200 r/min. Viable counts of the culture broth sampled at the end of the logarithmic phase (11 h) were analyzed by the plate count method. One liter of nutrient broth medium contained 10 g peptone, 3 g beef extract, and 5 g NaCl, with pH adjusted to 7.4.

2.8. Biodegradation of LMB

The mycelium of LMB-E stored in nutrient agar slants was inoculated into potato dextrose (PD) medium and then cultured at 25 °C and 200 r/min for 24 h. The broth was then inoculated into a fresh PD medium with a 10% inoculation ratio and cultured at 25 °C and 200 r/min for 72 h. Then, sterilized LMB inoculated with LMB-E culture broth (containing 3.5 g wet mycelium/mL) was cultured steadily at 25 °C for 144 h, and the biotreated LMB samples were weighted every 24 h. The LMB sample prepared with the same procedure without inoculation was used as the control.

To study the fungal mycelium's reusability, fresh LMB was supplied at the end of each batch. This process was repeated for ten batches.

3. Results

3.1. Isolation and screening of LMB biotreatment strains

Six morphologically distinct strains (LMB-A, LMB-B, LMB-C, LMB-D, LMB-E, and LMB-F) grew well on solid nutrient agar plates containing 10 g/L lincomycin (Fig. 1). They could use lincomycin as the sole carbon and energy source, suggesting that they may have the ability to degrade lincomycin. The lincomycin degradation ability of these isolates was further demonstrated with a lincomycin biodegradation study. After a 48-hour culture in BMM containing 3.5 g/L lincomycin, the six strains' lincomycin removal rates were in the range of 2.26–28.79%. The strain LMB-D had the best performance with a lincomycin degradation rate of 28.79%, and the strain LMB-A had a lincomycin removal rate of 26.45%. Additionally, the strain LMB-E could degrade LMB directly.

3.2. Identification of LMB-biotreatment strains

The strains with ideal lincomycin or LMB degradation capability were subjected to polyphasic taxonomic analyses based on their phenotypic characteristics and phylogenetic analysis (Fig. 1). Colonies of LMB-A are round and white with irregular edges (Fig. 1a), and its cells are lathy baculiform with spores of 0.4–0.8 μm wide by 1.2–2.4 μm long (Fig. 1b). Strain LMB-D has light orange round colonies with moist surface and viscous texture (Fig. 1c), and its cells are ellipse with the sizes of 2.0–2.3 μm wide by 2.3–2.8 μm long (Fig. 1d). The colony of strain LMB-E is velvety. Its initial color is white, and then its color turns carneous gradually, and the medium background of the petri dish looks dark white (Fig. 1e). Microscopy observation showed that LMB-E had septate hyphae with branched conidiophores (3.0–4.5 μm wide by 4.5–6.0 μm long), swollen phialides and unicellular conidia, with rough oval conidia forming long chains (Fig. 1f).

The 16S rRNA sequence of LMB-A exhibited 99% identity with a *B. subtilis* strain (EU882846). The18S rRNA sequence of LMB-D revealed 100% identity with a *Rhodotorula mucilaginosa* strain (MH023288). The 18S rRNA sequence of LMB-E showed 99.28% identity with a *Penicillium oxalicum* strain (SCSGAF0104). LMB-A was located in *Bacillus* clade comprising of eight strains, and the *B. subtilis* H10-5-5 strain was its closest relative (Fig. 2a). LMB-D was found in *Rhodotorula* clade with 13 *Rhodotorula* sp. strains (Fig. 2b), and *R. mucilaginosa* strain GITA 1443 was the closest strain. LMB-E was located in *Penicillium* clade (Fig. 2c), and its closest strain was *P. oxalicum* strain R1.

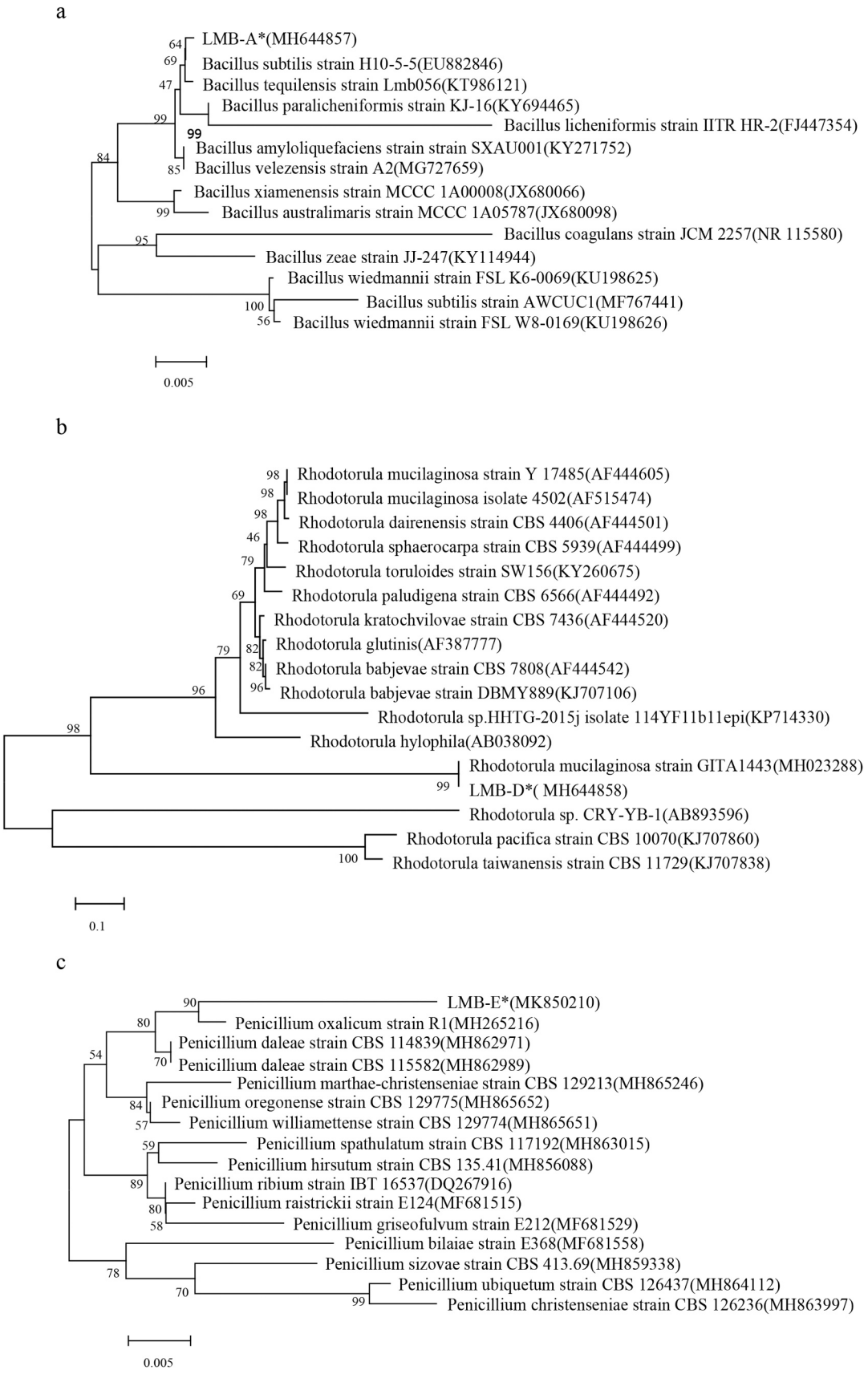

Fig. 2. Phylogenetic trees constructed by the Neighbor-Joining approach. (a) Strain LMB-A. (b) Strain LMB-D. (c) Strain LMB-E. The GenBank accession numbers of the strains are shown in the parentheses.

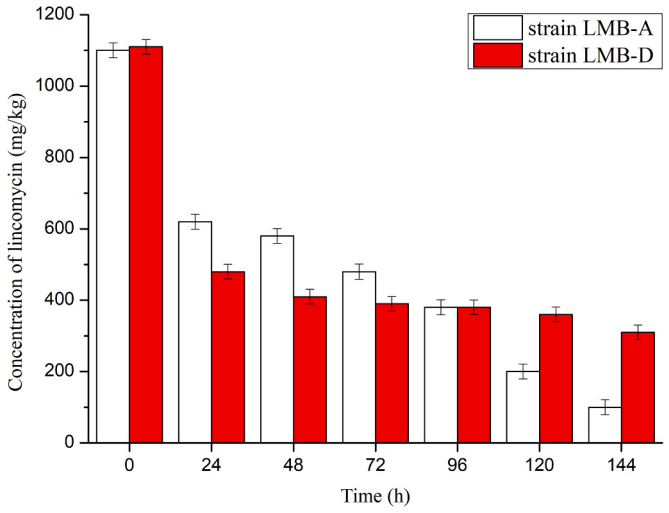

Fig. 3. Lincomycin biodegradation dynamic curves of LMB-A and LMB-D. Symbol legend: (□) LMB-A; (●) LMB-D.

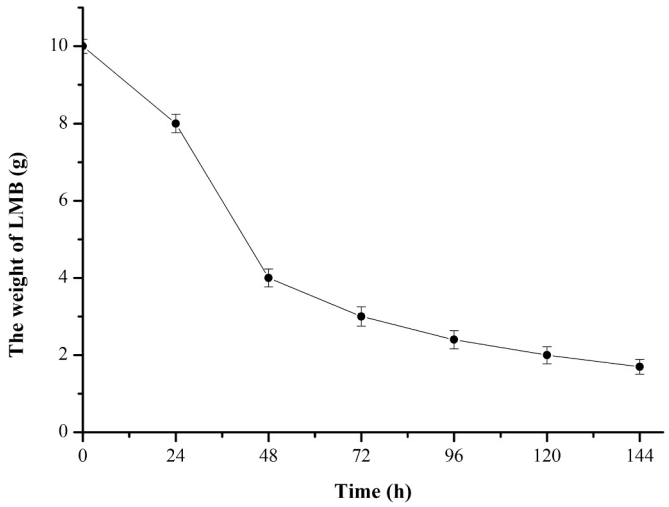

Fig. 5. LMB degradation dynamic curve of strain LMB-E.

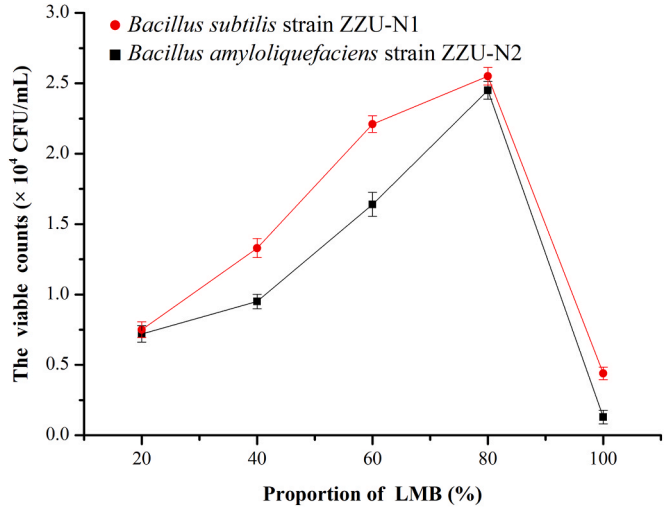

Fig. 4. Influence of decontaminated LMB proportion on the growth of halotolerant bacteria. Symbol legend: (●) *Bacillus subtilis* strain ZZU-N1; (■) *B. amyloliquefaciens* strain ZZU-N2.

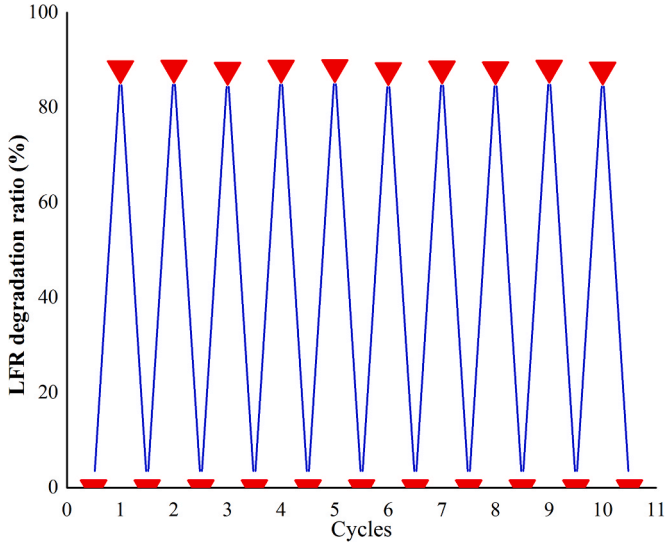

Fig. 6. Reusability of *penicillium oxalicum* mycelium in LMB degradation.

3.3. Degradation performance of lincomycin-biodegradation strains

The degradation curve of LMB-D declined faster than that of LMB-A at the initial stage (Fig. 3). After a 24-h treatment, the lincomycin degradation rate of LMB-D and LMB-A were 57.69% and 43.74%, respectively, showing that LMB-D was more efficient than LMB-A at the initial stage. However, the trend changed from the 2nd day. Although the degradation curves of both LMB-A and LMB-D declined at a smaller slope after a 24-h flattened out, the degradation curve of LMB-A descended faster than that of LMB-D, indicating that LMB-A was more effective than LMB-D with the extension of biotreatment time (Fig. 3).

Lincomycin biodegradation trends of LMB-A and LMB-D are similar. The lincomycin degradation rate was fast on the first day, and then slowed down after the second day (Fig. 3). The underlying potential reason for this trend might be that lincomycin was degraded fast by sufficient lincomycin degradation enzymes on the first day, but the degradation intermediates inhibited the enzymes from the second day. After a 144-h biotreatment, the residual lincomycin in LMB treated by LMB-A and LMB-D was 81.68 μg/g, and 289.98 μg/g, respectively, and their total nitrogen contents were 7.66% and 7.62%, respectively. For the LMB with 1117.55 mg/L residual lincomycin and 48.25% crude protein content, the lincomycin removal rates of LMB-A and LMB-D were 92.69% and 74.05%, respectively. LMB pretreated by LMB-A was selected for subsequent reclamation study because of its lower residual lincomycin concentration and slightly higher reserved nitrogen nutrition content.

3.4. Reclamation of pretreated LMB

The fermentation suspension with the highest viable counts was obtained by the halotolerant bacteria strains cultured in the 80% peptone-replaced broth medium (Fig. 4). Microbial biomass in the culture broth increased with the proportion of decontaminated LMB, except that all the peptone was replaced. The pretreated LMB might contain various trace amounts of nutritional factors. It might help the bacteria grow better in the pretreated LMB's nitrogen source than in the peptone. However, the peptone might contain necessary nutrients for bacterial growth, and it cannot be replaced entirely.

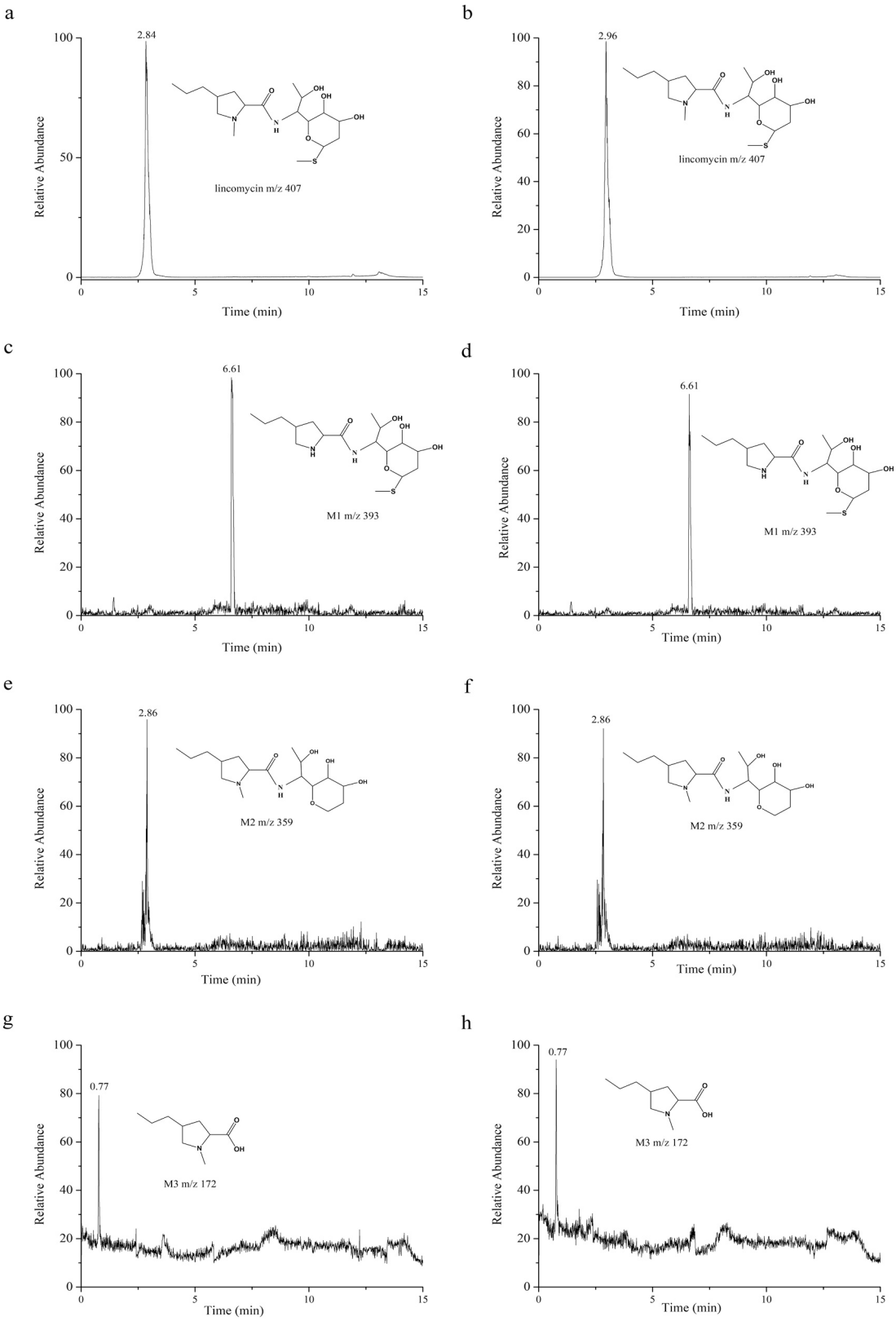

Fig. 7. LC-MS spectrum [M+H] + ions and possible lincomycin structures of lincomycin and its potential biodegradation intermediates. (a) LC-MS/MS spectrum [M+H] + ions and structures of lincomycin by LMB -A. (b) LC-MS/MS spectrum [M+H] + ions and structures of lincomycin by LMB -D. (c)LC-MS/MS spectrum [M+H] + ions and possible structure of M1 by LMB -A. (d)LC-MS/MS spectrum [M+H] + ions and possible structure of M1 by LMB -D. (e) LC-MS/MS spectrum [M+H] + ions and possible structure of M2 by LMB-A. (f) LC-MS/MS spectrum [M+H] + ions and possible structure of M2 by LMB-D. (g) LC-MS/MS spectrum [M+H] + ions and probable structure of M3 by LMB-A. (h) LC-MS/MS spectrum [M+H] + ions and possible structure of M3 by LMB-D.

3.5. Direct biodegradation of LMB

LMB biodegradation rate by LMB-E was the highest on the 2nd day (Fig. 5), with the LMB weight decreased from 8.11 g to 4.07 g at a biodegradation rate of 4.04 g/d. The LMB biodegradation rate slowed down from the 3rd day and kept almost unchanged till the sixth day (0.73 g LMB degraded every 24 h). Finally, 88.20% of LMB was degraded at the end of the 6th day. At the lag phase, fungal mycelium grew slowly on the 1st day and consumed few LMB. After entering the logarithmic phase on the second day, LMB-E grew faster and consumed LMB at a high speed. LMB-E entered a stable phase on the third day, and it consumed LMB at a slow rate.

P. oxalicum has excellent potential in pollutant degradation. It could reduce harmful effects of heavy metal, such as Cr, Pb and triclosan (TCS) (Tian et al., 2018; Ye et al., 2018). A total of 5 mg/L TCS could be degraded within 2 h, and most of the uptake took place within the first 10 min. In the degradation process, the *P. oxalicum* mycelium absorbed TCS from the initial 5 mg/L to 0.41 mg/L within the initial 10 min, and further degraded TCS to 0.05 mg/g within 60 min (Tian et al., 2018). Both biosorption and biodegradation were involved in the gentamicin degradation of fungi, for example, the fungal strain of *A. terreus* FZC3 absorbed gentamicin and subsequently degraded it (Liu et al., 2016). In this study, *P. oxalicum* may absorb LMB first, and then consume it slowly during growth. The fungal mycelium could be reused for at least ten batches with similar biodegradation efficiency (Fig. 6). At the end of each fermentation batch, the residual fungal mycelium could be used as an inoculum for the next batch.

3.6. Lincomycin biodegradation mechanism of LMB-A and LMB-D

HPLC-MS analysis results showed that lincomycin degradation products catalyzed by strain LMB-A and LMB-D were the same (Fig. 7). Molecular ions retention time of lincomycin and its degradation products (M1, M2 and M3) were eluted at 3 min, 6.61 min, 2.86 min and 0.77 min, respectively. Among the degradation products, M1 with a molecular ion (m/z) of 393 was identified as N-Demethyl lincomycin, a precursor substance in the biosynthesis of lincomycin. The lincomycin degradation step catalyzed by demethylase might be the reverse direction of the lincomycin synthetic step (Ren et al., 2019). The methyl group connected to the pyrrolidine ring might be easily removed in the degradation process, leading to the most robust response of M1 compared with M2 and M3. M2 with a molecular ion (m/z) of 359 was formed by losing thiomethyl group connected to the pyranose ring (Wang et al., 2018a). M3 with a molecular ion (m/z) of 172 was identified as 2-propyl-N-methylproline, which may be a lincomycin degradation intermediate catalyzed by amidase (Wang et al., 2018b).

4. Discussion

In this study, six strains with lincomycin degradation ability were screened. Four of them could degrade more than 19.30% of the initial 3.5 g/L LHM after a 48-h treatment. In a previous study, a *Clostridium sp.* strain degraded 62.03% lincomycin with an initial concentration of 100 mg/L after a 10-day treatment, but the degradation ratio was only 15.61% when the initial concentration increased to 500 mg/L (Wang et al., 2018a), indicating that it was not sufficient for high concentration lincomycin degradation. It has been reported that a *G. geotrichum* strain can degrade 37.35% of lincomycin with initial concentration of 5.012 ppb in the fermentation dregs after a 15-day treatment (Zhang et al., 2014). Lincomycin degradation efficiency by LMB-D and LMB-A is higher than the two strains mentioned above (Wang et al., 2018a; Zhang et al., 2014; Zhong et al., 2014).

LMB-A, LMB-D and LMB-E were assigned to *B. subtilis, R. mucilaginosa* and *P. oxalicum*, respectively. *Bacillus* and *Rhodotorula* play essential roles in removing pollutants and sustaining ecological balance (Dai et al., 2010; Gadhave et al., 2018; Zhang et al., 2008). *R. mucilaginosa* strain IM-2 could degrade acetamiprid and thiacloprid in sucrose mineral salt medium (Dai et al., 2010). *P. oxalicumis* is useful in heavy metal pollution remediation. *P. oxalicum* strain SL2 could remove Pb^{2+} with high efficiency (Ye et al., 2018). An endophytic strain *P. oxalicum* B4 isolated from Artemisia annua could degrade TCS into low toxic products effectively (Tian et al., 2018). The inoculation of isolated strains improved gentamicin degradation ability (Liu et al., 2017b), indicating that adding our isolated strains to the lincomycin wastewater treatment microbiota might augment LMB disposal effects. AMB pollutants could be decontaminated by the screened strains and then reused as a nitrogen source to culture salt-resistant bacteria, turning the waste into a resource. ARGs and horizontal gene transfer (intI1) in antibiotic-degradation strains are potential environmental risk factors. No study on such risk factors of *R. mucilaginosa* and *P. oxalicum* has been reported yet. Therefore, an in-depth research on lincomycin degradation mechanism and resistant genes of the strains should be carried out in the future. Alternatively, the safest disposal could be achieved through degraded LMB directly by strain LMB-D.

Table 1
Experimental inputs, energy consumption, cost, and product for batch test (10 cycles per test, Figs.6), 1 L *P. oxalicum* culture broth was used for LMB biodegradation. Data based on current market price (Sep. 2020) or from literature.

Materials	Flow per batch	Cost/US$ per ton
(1) Experiment inputs, energy consumption and product generated for one batch (10 cycles)		
Glucose (g)	2.5	400
Potato (g)	20	257
Peptone (g)	1	5714
Beef extract (g)	0.3	5000
NaCl (g)	0.5	107.14
Agar (g)	1.5	10,000
Treated LMB (g)	20,000	
Current alternative (disposal in hazardous site)	–	260
Electricity for 1 L *strain culture* (kWh)	2.45	0.14 (kWh)
Electricity for LMB treatment (kWh)	57.6	0.14 (kWh)
(2) Cost and product value for one batch experiment		
Categories	Cost/US$	
Chemical, etc.	0.03	
Electricity	8.4	
Avoiding landfilling as hazardous waste	–5.2	

The expense of the batch test (ten turnovers) based on the results mentioned above is presented in Table 1. Approximately, 20,000 g LMB could be treated by 1 L microbial cultures (LMB/cultures ratio volume/weight is 0.5, and ten cycles in this study), resulting in a $5.2 reduction from hazardous waste treatment. The electricity expense is $8.4 and the electricity for LMB treatment dominates the contribution. Although the batch test is not economically profitable at the lab-scale, the processing expense could be further reduced because the cost for microbial culture and LMB treatment can be significantly reduced in case of industrial-scale implementation (e.g., decrease the LMB/cultures ratio, more turnover due to the high reusability) (Liang et al., 2019, 2020; Wei et al., 2020). The process should be further optimized and a more precise evaluation for both economic and environmental aspects could be performed based on the pilot-scale test data.

5. Conclusion

Three effective LMB biotreatment strains were screened. Two of them could degrade the residual lincomycin in LMB, and the other could degrade LMB directly. After degradation of lincomycin in LBM by LMB-A and LMB-D, the treated LMB could be reused as a nitrogen source to culture useful bacteria. Besides, economic assessment demonstrated the great potential of the strains toward organic pollutant degradation. In the future, the strategies established here would be applied for the biotreatment of other antibiotic fermentation manufacturing biowastes.

Credit author statement

Hong-Min Liu and Yonghong Li contributed to the conception of the study. Luping Fu performed the experiment. Yun Wang and Xuan Li contributed significantly to analysis and manuscript preparation. Yongjun Wei and Jinfeng Tang helped perform the analysis with constructive discussions.

CRediT authorship contribution statement

Yonghong Li: Investigation, Validation, Methodology, Resources, Supervision. **Luping Fu:** Project administration, Writing-Original draft. **Xuan Li:** Data curation, Writing-Reviewing and Editing. **Yun Wang:** Data curation, Writing-Reviewing and Editing. **Yongjun Wei:** Conceptualization, Data curation, Writing-Reviewing and Editing. **Jinfeng Tang:** Conceptualization, Data curation, Formal analysis**. Hongmin Liu:** Conceptualization, Investigation, Validation, Methodology, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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