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Isolation and application of *Bacillus thuringiensis* LZX01: Efficient membrane biofouling mitigation function and anti-toxicity potential

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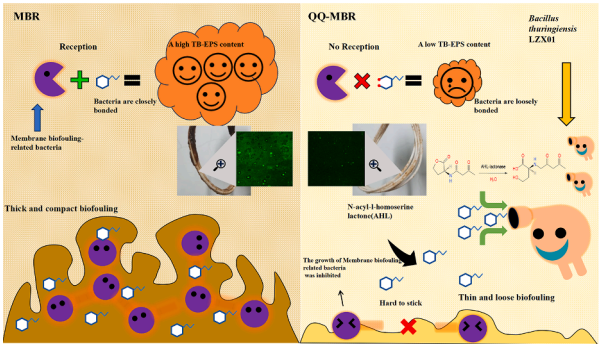
HIGHLIGHTS

- A new quorum quenching strain LZX01 was isolated and produce intracellular lactonase.
- LZX01 can rapidly degrade AHL and effectively mitigated membrane biofouling.
- LZX01 decreased activated sludge TB-EPS and enhances its hydrophilicity.
- LZX01 affected the growth of biofilm-associated bacteria but not MBR performance.
- LZX01 still has high QQ activity in the environment rich in some toxic substances.

GRAPHICAL ABSTRACT



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ABSTRACT

Significant progress has been made in mitigating membrane biofouling by microbial quorum quenching (QQ). More efficient and survivable QQ strains need to be discovered. A new strain named *Bacillus thuringiensis* LZX01 was isolated in this study using a low carbon source concentration "starving" method from a membrane bioreactor (MBR). LZX01 secreted intracellular lactonase to enable QQ behavior and was capable of degrading 90 % of C8-HSL (200 ng/mL) within 30 min, which effectively delayed biofouling by inhibiting the growth of bacteria associated with biofouling and improving the hydrophilicity of bound extracellular polymeric substances. As a result, the membrane biofouling rate of MBR adding LZX01 was four times slower than that of the control MBR. Importantly, LZX01 maintains its QQ activity even in environments contaminated with typical toxic pollutants. Therefore, with high efficiency, toxicity resistance, and easy culture, LZX01 holds great potential and significant promise for biofouling control applications.

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1. Introduction

MBR technology offers several benefits, including a high organic matter removal rate, a compact footprint, and ease of operation and management. However, the prevalent challenge in its widespread application is membrane biofouling. Membrane biofouling results from intricate physical and chemical reactions between various influent components as well as between these substances and the membrane surface (Shi et al., 2019a). During biofilm formation, microorganisms release extracellular polymeric substances (EPS) and soluble microbial products (SMP) by utilizing substrates for endogenous respiration in degraded environments or under environmental stress conditions. EPS and SMP cause membrane biofouling due to the adhesive effect on the membrane's surface including deposition, polymerization, and adsorption. The pore blockage blocks liquid filtration and reduces membrane flux (Shi et al., 2019b).

Bacteria regulate their interspecies communication through autoinducer molecules that depend on cell density, a process known as quorum sensing (QS) (Whiteley et al., 2017). QS regulates bacterial secretion of metabolites, including proteins and polysaccharides which possess adhesive properties, the process is the initial stage for biofilm formation (Weerasekara et al., 2016). Quorum quenching (QQ), as a process that disrupts the expression of QS-related genes, can effectively block the expansion of QS bacteria and thus inhibit biofilm formation (Huang et al., 2019a). At present, QQ has been applied in MBR and has effectively mitigated membrane biofouling (Kim et al., 2013). N-acyl-l-homoserine lactones (AHL) serve as a common autoinducer in MBRs, and QQ primarily focuses on degrading AHLs to disrupt cell communication. *Rhodococcus* sp. BH4 is a QQ strain that has been isolated from an MBR system and is used to control MBR's biofouling, and has been extensively studied for its effective QQ activity (Lan et al., 2021; Li et al., 2023).

Many previous studies have focused on enhancing the immobilization media for QQ bacteria (Iqbal et al., 2021; Shah et al., 2023). The material used to encase QQ bacteria serves to shield them from the complex environment, thus extending their service life. However, bacteria with QQ ability to disrupt AHLs are considered to be abundant in the natural environment, but only a few QQ strains have been used to mitigate biofouling. The degradation effect of the signal molecule AHL varies among different QQ strains and their AHL degradation effect in 30 min ranged from 30 % to 90 % (Kampouris et al., 2018; Noori et al., 2022). Isolating QQ strains with great environment adaptability and high AHL degradation effect can enhance their effectiveness for mitigating membrane biofouling in practical applications. Additionally, the application of QQ is not only limited to MBRs for domestic sewage treatment, and it can also mitigate biofouling in other filtration technologies including anaerobic membrane bioreactor, biofilters, reverse osmosis membrane (Huang et al., 2019c; Liu et al., 2019; Wang et al., 2023). This means that QQ strains need a strong environmental tolerance to cope with wastewater in a variety of environments, but few studies on the point have been conducted to date. Therefore, exploring the potential applications of isolated QQ strains can lay the foundation for their wide application.

This study aims to develop a new isolation method for quorum quenching (QQ) strains and evaluate the potential application of the new QQ strain isolated from MBRs in mitigating membrane biofouling. Different from previous studies, setting a medium with a low concentration of carbon source to isolate the QQ strain was not only cost-saving but also simple to operate, and a strain of *Bacillus thuringiensis* with strong QQ activity was effectively isolated. *Bacillus thuringiensis* is often used as a bio insecticide in agricultural production, and this work seems to have found its application prospects as a multifunctional strain. To explore more potential applications of the novel QQ strain in the field of water pollution control, the anti-toxicity of the strain was also studied, and its biofouling inhibition ability in the presence of common toxic pollutants (such as naphthalene, phenol, and toluene) in petroleum and printing and dyeing wastewater was evaluated. A lab-scale MBR system was established to evaluate the efficacy of the novel QQ strain in controlling biofouling by recording the variation of transmembrane pressure (TMP) and observing the degree of membrane biofouling. The analysis of the content and composition of SMP and EPS, along with the examination of changes in microbial community composition, was conducted at a micro level to provide a more comprehensive understanding of the macro phenomenon of membrane biofouling.

2. Material and methods

2.1. Isolation of quorum quenching (QQ) bacteria

QQ bacteria were isolated from activated sludge obtained from the aeration tank of the MBR process at the Guo Zhen sewage treatment plant in Changsha, Hunan, China. Sludge samples (5 mL) were inoculated in an LB medium and allowed to grow for 24 h. The bacterial suspensions (1 mL) were added to 20 mL of a sterile minimal medium, which contained 22 μM N-octanoyl-L-homoserine lactone (C8-HSL) as the sole carbon source. C8-HSL is a commonly found signaling molecule in sewage, and its utilization as the exclusive carbon source enables the isolating of bacteria capable of degrading it. The minimum medium (Chan et al., 2009) was utilized as a source of inorganic salts, which contained 1 g/L NaCl, 0.5 g/L KCl, 0.4 g/L $MgCl_2$, 0.1 g/L $CaCl_2$, 0.15 g/L Na_2SO_4, 2 g/L KH_2PO_4, and 2.25 g/L Na_2HPO_4. The minimal medium was adjusted to pH 5.5 using HCl, and trace elements were added at a concentration of 1 mg/L $FeCl_3$, 100 mg/L $MnCl_2$, and 46 mg/L $ZnCl_2$. After incubation for one day, 1 mL of the bacterial medium was transferred to fresh C8-HSL minimum medium to select strains capable of metabolizing C8-HSL. This process was repeated twice. Finally, the cultured bacterial solution was transferred to LB agar using an inoculation ring, and single colonies were selected and purified through plate streaking.

2.2. Strain identification

The isolated strains were cultured and DNA extraction was performed using the TSINGKE plant DNA extraction kit. Subsequently, 16SrRNA PCR was conducted using universal primers 27F and 1492R. Following successful PCR product detection, the target bands were cut for purification and recovery. The recovered products were sequenced by the Sanger method, sequencing results were spliced using ContigExpress, and the inaccurate regions at both ends were removed. After that, the BLASTn algorithm was utilized to analyze the sequences and compare them with the Nucleotide Sequence Database.

2.3. Detection of C8-HSL concentration

C8-HSL concentrations were determined using biosensor strain A. *tumefaciens* A136 (Oh et al., 2012) 10 mL of A136 bacterial suspensions were mixed with 90 mL LB agar containing Spectinomycin, tetracycline, and X-gal. After cooling and solidifying in a petri dish, 6 μL of the sample was spotted on LB agar. The medium was incubated at 30 °C for 15 h and then chromogenic reaction was observed. The concentration of C8-HSL for each sample was calculated based on the diameter of the green circle, and a standard curve was constructed using the known concentration solutions. ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was also used to quantify the C8-HSL concentration. In brief, after 5 mL of methanol and deionized water activated the SPE column, the sample was passed through the column to remove the salt from the solution, and the column was recovered with 5 mL of acetonitrile, followed by nitrogen blowing to completely volatilize the acetonitrile, redissolved with aqueous methanol ($V_{methanol}$:V_{water} = 1:1, 0.1 % formic acid), and determined by UHPLC-MS/MS. Details are provided in see e-supplementary data (Pang et al., 2023). E-supplementary data for this work can be found in the e-version of this paper

online.

2.4. Detection of quorum quenching activity

The QQ activity of the isolated strains was detected based on the degradation rate of C8-HSL. The strains were cultured for 18 h and then centrifuged at 6500 rpm for 15 min. The cell pellets were resuspended in a sterile buffer solution containing Tris-HCl until OD_{600} = 0.25 was obtained. 12.25 mL of bacterial suspension was mixed with 0.25 mL of C8-HSL (10 μg/mL) (The final concentration of C8-HSL was 200 ng/mL), and then incubated in a shaker. The samples of 1 mL were taken at intervals during incubation, and the concentration of C8-HSL in these samples was determined by UHPLC-MS/MS and the A.*tumefaciens* A136 bioassays.

2.5. Strain enzyme analysis

Bacillus thuringiensis LZX01 was inoculated in an LB medium for 18 h, and the obtained bacterial solution was centrifuged at 8000 rpm for 15 min. LZX01 was precipitated by centrifugal force, but the rotational speed was not enough to precipitate the enzyme. The QQ activity of supernatant and cell pellets was measured by the A136 bioassays to determine whether QQ enzymes are produced extracellularly or intracellularly. In order to determine whether the QQ enzyme produced by the QQ strain was lactonase or acyltransferase, the repair of the lactone ring under acidic conditions thus restores AHL activity to determine whether the lactone ring has been destroyed (Shah & Choo, 2020).

2.6. MBR setup

The activated sludge from the MBR aeration tank was acclimated for 5 days in a lab-scale MBR system. The main body of the MBR system was two 10 L aeration tanks (see e-supplementary data). After acclimation, the sludge was evenly distributed into the two reactors to ensure the same initial state. The liquid level relay controlled the metering pump according to the water level of the aeration tank to pump the influent, which was synthetic urban wastewater with the following components: 400 mg/L glucose, 14 mg/L yeast powder, 115 mg/L tryptone, 105 mg/L $(NH_4)_2SO_4$, 21.75 mg/L KH_2PO_4, 15.63 mg/L $MgSO_4$, 2.45 mg/L $CaCl_2$, 0.18 mg/L $MnSO_4$, and 256 mg/L $NaHCO_3$. Polyvinylidene Fluoride (PVDF) (Huang et al., 2019a) hollow fiber membranes (KOCH, USA) with an effective area of 0.02 m^2 and 0.03 μm nominal pore size were immersed in aeration tank, with the membrane modules connected to the effluent pipe. Membrane filtration was driven by a peristaltic pump, maintaining a constant effluent flow rate of 8 mL/min by adjusting the pump's rotational speed. The aeration tank had an air diffusion ring at the bottom, maintaining dissolved oxygen (DO) in the range of 2–4 mg/L. The aeration rate was regulated by a gas flow rotor. *Bacillus thuringiensis* LZX01 was regularly added into the QQ-MBR, the dosage was 15 mg/L·d through three continuous operation stages. The dosage of strain was referred to previous studies (Shu et al., 2022b). TMP was real-time monitored by the vacuum pressure gauge (TPI-665L, Summit, Korea), When the TMP of the two MBRs reached 40 KPa, the membrane modules were drawn out and then cleaned.

2.7. Extraction and analysis of EPS and SMP

The extraction method of EPS and SMP is referred to in the description of previous studies (Yi et al., 2023). The protein and carbohydrate in SMP and EPS were measured using the Lowry method and phenol–sulfuric acid method, respectively. To further understand the composition of membrane biofouling, the protein, and humus in SMP and EPS were analyzed by Excitation—Emission—Matrix Spectra (EEM). The EEM spectra were gathered with corresponding scanning emission (Em) spectra from 200 nm to 500 nm at 5 nm increments by varying the excitation (Ex) wavelength from 200 nm to 500 nm at 5 nm increments. The Ex and Em slits were both maintained at 5 nm, and the scanning speed was set at 4800 nm/min for all the measurements.

2.8. MBR performance test and membrane biofouling detection

Chemical oxygen demand (COD), ammonia nitrogen (NH_4-N) values in influent and effluent samples, mixed liquor suspended solids (MLSS), and sludge volume index (SVI) values of activated sludge were measured according to the standard method every two days. The biofilm attached to membrane modules was visualized using a confocal laser scanning microscope (CLSM, LSM 700, Carl Zeiss, Germany). The membrane modules were cut into sheets (1 × 1 cm) and stained with a Live/Dead BacLight Bacterial Viability kit (L7012 Live/Dead BacLight, Molecular Probes, USA) for 15 min in the dark and then observed with a CLSM after its careful washing in deionized water. The fluorescence of each membrane sheet was measured by using ~485 nm as the excitation wavelength and ~530 nm as the emission wavelength (emission1, green); ~485 nm as the excitation wavelength and ~630 nm as the emission wavelength (emission2, red).

2.9. Microbial community analysis

The activated sludge samples were taken from phase 1 of the control MBR and QQ-MBR on the 7th and 15th days for community analysis. Methods referring to a previous study (Liu et al., 2023), QJ magnetic beads were used to extract DNA from activated sludge, and the extracted genomic DNA was checked by 1 % agarose gel electrophoresis, and then PCR amplification was performed using primers 515F and 907R. PCR products were quantified using the QuantiFluor™ -ST Blue fluorescence quantification system (Promega), and were performing Illumina sequencing by sequencing platform novaseq 6000.

2.10. Test for QQ activity in toxic substance environment

Four sets of 100 mL of artificial wastewater were prepared, one of which served as a control, and the remaining three sets were added to 500 mg/L phenol, 100 mg/L naphthalene, and 100 mg/L toluene, respectively. All sets were inoculated with 20 μL of the bacterial suspension. It is important to note that naphthalene was dissolved in acetone before being added to the set. The four sets were cultured at 30 °C for 2 days, and then centrifuged and re-suspended twice. 1 mL bacterial solution was taken from each set to degrade C8-HSL, and the degradation rate of C8-HSL within 2 h was measured.

2.11. Evaluation of membrane biofouling formation

The biofilm content on PVDF membrane sheets submerged in sequencing batch reactors (SBR) was measured. The method was referred from previous studies (Huang et al., 2019b). Specifically, 8 SBRs of 200 mL with the same aeration intensity and sludge concentration were run for 7 d, with 8 PVDF membrane sheets added into the above-mentioned batch reactors, respectively. All SBRs were classified into two groups: the QQ group and the control group. In the QQ group, 5 mL *Bacillus thuringiensis* LZX01 suspension was added to three reactors, while the remaining one without LZX01 was used as a blank group. In the QQ group, 500 mg/L phenol, 100 mg/L toluene, and 100 mg/L naphthalene were each added into three different groups with LZX01. The only difference between the control group and the QQ group was that LZX01 was not added. During continuous aeration for one week, both groups of reactors were supplemented with an equal amount of nutrient solution and discharged with 10 mL of activated sludge daily. On 2nd and 5th day, three PVDF membrane sheets were drawn out from each reactor group. Initially, the samples were stained with 10 mL of 0.1 % (w/v) crystal violet for 30 min. Subsequently, the membrane sheets were rinsed with sterile normal saline to wash off the surface floating color and then immersed into 10 mL of 95 % (w/v) ethanol for 15 min to

release the crystal violet combined with biofilm. Finally, the OD_{590} of the ethanol solution was measured using an ultraviolet–visible spectrophotometer to assess the quantity of biofilm formed.

3. Results and discussion

3.1. Isolation and identification of a novel indigenous quorum quenching strain

The indigenous QQ strain capable of degrading AHL was isolated from activated sludge withdrawn from the local MBR system. In contrast to the usual isolation method, a low concentration of C8-HSL was added as the only carbon source in the culture medium. Through repeated "starving" selection, a strain capable of efficient carbon source utilization was isolated, and this method resulted in the isolation of a strain capable of rapidly degrading C8-HSL. The strain was named *Bacillus thuringiensis* LZX01 through 16S rRNA sequencing, revealing a 99.25 % sequence identity with known sequences of *Bacillus thuringiensis*. The evolutionary tree is provided in e-supplementary data. The nucleotide sequence of the QQ strain LZX01 has been recorded in the GenBank database under accession number OP714526. The morphological characterization of LZX01 was consistent with that of *Bacillus thuringiensis* screened by previous studies (Gangireddygari et al., 2017). LZX01 is a Gram-positive bacterium with rod-shaped and long chain distribution of cells, forming pale yellow colonies on agar medium (see e-supplementary data). The growth curve of LZX01 in LB medium is shown in e-supplementary data, LZX01 grown in LB medium reached the maximum population size 13 h later.

3.2. The quorum quenching function analysis of strain Bacillus thuringiensis LZX01

To confirm the ability of LZX01 to inhibit membrane biofouling, the effect of LZX01 in degrading AHL was first investigated. The degradation efficiency of LZX01 to C8-HSL was measured by two common methods to determine its QQ ability. As shown in Fig. 1, LZX01 demonstrated a high level of efficiency in degrading C8-HSL. The results of A136 bioassays showed that LZX01 degraded 87 % of C8-HSL within 30 min, and only less than 5 % of C8-HSL remained after 60 min. Additionally, the results of HPLC-MS/MS also showed that LZX01 degraded more than 94 % of C8-HSL within 30 min and almost completely degraded C8-HSL within 60 min. The results of the two methods were consistent, indicating that LZX01 had extremely high C8-HSL degradation ability (*Rhodococcus* sp.BH4 was determined for C8-HSL in a previous study, BH4 degraded 85 % of C8-HSL after 4 h, and its degradation ability was weaker than that of LZX01 (Pang et al., 2023)).

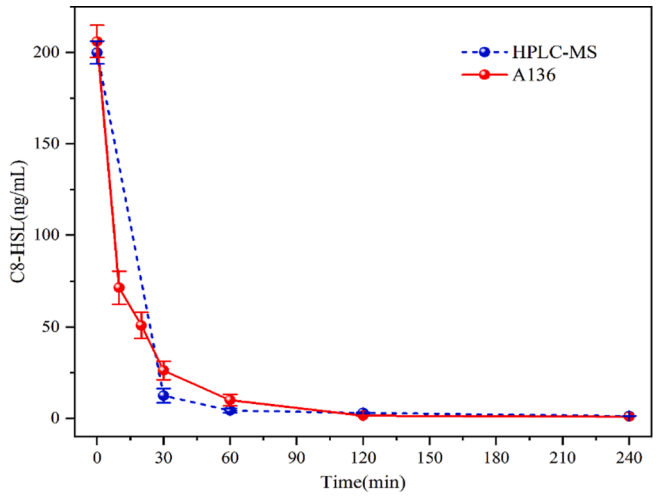

Fig. 1. Degradation of C8-HSL concentration by isolated strain LZX01 as a function of time (vertical bars denote standard deviations of n = 3).

The HPLC-MS/MS results directly show the concentration change of C8-HSL, and confirm that LZX01 achieves QQ behavior by degrading AHL. The ability of LZX01 to degrade C8-HSL can be attributed to the QQ enzyme it produces, and the QQ enzyme mechanism needs to be further elucidated. The QQ activity of the supernatant and cell pellets were measured respectively after separating the bacteria from the culture medium (see e-supplementary data). A136 bioassays showed that 90 % of C8-HSL was degraded by cell pellets within 30 min and only 10 % of C8-HSL was degraded by supernatant after 2 h. The results showed that LZX01 has an intracellular QQ enzyme and absorbs AHL into the cell for degradation, a behavior similar to the well-known QQ strain *Rhodococcus* sp.BH4 (Oh et al., 2013). Future research involving encapsulating LZX01 may require paying attention to the high permeability of the material. The use of adsorbent materials makes it easier for AHL to enter the cells, in which the intracellular QQ enzyme is more likely to play a role in significantly improve the QQ efficacy of the packaged QQ strain.

QQ enzymes have been classified into AHL-lactonase and AHL-acylase. The former reversibly hydrolyzes the lactone ring of AHL, while the latter hydrolyzes the amide bond connecting the lactone ring to the side chain, irreversibly degrading AHL. This research examined lactone ring repair after acidification (see e-supplementary data). After mixing LZX01 with a C8-HSL solution, C8-HSL was completely degraded within 2 h. Then, HCl was added to the solution for acidification, and C8-HSL was restored after 30 min, indicating the lactone ring was repaired by acidification. After 48 h, the C8-HSL concentration almost recovered to the original level. LZX01 showed AHL-lactonase activity, which aligns with the findings of previous studies, that *Bacillus thuringiensis* carries a gene fragment for AHL-lactonase (Lee Sang et al., 2002). AHL lactonase is more practical in that it can degrade a wide range of AHLs with short and long acyl chains (Ham et al., 2018), such as C8-HSL and C6-HSL, which are widely found in activated sludge.

3.3. Mitigation of MBR membrane biofouling

LZX01 can efficiently degrade AHL, but its ability to inhibit biofilm formation needs to be further evaluated. To more visually evaluate without the help of external factors the ability of LZX01 to mitigate membrane biofouling, LZX01 was added without encapsulating into the lab-scale MBR by direct injection in this study, and three cycles were designed to assess the persistence of LZX01. Two parallel MBRs were established, and the MBR with LZX01 was named QQ-MBR and the remaining one was the control. Fig. 2(e) shows trends in the increase of TMP in the two MBR groups. The TMP of QQ-MBR did not increase significantly in the first week of operation, while that of the control MBR did not have this buffer, and exhibited a rapid rise from the start. It may be that the membrane pores of the membrane modules in the control MBR were gradually blocked. The running time for QQ-MBR and the control MBR was 22 and 9 days, respectively. The LZX01 added extended the service life of the membrane by about 236 % compared to the control MBR. End of the first phase, after cleaning membrane modules continued to be put into use. During phase 2, the control MBR ran for only six days, while the QQ-MBR had a run time of ten days, extending its operational duration by 166.7 % compared to the control MBR. Notably, the mitigating effect of LZX01 on membrane biofouling during phase 2 was not as pronounced as in phase 1. During phase 3, after the membrane modules were cleaned again, biofouling increased more rapidly, and the TMP of both groups rose faster than in phases 1 and 2, indicating that repeated cleaning of membrane modules can lead to irreversible damage and greatly reduce the service life of membrane modules. This issue may stem from the fact that chemical and physical cleaning cannot restore the membrane modules to their initial state, leaving some stubborn biofouling strains remaining on the membrane surface (Li et al., 2022). The organic pollution that accumulates during

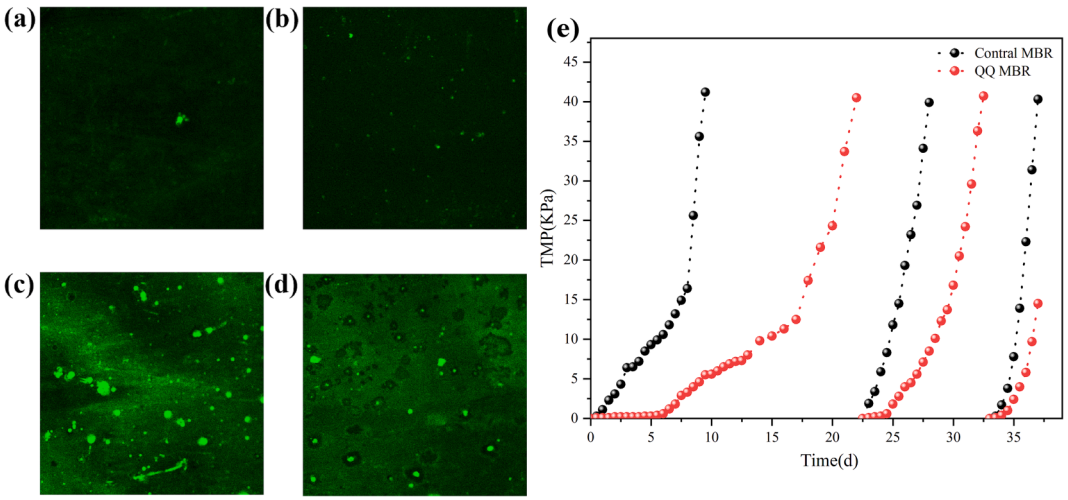

Fig. 2. LZX01 effectively controlled membrane biofouling. CLSM images of membrane modules of QQ-MBR and the control MBR (magnification: 20×). (a, b) the surface images of the membrane modules of QQ-MBR; (c, d) the surface images of the membrane modules of the control MBR. (e) TMP changes of QQ-MBR (MBR with added) and the control MBR at three phases.

the membrane filtration process becomes a source of nutrition for stubborn strains, leading to their rapid growth. However, LZX01 degraded AHL to ensure that the membrane scaling rate in the QQ-MBR remains slower than that in the control MBR during the third stage. In summary, QQ strains mainly play a role in the early stage of membrane biofouling. At the same time, when the membrane is repeatedly cleaned, it tends to get fouled faster and the ability of QQ to mitigate its fouling also weakens. However, compared to a membrane without QQ strain, its lifespan still gets extended. More importantly, prolonging the scaling time of the membrane can reduce its cleaning times, which is positive feedback.

When the TMP of the control MBR in phase 3 reached 40 KPa, the biofilm on the membranes of both groups was observed through CLSM. The pictures of CLSM are shown in Fig. 2. The membrane modules in the control MBR exhibited significantly higher biofouling compared to that in the QQ-MBR. The sludge layers attached to the membrane surface were thick and more densely distributed in the control MBR. All in all, TMP rose slowly in the early stage of pollution and then jumped after a slow rise. CLSM images also showed that thick biofilm accumulated on the surface of the membrane that reached the peak TMP, which reflected that biofilm accumulation and mud cake layer were the main factors causing membrane pollution. LZX01 effectively inhibited the formation of biofilm and delayed the rapid rise of TMP in the MBR, but the specific mechanism needs to be further explored.

3.4. Analysis of EPS and SMP in MBR

EPS and SMP secreted by microorganisms are key substances for biofilm formation. In the first phase of MBR operation, the concentration of protein and carbohydrate in EPS and SMP were measured (Fig. 3). In general, the SMP content in the control MBR was slightly higher than that in the QQ-MBR. Most of the time, however, LB-EPS in the QQ-MBR was higher than in the control MBR. This may be because the microbial content in the control MBR was higher than that in QQ-MBR, which maintained a higher concentration of mixed liquor-suspended solids (see e-supplementary data). This phenomenon was consistent with a previous study (Shu et al., 2022a). The total concentration of TB-EPS (Sum of protein and carbohydrate concentrations) in QQ-MBR decreased by 2

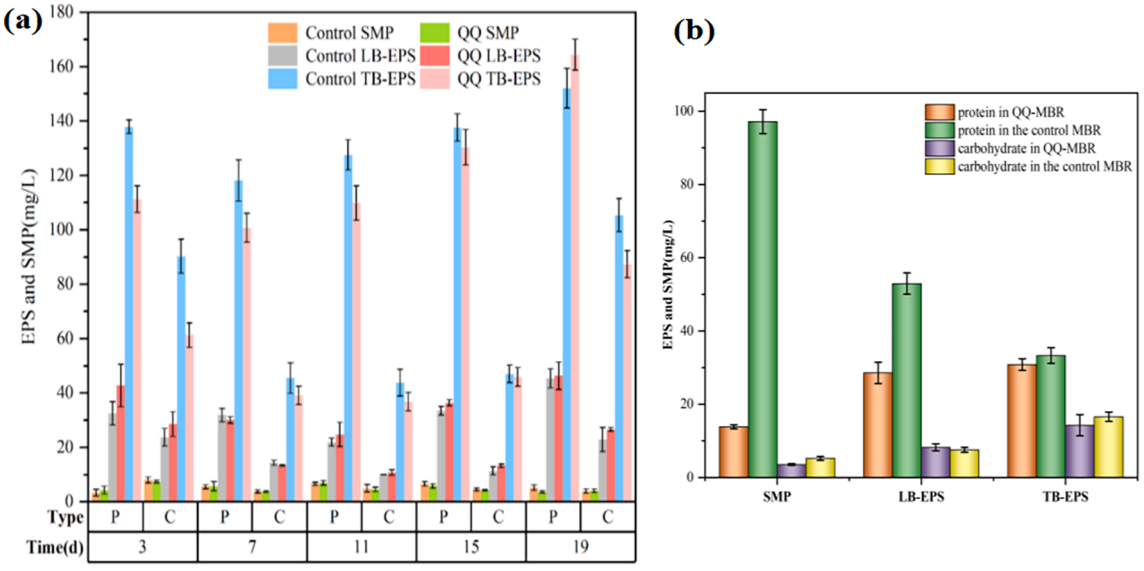

Fig. 3. SMP and EPS analysis. (a) SMP and EPS of the activated sludge in QQ-MBR and the control MBR, including concentrations of protein (P) and carbohydrate (C); (b) SMP and EPS of the membrane surface in QQ-MBR and the control MBR (vertical bars denote standard deviations of n = 3).

%–18 % compared with the control MBR. TB-EPS has a high molecular weight but also has a strong adsorption capacity, flocculation makes bacteria aggregate to form large particles, more easily bond to each other for biofilm (Zhang et al., 2014). This indicated that the mitigation of membrane biofouling by LZX01 resulted in a reduction in TB-EPS. Lower TB-EPS levels indicate reduced viscosity around the bacteria, making it challenging for them to bond with each other. Consequently, this decelerates the rate of biofilm formation. In addition, EPS and SMP concentrations on the membrane surface were measured to assess the biofilm content on each MBR membrane surface. Different from the EPS measured in activated sludge, the membrane surface of the control MBR had a high SMP concentration, which may be because the ultrasonic process dispersed part of the TB-EPS and converted it into SMP. In general, the contents of EPS and SMP in the MBR of the control group were higher than those of QQ-MBR, indicating that LZX01 inhibited the formation of biofilm on the membrane surface.

Furthermore, after the first phase of MBR operation, compositions of EPS and SMP were also analyzed by EEM to understand the mechanism of membrane biofouling mitigation (Fig. 4). Different from the SMP of the QQ-MBR, there was a small amount of humic acid (Em = 380–420) in that of the control MBR. Due to the hydrophobic attraction of humus substances, the membrane pore narrows in the early stage, which may be the reason for the rapid increase of TMP in the control MBR (Yu et al., 2020). There were four peaks (c1: Ex/Em = 225/300; c2: Ex/Em = 225/340; c3: Ex/Em = 275/300; c4: Ex/Em = 275/345) in TB-EPS of the control MBR, among them, c1 and c2 peaks are related to aromatic proteins, and c3 and c4 peaks are related to tryptophan. Peak c2 in QQ-MBR was red-shifted by 5 nm along the emission axis and peak c4 was red-shifted by 5 nm along the excitation axis and was blue-shifted by 5 nm along the emission axis. The red shift is related to the increase of carboxyl, hydroxyl, amino, and other components, while the blue shift is related to the decrease of aromatic rings and conjugated bonds. The results showed that LZX01 enhances the hydrophilicity of some structures in TB-EPS, and hydrophilic substances did not easily cause membrane hole blockage. In addition, EEM analysis results of EPS and SMP on the membrane surface (see e-supplementary data) showed that the fluorescence intensity and the humus content of QQ-MBR were lower than that of the control MBR, indicating that the biofouling on the membrane surface of QQ-MBR was lesser. It's worth noting that the peak of SMP of QQ-MBR showed an obvious red shift, which also corresponded to the conclusion that the hydrophilicity of TB-EPS in activated sludge increased (Due to the ultrasonic process, a portion of SMP is derived from TB-EPS). In summary, LZX01 inhibited the secretion of more TB-EPS by microorganisms and enhanced the hydrophilicity of protein substances in EPS, making it more difficult to form biofilms.

3.5. *Effects of Bacillus thuringiensis LZX01 on indigenous microbial communities*

The changes in the microbial community are closely linked to how well the MBR purifies water, and these changes can reflect the potential mechanism of QQ strain to mitigate membrane biofouling. Adding bacteria directly to the MBR leads to more noticeable changes in the microbial communities compared to encapsulating them. This approach allows for a clearer observation of how isolating QQ strains affects the native microbial community, a facet not previously explored in isolation studies. The Simpson index and Shannon index of QQ-MBR on day 7 were higher than that of the control MBR, which indicated QQ strain may weakened the number of dominant strains in the original MBR, making the number of different species tend to be evenly distributed. Meanwhile, the analysis of α-diversity proves that LZX01 would not destroy the microbial diversity of the original activated sludge and had no effect on the efficiency of wastewater treatment by the activated sludge (see e-supplementary data).

There was no significant disparity observed between the QQ-MBR and the control MBR regarding the composition of microbial communities at the phylum level (Fig. 5(a)), and this showed that LZX01 had no significant impact on the microbial structure of activated sludge. *Proteobacteria* and *Bacteroidetes* dominated in both MBRs, constituting approximately 70 % to 80 % of total OTU counts. After a duration of 7 days, the MBR membrane of the control group was seriously contaminated, and the relative abundance of *Bacteroides* in the MBR of the control group was more than that of QQ-MBR. After running for 15 days, the relative abundance of *Bacteroides* in QQ-MBR increased by 3.8 %,

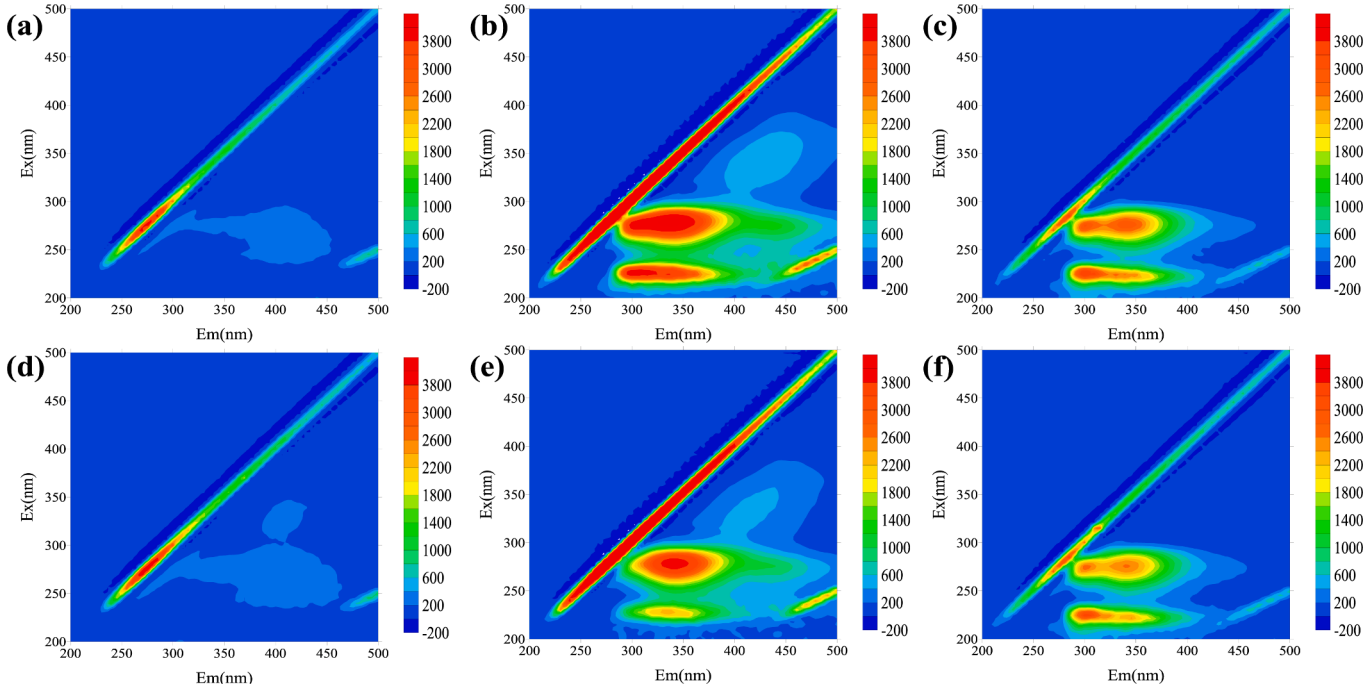

Fig. 4. EEM spectra of SMP and EPS in activated sludge. (a) SMP of QQ-MBR; (b) LB-EPS of QQ-MBR; (c) TB-EPS of QQ-MBR; (d) SMP of the control MBR; (e) LB-EPS of MBR in the control MBR; (f) TB-EPS of the control MBR.

while that of *Proteobacteria* decreased by 2.6 %. *Bacteroides* are thought to be a primary contributor to membrane biofouling by secreting extracellular proteins and adhering to the membrane surface (Ding et al., 2015; Guo et al., 2015). It can be found that *Bacteroidetes* in the two groups of MBRs increased when the membrane biofouling deepened and the microbial community structure of QQ-MBR on day 15 was similar to that of the control MBR on day 7. Interestingly, much like the TMP trend, the addition of LZX01 appeared to delay changes in certain microbial communities.

To provide a more comprehensive illustration of the dynamics of microbial community changes, the study analyzed the top 30 relative abundance genera with relative abundance in both MBRs (Fig. 5(b)). *Alysiophaera*, *Luteimonas*, and *Microscillaceae* emerged as dominant species in the sludge. *Microscillaceae* played a crucial role in the biological cake, and its secreted metabolites provided nutrition for the inner layer strains of the biological cake (Yao et al., 2021). When the TMP value peaked, the relative abundance of *Microscillaceae* reached about 5.5 % in both MBRs. However, the relative abundance of *Microscillaceae* in QQ-MBR did not increase to about 5.5 % until day 15; furthermore, on day 7, the relative abundance of *Microscillaceae* in QQ-MBR was significantly lower than that in the control MBR. This is like the pattern of microbial community changes shown at the phylum level. *Sphaerotilus* and *Leptothrix*, filamentous bacteria closely correlated with promoting early colonization of microorganisms on the membrane surface (Deng et al., 2020). On day 7, exhibited a lower abundance of *Sphaerotilus* and *Leptothrix* in QQ-MBR compared to that in the control MBR (0.7 % vs 1.2 %; 2.1 % vs 2.4 %). In addition, other bacteria that contribute to membrane biofouling have shown similar changes in relative abundance, *Saprospiraceae* is a typical membrane-fouling bacterium belonging to *Bacteroidetes* (He et al., 2022), and *Terrimonas* has strong flocculation ability and can participate in forming the skeleton of biofilm formation (Zhao et al., 2019). The relative abundance of *Saprospiraceae* and *Terrimonas* in the control MBR on the 7th day of MBR was similar to that in QQ-MBR on the 15th day. Moreover, some

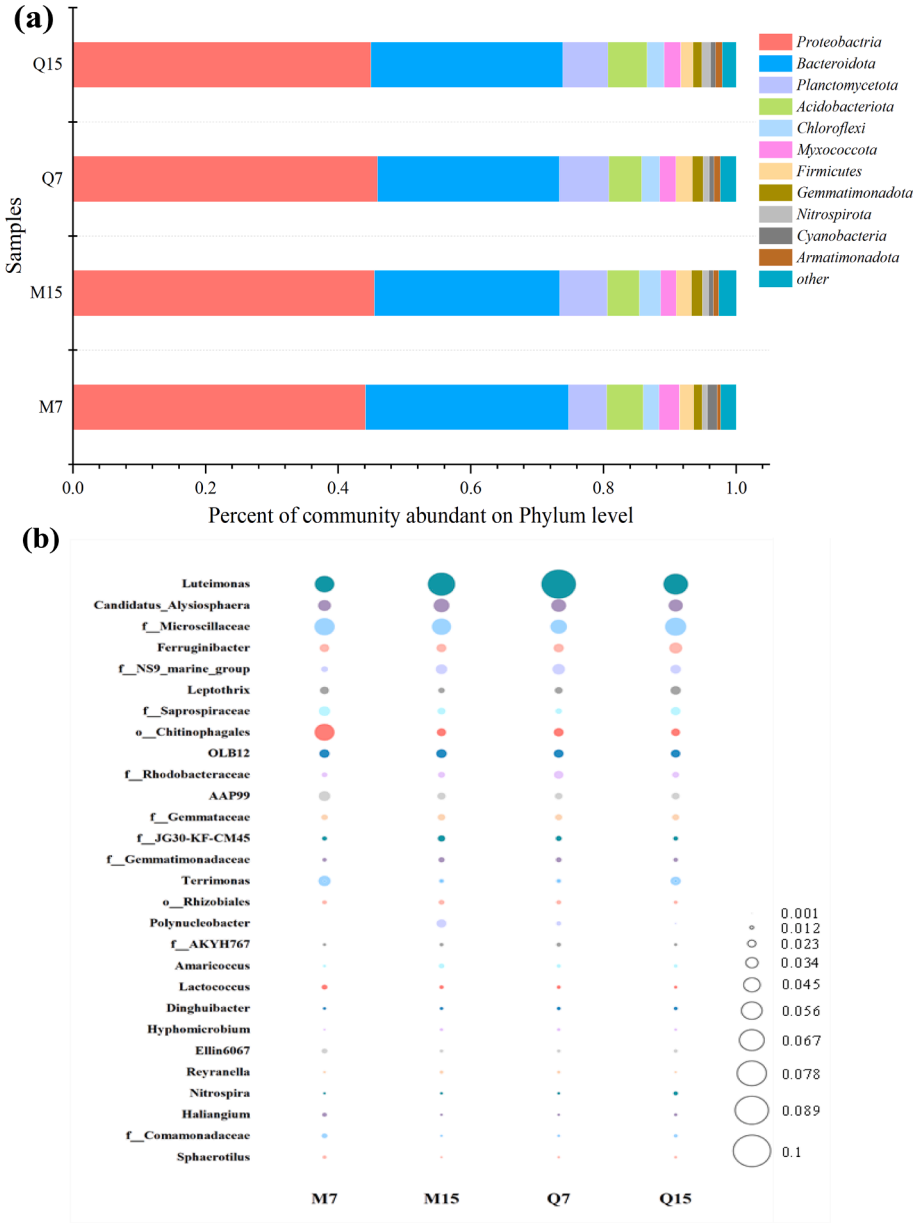

Fig. 5. (a) Microbial community composition on phylum level of QQ-MBR (Q7: QQ-MBR on the 7th day; Q15: QQ-MBR on the 15th day) and the control MBR (M7: the control MBR on the 7th day; M15: the control MBR on the 15th day); (b) Microbial community composition of QQ-MBR on genus level (Q7; Q15) and the control MBR (M7; M15) (The right circle is the relative abundance standard).

denitrification-involved bacteria were also affected by the QQ strain. *Amaricoccus* and *Nitrospira*, as typical nitrifying bacteria, showed *Amaricoccus* reduced abundance in QQ-MBR and *Nitrospira* increased abundance in QQ-MBR compared with the control group. However, the QQ-MBR still maintained a high ammonia nitrogen removal rate (see e-supplementary data).

Combined with the above analysis results of membrane contamination analysis, it is inferred that the main cause of membrane contamination is the formation of a cake layer. Abundant TB-EPS makes the microorganisms on the surface of the membrane bond to each other, and promotes the rapid growth of some biofouling-related bacteria, thus forming thick biofilm and further accumulating into cake layer. In addition, membrane pore blockage also contributed to the early stage of membrane contamination. There was humus on the membrane surface in the control MBR, and due to its strong hydrophobicity, it was easy to block the membrane pore, so the TMP of the control MBR began to rise at the early stage.

3.6. Resistance of *Bacillus thuringiensis* LZX01 to toxic environment

Compared to the simulation environment, QQ strains in practice often will encounter a harsher environment, its potential of mitigating biofouling in complex environments is closely related to the harmful material for tolerance. The effects of common toxic substances on the activity of LZX01 were preliminarily evaluated. Even in wastewater rich in three toxic pollutants, strain LZX01 exhibited strong QQ activity, as shown in Fig. 6(a). In the wastewater containing phenol, toluene, and naphthalene, strain LZX01 was able to degrade 92 %, 88 %, and 91 % of C8-HSL, respectively, within 20 min. When compared to the control group without toxic pollutants (strain LZX01 degraded 90 % of C8-HSL within 20 min), there was no significant change in the QQ activity of the strain. This demonstrates that strain LZX01 retains its QQ capability in environments with these three toxic pollutants, indicating its potential to mitigate membrane biofouling in such settings.

To further explore its application prospect in toxic wastewater, sequential batch reactors were used to simulate the generation of actual membrane biofouling. The membrane biofouling in both the QQ group and the control group is shown in Fig. 6(b, c). For the blank samples, no significant difference in membrane biofouling degree was observed between the two groups, indicating that the biofouling background of the two groups was similar. Three groups with toxic pollutants added in the control group led to a slightly lower biofouling degree compared to the blank sample, indicating that these three toxic substances have certain harmful effects on the microorganisms in the activated sludge. In the QQ group, the membrane biofouling of the three groups supplemented with strain LZX01 was significantly relieved. Compared with the three groups containing phenol, toluene and, naphthalene in the control group, the degree of membrane biofouling decreased by 11 %, 14 %, and 27 % on the second day, and by 15 %, 11 % and, 4 % on the fifth day, respectively. This indicated that the QQ strain LZX01 retained its capability to relieve membrane biofouling. In summary, the QQ activity of LZX01 remained unaffected by phenol, toluene, and naphthalene, and it still

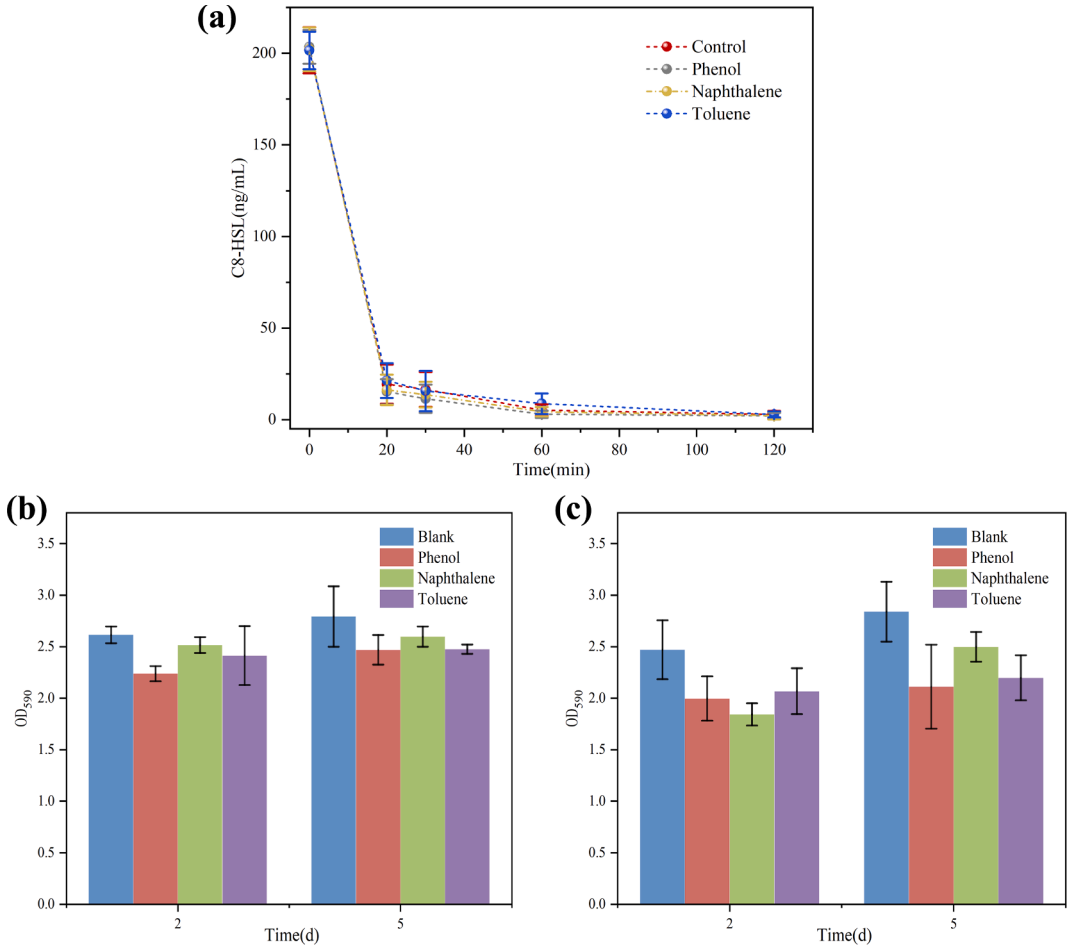

Fig. 6. QQ function of LZX01 in toxic environments rich in phenol, toluene and naphthalene. (a) QQ activity of QQ strain LZX01 in wastewater rich in phenol, toluene and naphthalene (vertical bars denote standard deviations of n = 3); Simulated biofilm mitigation experiment of QQ strain LZX01. (b) Reactors without adding strain LZX01; (c) Reactors in which strain LZX01 was added (vertical bars denote standard deviations of n = 3) (OD_{590} is absorbance, and the higher the absorbance, the more biofilm).

maintained excellent membrane biofouling mitigation effect in the sequencing batch reactor even when these three typical toxic substances were present. Therefore, QQ strain LZX01 has an excellent application prospect in the treatment of wastewater containing phenol, toluene, and naphthalene.

4. Conclusions

A novel QQ strain, *Bacillus thuringiensis* LZX01, was isolated from the actual MBR. LZX01 produces intracellular lactonase to degrade AHL efficiently, LZX01 reduced the content of TB-EPS in activated sludge, enhanced its hydrophilicity, and inhibited the growth of biofilm-associated bacteria thereby mitigating the formation of membrane biofouling. LZX01 stabilized the activated sludge state without affecting the wastewater treatment efficiency and microbial diversity of MBR. In addition, LZX01 maintains high QQ activity in an environment rich in some common toxic substances. Overall, LZX01 appears to be very promising for biofouling mitigation.

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CRediT authorship contribution statement

Zhexi Liu: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Haoliang Pang:** Writing – original draft, Investigation, Data curation, Conceptualization. **Kaixin Yi:** Validation, Software, Data curation. **Xia Wang:** Validation, Investigation. **Wei Zhang:** Investigation. **Chenyu Zhang:** Visualization. **Si Liu:** Project administration. **Yanling Gu:** Resources. **Jinhui Huang:** Writing – review & editing, Funding acquisition. **Lixiu Shi:** Writing – review & editing, 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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130272.

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