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The removal of methyl violet from dye wastewater by *Pseudomonas* sp.: the effect of extra- and intra-cellular substances

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ABSTRACT

Sorption and degradation are considered two primary modes of pollutant removal by microorganisms, and extracellular polymeric substances have been shown to play an important role in biological processes. However, the way sorption and degradation by microorganisms work to remove refractory organic pollutants and the effects of intracellular substances remain unclear. In this study, we investigated both the removal mechanisms and the intracellular substances involved in removal of pollutants by bacteria in the system of methyl violet (MV) dye pollutant removal by *Pseudomonas sp.* bacteria. The results, obtained using Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy analyses, acid-base titration and conductivity analysis, indicated that the MV was mainly adsorbed rather than degraded by bacteria. Moreover, various bacterial components were extracted and utilized for MV removal. We found that the major bacterial component involved in MV adsorption was the cell wall and membrane fraction, accounting for more than 80% of total MV removal. Tightly-bound extracellular polymeric substances accounted for about 10%, and there was no obvious influence of other components. Protein played the largest role in MV adsorption, and polysaccharides had no adsorption effect on MV. Furthermore, a possible mechanism of MV removal by bacteria is proposed.

Keywords: Biosorption; Extracellular polymeric substances; Intracellular substances; *Pseudomonas* bacteria; Methyl violet

1. Introduction

Wastewater produced by the dyestuff industry is generally characterized by large volume, deep color, complex composition, and persistence in the environment [1,2]. For instance, the half-life of the dye Reactive Blue 19 can be up to 46 years in the environment [3]. In addition, the chromaticity of dyes, which can affect human senses even at low levels, contributes to the large impact of dye wastewater [4]. Current removal methods include chemical flocculation, adsorption, filtration, advanced oxidation processes, electrochemical methods and biological methods [5]. Compared with other methods, biotreatment is regarded as cost-effective, operationally convenient, environmental friendly, and less likely to produce secondary pollution; it has therefore been widely investigated [6].

Various bacterial strains have been used in the treatment of dyestuff-contaminated wastewater. Pure bacterial strains, mixed bacterial strains or bacterial enzymes can be employed for this purpose [7]. A number of bacterial strains have been selected and used as pure strains specializing in the removal of a particular contaminant. Meanwhile, mixtures of bacteria can offer improved overall bioremediation abilities; thus, pollutants can be removed more effectively. Certain enzymes with high catalytic activity can efficiently degrade pollutants. However,

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enzyme activity is susceptible to environmental factors and may be inhibited by specific environments to some extent, especially when the enzyme is not surrounded by extracellular polymeric substances (EPS). EPS are generally defined as metabolites or autolysates attached to or around the surface of microorganisms (mainly bacteria). EPS are mainly composed of proteins, polysaccharides, and smaller amounts of nucleic acids and other substances; they protect microbial cells and provide a food source for microbial life under conditions of food scarcity [8]. Based on the strength of its association with the microorganism's surface, EPS can be classified into loosely-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) [9].

The importance of EPS in bioremediation cannot be ignored, as these are the first contact materials for microbial adsorption and degradation of pollutants. In recent decades, the functional groups responsible for adsorption and the main components of EPS responsible for pollutant removal have been extensively studied. Liu et al. [10] used titration and FTIR to verify the functional groups associated with the proton adsorption sites present on the bacterial surface and found that they include carboxyl, phosphate, hydroxyl, and a mine groups, among others. Yin et al. [11] using Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) analysis, pointed out that the EPS extracted from anaerobic ammonium oxidizing bacteria and traditional activated sludge mainly contains compounds with amino groups, whereas the EPS produced by aerobic ammonia oxidation bacteria is rich in oxygen-containing compounds. However, other researchers obtained different or even contradictory results utilizing different types of microorganisms and different EPS extraction methods. Sheng et al. [12] removed the cationic dye toluidine blue from aerobic sludge (AE-EPS) and from anaerobic sludge (AN-EPS) by EPS adsorption. Their results showed that removal from AE-EPS was more effective than removal from AN-EPS due to the larger protein content of AE-EPS combined with the high abundance of adsorption sites in protein. Li et al. [13] found that even though the amount of EPS produced from anaerobic granular sludge was smaller, Ni (II) was removed by adsorption at a higher rate using anaerobic granular sludge than using aerobic granular sludge. They concluded that the protein in anaerobic granular sludge played a major role in Ni (II) adsorption, while the polysaccharide was the main substance responsible for adsorption in the aerobic granular sludge. However, because EPS used by previous researchers working to develop real applications have been derived mostly from anaerobic/aerobic granular sludge, traditional activated sludge and mixed bacteria, the impact of intracellular substances on pollutant removal has been neglected. In addition, researchers have rarely considered the distribution of pollutants in bacteria after removal.

Hence, the objectives of this study were (1) to understand the role of adsorption and degradation on bacterial removal of methyl violet (MV), a typical cationic dye, (2) to evaluate the effect of bacterial EPS on MV removal, and (3) to elucidate the MV distribution in bacteria after treatment and to determine the relative contributions of intracellular, extracellular and cell wall and membrane components of bacteria to MV removal.

2. Materials and methods

2.1. Chemicals

All experimental solutions were prepared using deionized water. A stock solution of 5000 mg/L MV was prepared by dissolving 5 g MV (analytic grade) in 1 L deionized water. Working MV solutions were prepared by appropriate dilution, and the pH of solutions was adjusted with 1 M NaOH and 1M HCl whenever necessary.

2.2. MV removal by bacteria

500 mL of autoclaved anaerobic nutrient solution was added to a 510-mL sterilized glass bottle. The pH of the solution was about 7, and the bottle was sealed with an aluminum cap and passed into N_2 before the bacteria were added. The *Pseudomonas sp*. bacteria were screened from the aerobic tank of SiBao sewage treatment plant in Hangzhou, China [14]. Glucose was the sole carbon source and electron donor. The bacterial cultures for the experiment were divided into two groups. In one group, bacteria and MV were added simultaneously; for the other group, the MV was added after culturing the bacteria for 24 h. Bacteria were inoculated at a volume ratio of 1/100. 2 mL samples were taken from each culture and centrifuged at 8000 g to determine the MV concentration at regular intervals. The control experiment was settled without bacteria. All experiments were carried out at a constant temperature of 33°C [14].

2.3. EPS extraction, cell disruption and MV adsorption

EPS extraction: Bacteria were cultivated in 2 L bottles in a shaking incubator maintained at 33°C and 160 rpm for 24 h. The medium used for bacterial growth was composed of the following: glucose (1000 mg/L), $(NH_4)_2SO_4$ (96 mg/L), KH_2PO_4 (15 mg/L), K_2HPO_4 (10 mg/L), $MgSO_4$ (12.5 mg/L), and $FeSO_4$ (12.5 mg/L); phosphate buffer was utilized to maintain a pH of 7. The bacteria were obtained by discarding the supernatant after centrifuging at 8000 g for 10 min and washing twice with 0.05% NaCl solution. A modified heat extraction method was used to extract the LB-EPS and TB-EPS of the bacteria [15]. The bacteria were dewatered by centrifuging at 8000 g for 10 min, then were resuspended in a 15 mL 0.05% NaCl solution pre-heated to 70°C to ensure the bacteria suspension reached an immediate warm temperature of 50°C. The bacterial suspension was sheared with a vortex mixer for 1 min and centrifuged at 8000 g for another 10 min, then the LB-EPS was obtained by filtering the supernatant through 0.22 μm cellulose acetate membranes.

The TB-EPS were collected by further treating the bacteria without LB-EPS according to the following steps: 1) the bacteria without LB-EPS were resuspended again in 15 mL 0.05% NaCl solution; 2) the bacteria were heated to 60°C in a water bath for 30 min; 3) the bacterial mixture was centrifuged at 12,000 g for 30 min and filtered through 0.22 μm cellulose acetate membranes, and the supernatant was regarded as the TB-EPS. The sediment obtained, consisting of bacteria without EPS, is referred to as EPS-free bacteria.

Cell disruption: EPS-free bacteria were resuspended in 2.5 mL of phosphate buffer at pH 7, then disrupted using an ultrasonic cell disruptor in an ice water bath for 30 min (power 200 W). The intracellular substances were obtained by centrifuging the liquid at 8000 g for 5 min and filtering the supernatant through 0.22 μm cellulose acetate membranes.

MV adsorption: The bacteria and EPS-free bacteria were distributed into separate tubes for the MV adsorption experiment. A certain volume of each of the above materials was added to a 10 mL tube containing phosphate buffer, then MV solution was added to ensure that the final concentrations of MV were 25, 50, 100, 200, 300, 400 and 500 mg/L. For TB-EPS, LB-EPS and intracellular substances, a certain volume of each material was added to a 10 mL tube for a final MV concentration of 20 mg/L. The tubes were sealed in a constant temperature shaker maintained at 33°C with an oscillation speed of 200 rpm. After 24 h of adsorption, the supernatant was centrifuged to determine the MV content. The whole experiment was repeated three times.

2.4. Analysis methods

Samples were periodically collected and centrifuged at 8000 g for 10 min, then the supernatant was analyzed for the residual MV concentration using a UV-vis spectrophotometer at λ_{max} (580 nm).

The amount of MV adsorbed by bacteria at equilibrium was calculated as:

$$q_e = v\,(C_0 - C_e)/M \quad (1)$$

where q_e is the biosorbent sorption capacity at equilibrium (mg/g), C_0 is the initial dye concentration in the solution (mg/L), C_e is the liquid phase dye concentration at equilibrium (mg/L), V is the volume of solution (L), and M is the weight of biosorbent used (g).

Acid-base titration: EPS-free B. and B. were added to 40 mL of 0.01 mol/L KNO_3 solutions to prepare a suspension with a dry weight of about 580 mg/L. After maintaining the temperature at 25°C in the bacteria suspension, acid-base titration was started with 0.04865 mol/L (1.946 g) NaOH and 0.5270 mol/L (9.23 g/L) HCl solution. At the beginning of the experiment, the pH was adjusted to 2.5 with HCl, the bacteria were equilibrated for 40 min, and acid titration was carried out with NaOH. The next titration was initiated when the conductivity was stabilized at 0.1 mv/s during each titration.

Conductivity measurement: A certain amount of bacterial solution was added to 100 mL of 100 mg/L MV to give a final OD600 of 0.1 and a dry weight of about 77.5 mg/L of bacteria. The solution was shaken at 33°C, and a 1 mL sample was centrifuged to determine the change in conductivity over time during the reaction.

Analysis of fluorescence spectra of EPS and MV: 2 mL of EPS was added to 8 mL of different concentrations of MV to ensure the final concentrations of MV were 0, 5, 10, 15, 20 and 30 mg/L. The mixture was allowed to stand for 1 h and then analyzed by fluorescence spectroscopy. The excitation wavelength was settled in 280 nm with the scanning interval between 300–500 nm.

Other analytical methods: The protein content of EPS was determined by the Coomassie brilliant blue method, and the glucose content was determined by the phenol-sulphuric acid method [16].

The functional groups on the bacterial surface were tested using Fourier transform infrared spectroscopy (FTIR) (Nicolet 6700, USA). Bacterial surface elements were measured by X-ray photoelectron spectroscopy (XPS) (Escalab 250Xi, UK). The Zeta potential of the bacterial surface was measured using a nano-particle size analyzer (ZET-3000HS, UK). The morphology of the bacteria before and after the adsorption of the dye was analyzed by Bio-SEM (SU8010, Japan).

2.5. Adsorbent adsorption experiments using substances similar to bacterial polysaccharide, protein and lipid

Concentrations of 0, 0.2, 0.4, 0.8, and 1.0 mL of 100 μg/mL glucose, bovine serum albumin or lecithin were used to adsorb MV. The buffer was supplemented to ensure the final concentration of 10 mL MV was 50 mg/L. The solution was maintained on a shaker at 33°C and 200 rpm for 24 h, then was passed through a 0.22 μm filter to determine the MV concentration in the supernatant.

3. Results and discussion

3.1. MV removal by bacteria

In order to investigate the MV removal mechanism in microbial treatment, *Pseudomonas sp.* bacteria were continuously cultured for 7 d in different MV concentrations. Fig. 1a shows the concentrations of MV over 7 d of continuous culturing with bacteria; it is clear that the MV concentration decreased over time, rapidly for the first few days and then more slowly. We also investigated the bacterial content OD600 (Fig. 1b) and pH value (Fig. 1c) over time. The results showed that in the treatments with higher initial MV concentrations, a lag in bacterial growth occurred. This hysteresis phenomenon was more obvious at higher initial MV concentrations. In each treatment, the bacterial content decreased after reaching an OD600 of approximately 0.3, which directly led to changes in the rate of removal of MV. At the same time, pH values decreased from 6.95 to 6.75–6.8; the tendency was similar to the trend for MV removal. The MV concentration and pH were unchanged in the control group, in which 10 mg/L MV was added without bacteria. Furthermore, no new peaks appeared in ultraviolet-visible spectrophotometer scanning in full wave, which indicated that the bacterial MV removal we observed might be ascribed to adsorption rather than degradation. In general, microorganisms do not perform well in degrading pollutants unless they have been domesticated for this purpose, but in most cases sorption is a significant removal mechanism for recalcitrant pollutants [17].

Taking into account the inhibitory effects of MV on bacterial growth, we performed a second experiment in which we cultured bacteria for 24 h and then added different concentrations of MV. Due to the large initial

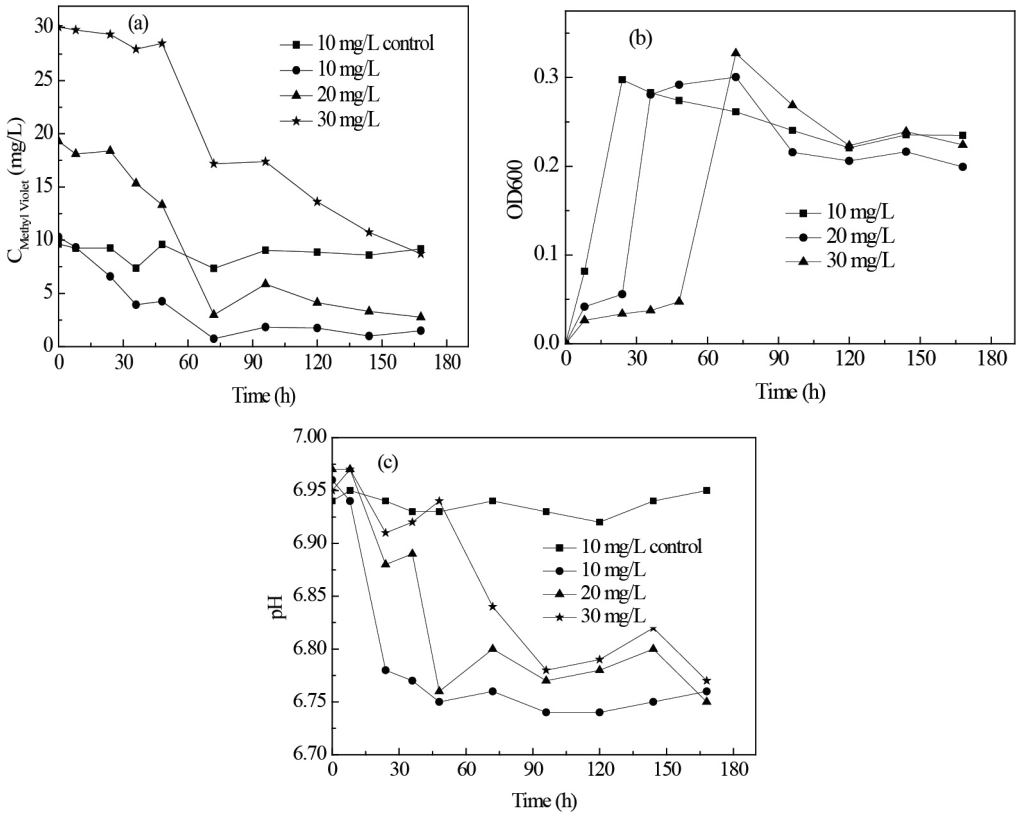

Fig. 1. MV removal experiment with bacteria and MV added simultaneously; graphs: display a) concentration of MV, b) bacterial density (OD600), and c) pH of the solution over time.

bacterial concentration, the MV adsorption was completed quickly and gradually leveled off (Fig. 2a). However, the bacteria continued to grow and fluctuate as the availability of nutrients allowed. For example, the OD600 fluctuated between 0.2 and 0.3 in a 10 mg/L MV solution (Fig. 2b). In order to remove the effect of nutrient availability, the bacterial MV absorption experiment was carried out in a buffer of pH 7. We additionally investigated the effect of EPS extracted from solutions of bacteria on MV removal. MV adsorption by EPS-free bacteria and bacteria is shown in Fig. 3. It is apparent that the amount of MV adsorption on the bacteria was greater than on the EPS-free bacteria.

Langmuir [18] and Freundlich [19] isotherm models were used to describe the batch sorption experimental data. The Langmuir equation is based on the assumption that the maximum adsorption corresponds to a saturated mono layer of adsorbate molecules on the adsorbate surface with a constant energy, and there is no transmigration of adsorbate in the plane of the adsorbate surface. The saturated mono layer isotherm can be represented as:

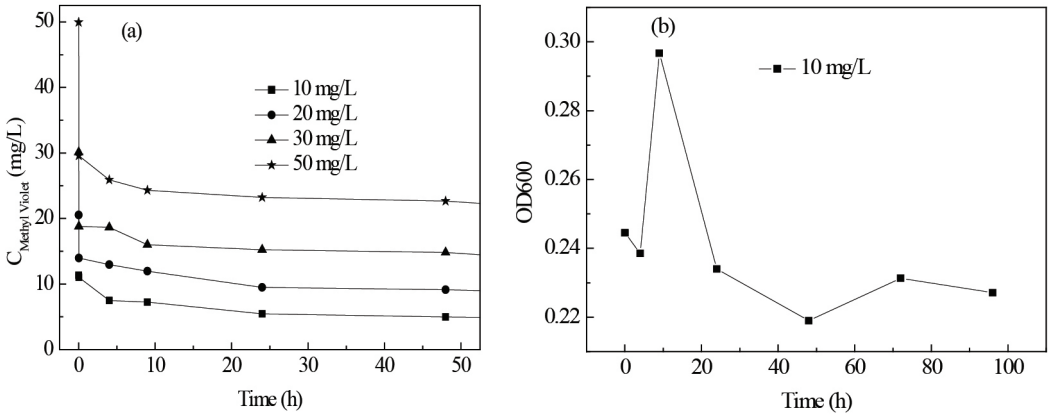

Fig. 2. MV removal experiment with MV added after 24 h of bacterial culture; graphs display a) concentration of MV; b) bacterial density (OD600).

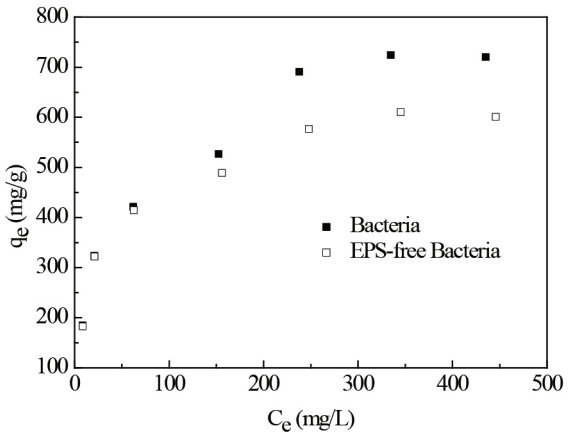

Fig. 3. Adsorption isotherms for MV removal by bacteria.

$$q_e = \frac{K_L C_e}{1 + a_L C_e} \tag{2}$$

A linear form of this expression is:

$$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e \tag{3}$$

Meanwhile the dimensionless expression is

$$R_L = \frac{1}{1 + a_L C_0} \tag{4}$$

where the K_L ($L \cdot g^{-1}$) is the Langmuir equilibrium constant and a_L ($L \cdot mg^{-1}$) is the Langmuir constant related to the energy of adsorption. The K_L/a_L gives the theoretical monolayer saturation capacity, Q_0 ($mg \cdot g^{-1}$).

The Freundlich equation is the earliest relationship for describing the adsorption equation. The isotherm can be used for nonideal adsorption on a heterogeneous surface as well as multilayer sorption and is expressed as the following equation:

$$q_e = K_F C_e^{1/n} \tag{5}$$

A linear form of this expression is:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \tag{6}$$

where q_e ($mg \cdot g^{-1}$) and C_e ($mg \cdot L^{-1}$) are the amount of the dye adsorbed per unit weight of the adsorbent and the amount of dye left in solution at equilibrium, respectively. The K_F ($mg^{1-1/n} \cdot L^{1/n} \cdot g^{-1}$) is the Freundlich constant and n ($g \cdot L^{-1}$) is the Freundlich exponent.

Compared with the Freundlich isotherm, adsorption of MV by both materials was better fitted with the Langmuir adsorption isotherm; R^2 could reach more than 0.99 (Table 1). The maximum mono layer adsorption capacities of bacteria and EPS-free bacteria were 769 and 625 mg/g, respectively. Wei et al. [20] reported that untreated bacteria were more effective than EPS-free bacteria for cadmium adsorption; they indicate that this is likely because the removal of EPS from bacteria only decreased the amount of adsorption binding sites and did not change the functional groups on the bacterial surface. Furthermore, we found that the Zeta potentials of these two materials were –26 ± 0.6 mv (bacteria) and –24 ± 0.7 mv (EPS-free bacteria) (Table 2). The Zeta potential was increased slightly after the EPS was extracted; this result was similar to the findings of Tsuneda et al., who reported that EPS could decrease the negative surface charge density of the cell surface [21]. MV is a cationic dye that has a positive charge after hydrolysis and is easily absorbed by materials with negative charge on the surface. Therefore, the lower the electro negativity on the bacterial surface, the more effective the bacteria are for MV adsorption. Khunjar et al. [22] attributed this phenomenon to electrostatic interactions. They investigated the ability of four kinds of biomasses (pure ammonia oxidizing bacteria, two kinds of intensive heterotrophic bacteria with different degrees of oxygenase activity, and full nitrification activated sludge (NAS) bacteria) to remove pollutants, including Pakistan imipramine (CBZ), iodine the general a mine (IOP), trimethoprim (TMP) and 17a-ethinylestradiol (EE2). They found that the correlation between Zeta potential and EE2 removal ability was not significant, but the relationship with TMP removal ability was relatively large, which indicated that biomass adsorption of TMP was mainly influenced by electrostatic interaction. Moreover, the adsorption removal rate of EE2 and TMP by biomasses was decreased by about 50% when the EPS was extracted.

We also compared our adsorbents with those investigated by other research teams for dye removal. The results, listed in Table 3, show that the dye adsorption capacities of our bacteria fall near the middle of the range covered by these adsorbents.

Table 1
Parameters for adsorption isotherm of MV by bacteria

Isotherm models	Constants	Adsorbent	
		Bacteria	EPS-Free bacteria
Langmuir	R_L^2	0.994	0.998
	K_L (L/g)	18.52	22.22
	a_L (L/mg)	0.019	0.036
	Q_0 (mg/g)	769	625
	R_L	0.077–0.624	0.053–0.529
Freundlich	R_L^2	0.984	0.970
	K_F ($mg^{1-1/n} \cdot L^{1/n}/g$)	101.86	116.68
	n (g/L)	2.98	3.52

Table 2
Zeta potentials of Bacteria and EPS-free bacteria

Sample	Zeta potential (mv)
Bacteria	–26 ± 0.6
EPS-Free bacteria	–24 ± 0.7

Table 3
Comparison of maximum dye adsorption capacities of various adsorbents

Adsorbent	Adsorbate	q_{max}(mg/g)	Reference
A. odoratissimus peel	Methylene Blue	184.6	23
	Methyl Violet 2B	137.3	
A. odoratissimus leaves	Methyl Violet 2B	139.7	24
NaOH-modified *A. odoratissimus* leaves	Methyl Violet 2B	1004.3	24
Breadfruit peel	Methyl Violet 2B	222	25
Breadfruit core	Methyl Violet 2B	307	25
Pu-erh Tea powder (40 mesh)	Methyl Violet	277.8	26
Peach gum	Methylene Blue	298	27
	Methyl Violet	277	
Magnetic GO	Methylene Blue	188.32	28
mGO/PVA-CG	Methylene Blue	270.94	28
MION-DMA	Congo Red	292.74	29
EPS of *Proteus mirabilis* TJ-1	Basic Blue 54	2005	30
Dekkera bruxellensis	Sulfur Black 1	527	31
Rhizopus oryzae	Sulfur Black 1	1107	31
Activated sludge	Maxilon Red BL-N	123.2	32
Pseudomonas sp. bacteria	Methyl Violet	769	This Work
Pseudomonas sp. bacteria without EPS	Methyl Violet	625	This Work

3.2. XPS and FTIR analysis of MV adsorption by bacteria

The molecular formula of MV is presented as $C_{25}H_{30}N_3Cl$. In order to characterize the MV adsorption behavior of the bacteria, two samples were freeze-dried and analyzed by XPS (Fig. 4). This analysis showed that abundant functional groups which existed on the surfaces of the bacteria were almost unchanged after MV adsorption. An elemental analysis is shown in Table 4. The results show that the nitrogen-to-carbon ratio (N/C) and the oxygen-to-carbon ratio (O/C) were lower in EPS-free bacteria than in bacteria, and the ratios were reduced even further after MV adsorption. Specifically, N/C and O/C decreased to 0.054 and 0.304, respectively, in the 50 mg/L MV treatment (MV50). The more MV was adsorbed, the smaller the ratios we observed. That is mainly because the MV has lower nitrogen content than the bacteria and no oxygen.

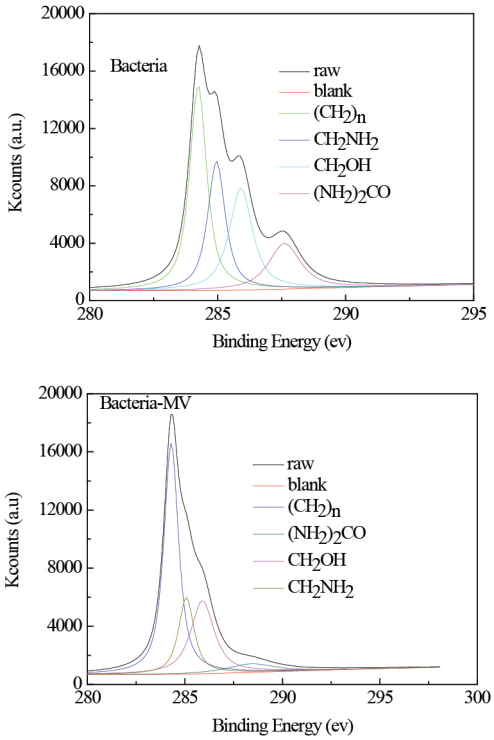

Fig. 4. Results of XPS analysis of bacteria with and without MV.

Table 4
Elemental proportions for bacteria with or without MV

Sample	Bacteria	EPS-Free bacteria	Bacteria-MV_{10}	Bacteria-MV_{50}
N/C	0.117	0.062	0.059	0.054
O/C	0.403	0.367	0.318	0.304

FTIR spectra of bacteria before and after the adsorption of dye are presented in Fig. 5. The functional groups from EPS-free bacteria were quite similar to those from bacteria. The peaks at 1550–1560 cm^{-1}, which could be due to stretching vibration of C-N and N-H groups in protein peptide bonds, showed obvious changes with the increase of MV concentration from 10 to 50 mg/L. Another peak at 1130–1160 cm^{-1}, caused by stretching vibration of C-O-C groups in polysaccharides, was nearly unchanged [33]. The analysis of the FTIR confirmed that MV adsorption was mainly ascribed to proteins rather than polysaccharides.

3.3. Conductivity change in the process of MV adsorption

MV can be ionized in solution. If electrostatic interactions occur between bacteria and this dye, the conductivity should change when bacteria are added to a MV solution [34]. Therefore, we investigated the conductivity of an MV solution before and after adsorption to bacteria. As shown in Fig. 6, conductivity changes in an MV solution were not obvious in the bacteria-free control. Conductivity decreased rapidly as soon as the bacteria were added to

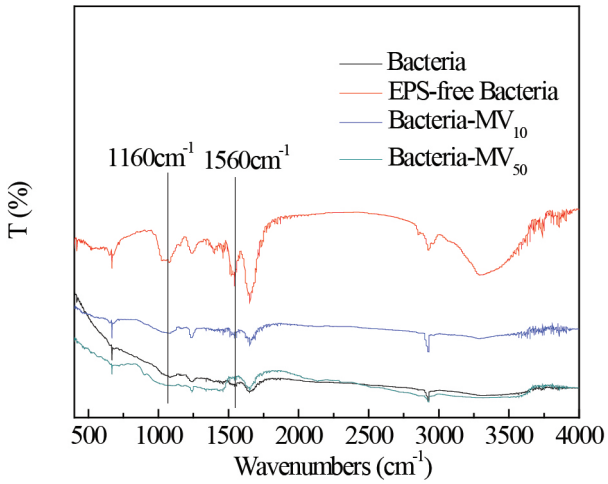

Fig. 5. Results of FTIR analysis of bacteria with and without MV.

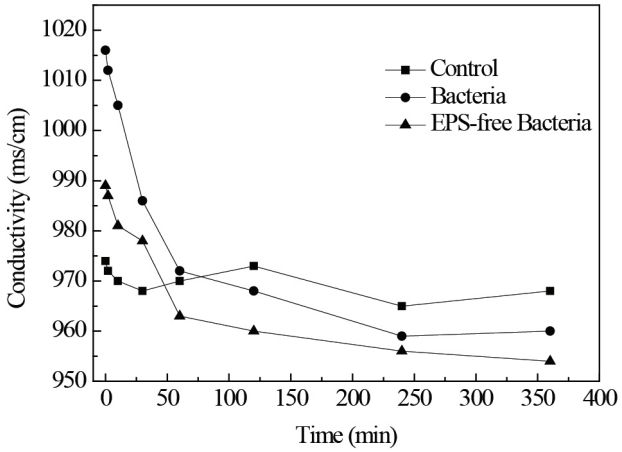

Fig. 6. Solution conductivity over time after addition of bacteria to a MV solution.

the MV solution, then slowed down. This process was very similar to MV removal. Compared with EPS-free B, the addition of B. decreased conductivity to a greater extent. These results are consistent with the conclusion that B. can adsorb more MV.

3.4. Potentiometric titration analysis of bacterial EPS extraction

Fein et al. [35] believed that potentiometric titration can determine the acidity constant and obtain the concentration of adsorption sites on the surface of bacteria. The potentiometric titration curve we generated shows that the acid-base buffering performance of the bacteria was reduced after EPS extraction (Fig. 7). The same conclusion was reached by Wei et al. [20] when they extracted EPS of *Bacillus subtilis* and *Pseudomonas putida* bacteria. The pKa value obtained from this potentiometric titration curve (Table 5) changed slightly after EPS extraction. Furthermore, the bacteria had plentiful functional groups such as carboxyl ($4 < pKa < 6$), phosphate ($pKa = 7$) and hydroxy acid ($9 < pKa < 11$). This is similar to the results reported by other research teams [36,37]. Bacteria containing rich functional

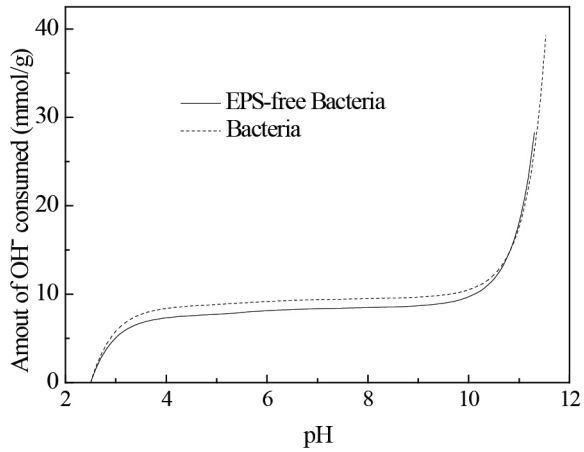

Fig. 7. Potentiometric titration curve for bacteria and EPS-free bacteria in the presence of 0.01 M KNO_3 at 25°C.

Table 5
pKa values for bacteria and EPS-free bacteria

Samples	pKa	Functional groups
Bacteria	4.03	Carboxylic ($4 < pKa < 6$)
	6.81	Phosphoric ($pKa \approx 7$)
	10.41	Hydroxy acids ($9 < pKa < 11$)
EPS-Free bacteria	4.11	Carboxylic ($4 < pKa < 6$)
	6.90	Phosphoric ($pKa \approx 7$)
	10.52	Hydroxy acids ($9 < pKa < 11$)

groups on their surfaces are useful for the adsorption of pollutants, especially cationic pollutants. This explains why the bacteria were effective in absorption of MV.

3.5. Fluorescence analysis of EPS after binding with MV

It is generally believed that EPS contain relatively rich substances such as carbohydrates, proteins, humic acids and so on. Many researchers have investigated applications of extracted EPS in the field of pollutant treatment. We investigated the interaction between MV and TB-EPS or LB-EPS by fluorescence spectroscopy. Fig. 8 shows fluorescent substances assigned to protein-like (PN-like) substances were richer in TB-EPS than in LB-EPS. Moreover, fluorescent substances in both types of EPS decreased with increased MV concentration; these results reveal that fluorescent substances in EPS interact with MV, causing quenching of fluorescence. The fluorescence we observed was mainly associated with protein, so these results could reflect an important role of protein in MV adsorption.

EPS are typically composed mainly of polysaccharides and proteins [37,38]; thus, we focused mainly on these two components. As shown in Fig. 9, the EPS secreted by bacteria was increased with increasing MV concentration. The results also demonstrated that the contents of both polysaccharide and protein were higher in TB-EPS than in LB-EPS. The results are potentially also supported by our SEM analysis (Fig. 10), which shows obvious filamentous

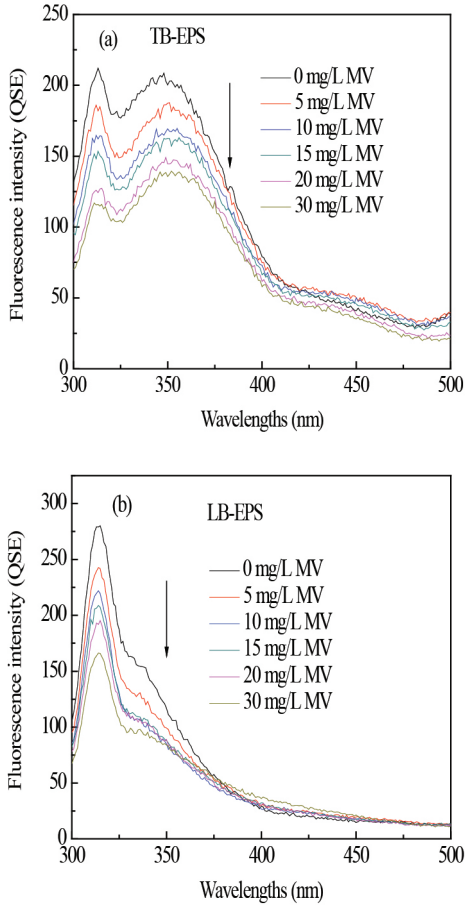

Fig. 8. Fluorescence spectra of the interaction between MV and a) TB-EPS; b) LB-EPS.

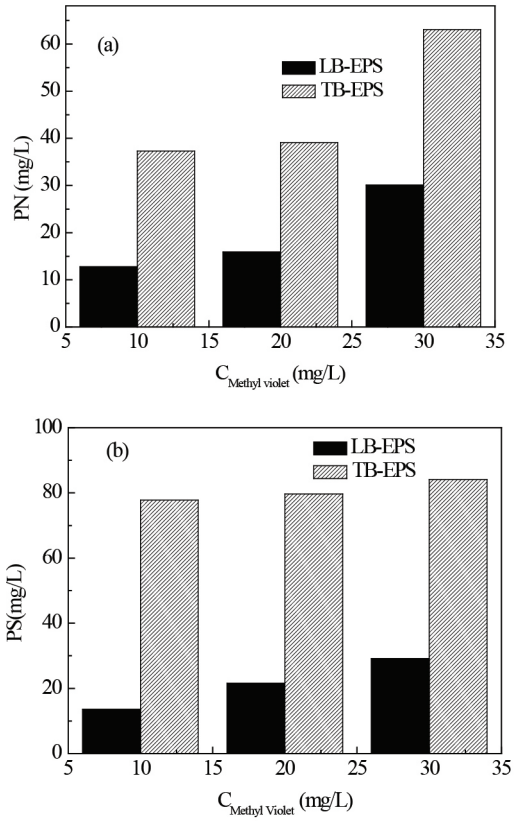

Fig. 9. Effect of MV concentration on bacterial secretion of EPS components (a) protein (PN); (b) polysaccharide (PS).

substances, which may be EPS or adsorbed dye, surrounding the bacteria in the presence of MV.

3.6. The distribution of MV in the bacteria

In general, macromolecular substances have difficulty entering cells and are mainly adsorbed on the surfaces of cells. The surfaces of bacteria, which have large functional groups and complex structures, needed to be simplified in the analysis process. Many researchers engaged in research on pollutant removal by bacteria have not separated the cell wall from the cell membrane, either because separating them requires complex methods or because the distribution of contaminants between these two structures was not relevant in the context of their experiments. In fact, Zhang et al. [39] proposed that adsorption of pollutants on the surfaces of bacterial cells can be attributed solely to the extracellular portion. Wei et al. described EPS and the bacterial cell wall as an outer envelope that shields the cell surface from pollutant exposure [20]. However, we isolated the cell wall and cell membrane together in this paper. We divided the bacterial cells into three components: EPS, cell walls and membranes, and intra-cellular substances. We investigated the contribution rate of each component to the removal of different concentrations of MV.

The adsorbed MV could be almost completely desorbed (above 98%) with 0.1 mol/L HCl in our primary experiment. Therefore, subsequent desorption was carried out using this desorbent. Fig. 11 shows the distribution of MV in bacterial cells. The results show that the cell wall and membrane component was mainly responsible for MV removal, accounting for more than 80% of the total MV removed. At the same time, TB-EPS contributed about 10% of the MV removal, while the contributions of LB-EPS and intracellular substances to MV removal were very small and could be neglected. However, this does not mean that intracellular substances have no effect on MV adsorption. The prerequisite for MV adsorption by intracellular substances is their ability to contact them. However, this process is hampered by EPS as well as cell walls and membranes. Liu et al. [40] pointed out that even though the genetically engineered bacterial strain *Escherichia coli YB* can express a high level of azo reductase in the cell, its azo dye decolorization effect is lower than that of two photosynthetic bacteria. Due to the lack of a transport system, the azo reductase protein cannot be transferred from the intra-cellular to the extracellular environment to decolorize the azo dyes.

In order to verify the reliability of the above results and investigate the adsorption of MV by intracellular substances, the impact of LB-EPS, TB-EPS, intracellular substances and cell wall and membrane on MV removal were investigated. The results, as shown in Fig. 12, show that the adsorption removal effects of the above substances

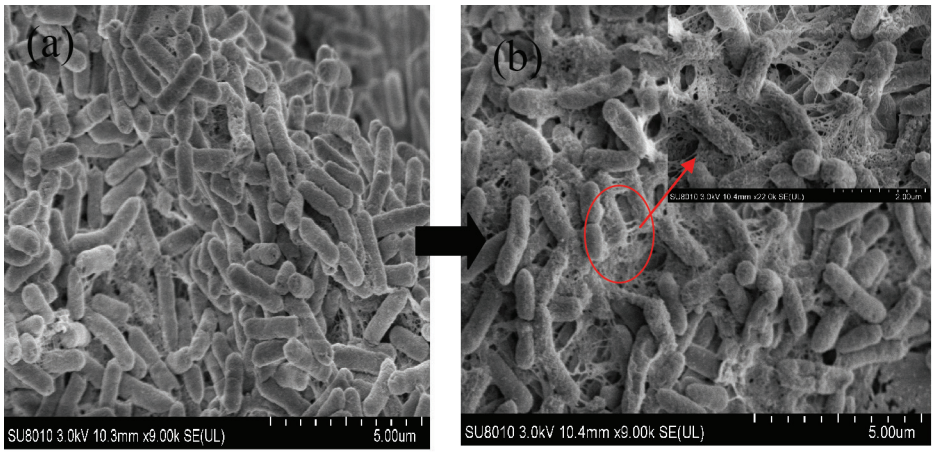

Fig. 10. SEM images of bacteria in the presence of a) 0 mg/L MV; b) 50 mg/L MV.

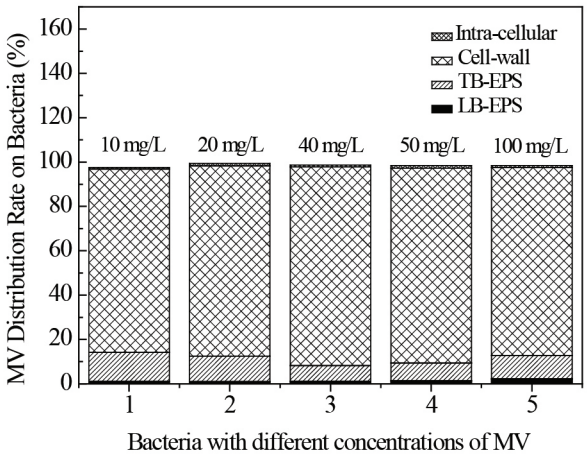

Fig. 11. Distribution of MV on bacteria.

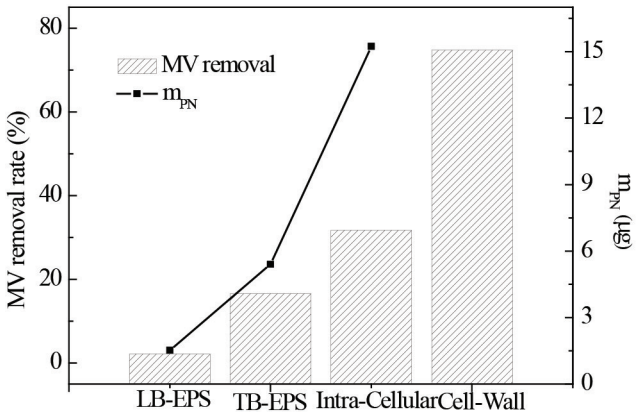

Fig. 12. Adsorption of MV by different extracted bacterial components.

were increased in turn. The adsorption effect of intracellular substances was significantly higher than that of EPS, which confirmed the prediction that the intracellular substances can effectively adsorb MV. Moreover, compared with the content of protein in the first three kinds of extracted substances, it was found that the protein content of the above four bacterial components increased in sequence. Intracellular substances were conducive to the adsorption of MV after cell disruption and the outflow of intracellular proteins.

Some research teams have utilized other substances [41] to simulate the polysaccharides, proteins and lipids produced by bacteria to make a simplified model of bacteria secretions. Similar substances were used in this paper to verify their effect on MV adsorption. Glucose was used in place of polysaccharides, bovine serum albumin was used instead of bacterial proteins, and lecithin was used to mimic bacterial lipids for the adsorption of MV. As expected, MV adsorption was mainly affected by the protein: the higher the protein content, the better the MV adsorption. The effectiveness of lecithin for MV adsorption was poor, and polysaccharide had basically no adsorption (Fig. 13). This result was the same as we observed with MV adsorption by bacterial extractives.

3.7. A possible mechanism of MV removal by bacteria

We propose a possible mechanism of MV removal by bacteria in Fig. 14. In this mechanism, the MV dye, which

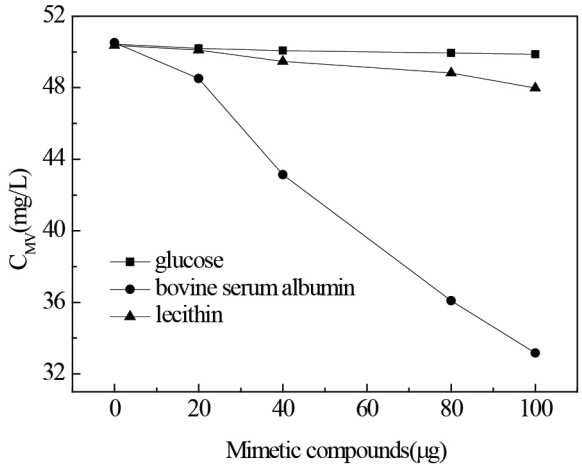

Fig. 13. Adsorption of MV by mimetic compounds.

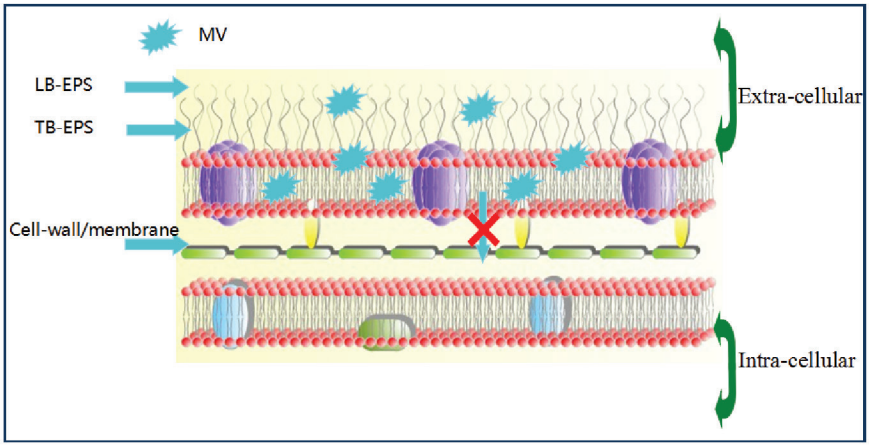

Fig. 14. Schematic diagram of proposed interactions between a bacterial cell and MV.

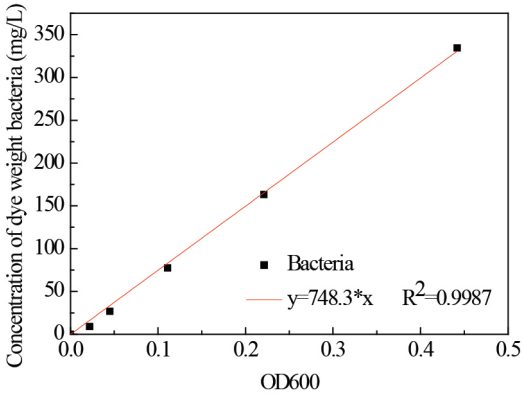

Fig. 15. The curve of OD 600 versus bacterial concentration.

hydrolyzes into positively charged ions, is first adsorbed by EPS when it comes in contact with bacteria. The contribution of TB-EPS to MV removal is much greater than that of LB-EPS. MV is then adsorbed by the cell wall and membrane. The main substances involved in MV removal are protein, then lipids; polysaccharides have almost no function in this process. The cell wall and membrane are the ultimate destination of MV, and almost no MV dye passes through the cell wall and membrane and enters the interior of the cell. Therefore, extracellular bacterial components, including the EPS and the cell wall and membrane, are largely responsible for MV removal.

4. Conclusion

In this study, we used XPS analysis, acid-base titration and conductivity analysis to determine that MV removal by a bacterial system was mainly due to adsorption rather than degradation. The adsorption process was rapid and fitted well with the Langmuir adsorption isotherm, and the maximum mono layer adsorption capacities of bacteria and EPS-free bacteria were 769 and 625 mg/g, respectively. Interaction between MV and proteins in EPS was demonstrated by fluorescence analysis. Various components of the bacteria were extracted and utilized for MV removal, and the results indicated that the cell wall and membrane were the main components responsible for MV absorption, accounting for more than 80% of the total removal. The TB-EPS also had some effect, probably accounting for about 10% of MV removal, while other components had no obvious effect. An adsorption experiment with similar substances used to mimic bacterial protein polysaccharides, and lipids supported the above results. Finally, we propose a possible mechanism of MV removal by bacteria.

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