Isolation of a Microorganic Strain for the High Volume Degradation of Aniline Blue and Its Application in Natural Sewage Treatment

Wenhao Li1*, Cheng Li1*, Yuxiang Xu1, Qin Wang1, Xin Ma1, Shouwen Chen1, Jun Yu2#, Yong Yang1#

1Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, Hubei University, Wuhan, China
2Tobacco Research Institute of Hubei Province, Wuhan, China

Email: ‘80196867@qq.com, ’519971800@qq.com

Abstract

Aniline blue, one of the triphenylmethane dyes, is the most commonly produced and used of these dyes yet it is also the most dangerous and the most serious cause of pollution amongst them. An exploration of aniline blue degradation is likely to facilitate an understanding of the degradation mechanism for a range of related dyes. In this study, we managed to isolate a particular strain of microorganism, identified to be Lysinibacillus fusiformis N019a, which showed a significant capacity for aniline blue degradation in both laboratory tests and natural sewage treatment. In analysis aided by a UV-Visible spectrophotometer, we found that 96.7% of aniline blue had degraded within 24 hours under laboratory conditions. When treating natural sewage, 80.1% of the aniline blue was removed after just 16 hours. Further analysis has shown that Lysinibacillus fusiformis N019a has a strong resistance to Cu2+, Mn2+, Zn2+, and Pb2+. We also found that the degradation product of aniline blue by Lysinibacillus fusiformis N019a was of reduced toxicity to plants and microbes.

Keywords

Aniline Blue, Dye Degradation, Lysinibacillus fusiformis N019a, Natural Sewage Treatment

1. Introduction

More than 0.7 million tons of triphenylmethane dyes are used annually in textile production and other related industries [1] [2]. However, synthetic dyes are no-
toriously difficult to degrade [3], so approximately 15% of synthetic dyes end up being released into the environment [4] [5] [6], with potentially harmful health and ecological outcomes [7]. In aqueous ecosystems, synthetic dyes can inhibit photosynthesis and in terrestrial ecosystems [8] [9], the primary broken-down molecules of synthetic dyes are known to have a detrimental effect on microorganisms [10], animals and grain crops [11] [12]. For human-beings, some synthetic dyes are potentially carcinogenic and mutagenic and can be harmful to the immune and respiratory systems [1] [2] [8] [13] [14].

In view of these health and ecological concerns [7] [15], chemical, physical and biological methods have been recruited to treat triphenylmethane dyes being harbored in the environment [2] [16]. Chemical and physical approaches, however, have proved relatively ineffective and expensive and a number of researchers have pointed to the significant secondary contamination produced by these treatments [6] [7] [17] [18].

Biological treatments are considered more environmentally friendly, less costly and more likely to succeed than physicochemical methods [4] [8] [12]. Microbial degradation of triphenylmethane dyes, in particular, has received a lot of attention [7] [19] [20]. The distinguishing features of this approach are lower treatment costs and higher efficiency [21]. This is because microorganisms are able to use triphenylmethane dyes as nutrients without the need for additional supplements [6] [22]. Fungi are considered to be the primary representative species capable of removing triphenylmethane dyes [23] [24] [25]. Amongst them, *Phanerochaete chrysosporium* [26], *Irpex lacteus* [27], *Pleurotus ostreatus* [27], *Aspergillus fumigatus* [28], *Penicillium fusiformis* and *Fusarium* have all been reported to successfully degrade triphenylmethane dyes [29] [30].

However, fungi are not well-adapted to aqueous ecosystems and this has limited the comprehensive and practical biological treatment of triphenylmethane dyes. Confronted with this problem, bacterium would seem to offer the optimum alternative [12] [16] [31]. To date, *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Aeromonas*, *Citrobacter*, *Escherichia*, *Desulphovibrio*, *Proteus*, *Schewanella* and *Alcaligenes* have had success in removing triphenylmethane dyes [4] [6].

Usually, microbiological removal of triphenylmethane dyes is primarily dependent on three kinds of essential enzymes. These are: laccase (Lac), ligninperoxidase (LiP), and manganeseperoxidase (MnP) [32] [33] [34]. In previous work, some authors have reported the pre-screening of pure single microorganisms for high-level enzyme activity so as to construct specific consortia for the removal of triphenylmethane dyes [35] [36]. Other authors have suggested that a microbial mixture will result in the simultaneous secretion of several kinds of enzymes, leading to a more complete removal of the dyes [2]. Others again have argued that prior high-level expression of the required enzymes is the most efficient way of breaking down the molecular structure of triphenylmethane dyes [37] [38] [39]. However, the role of enzyme activity in triphenylmethane dye degradation is not the absolute determinant. The efficiency of triphenylmethane dye removal results from having a balance of organism type, the specific condi-
tions of use and the character of the dyes themselves [4] [22].

In general, most investigators tend to pay more attention to representative triphenylmethane dyes, such as Crystal Violet (CV), Methyl Violet (MV), Malachite Green (MG) and Cotton Blue (CB). However, another important triphenylmethane dye, aniline blue, is largely overlooked. Yet, in fact, aniline blue is more widely used than the other members of the triphenylmethane dye family. It is used not only in the polymer, rubber, agricultural and dye industries [40], but also as a staining marker in molecular research [41]. At the same time, the presence of three sulfonic acid groups in aniline blue give it high polarity, making it very difficult to degrade with ordinary microbes [42]. So, aniline blue is capable of producing particularly dangerous and serious pollution [43]. In view of its greater resistance, exploration of aniline blue degradation may further facilitate understanding of the degradation mechanism for other triphenylmethane dyes. Research, here, may also be more broadly beneficial for the development of degradation microbes for bioremediation.

The core background to the work presented here is the exploration of how to approach the degradation of aniline blue with newly-screened microorganisms that have the capacity to bring about highly efficient removal of triphenylmethane dyes. There are thousands of millions of microorganisms in the environment and many of them are likely to offer effective possibilities for triphenylmethane dye removal.

2. Materials and Methods

2.1. Isolation and Identification of Microbes for Degradation of Aniline Blue

A wild-type strain of Lysinibacillus fusiformis N019a was isolated from decomposed timber in a wood-working factory in Enshi, Hubei province, China. The actual process of isolating this microbe has been previously described in [7] and [12]. The DNA sequence of the 16S RNA region of the microbe was blasted in NCBI and exported similar sequences used neighbor-joining method to construct phylogenetic tree by MEGA7.0. Throughout the duration of our experiments, strains were cultured in a Luria-Bertani (LB) medium at 37°C.

The medium used for testing degradation of aniline blue (DAB) contained (g.L⁻¹): Lactate 1.62, NaOH 0.3, NH₄Cl 1.5, KCl 0.1, NaH₂PO₄ 0.516, NaCl 5.85, yeast extract 0.5 and a trace mineral stock solution, The medium also contained 50 mg/L aniline blue (Sinopharm Chemical Reagent Co., Ltd.) and was adjusted to pH 7.0 unless otherwise mentioned. All of the Erlenmeyer flasks containing 50 ml of the DAB medium were autoclaved at 121°C for 20 min. 4% log phase cells of Lysinibacillus fusiformis N019a were inoculated into each flask. Each experimental sample was incubated in a thermostat oscillator at 37°C at 120 rpm for 36 hours. The cultured cells were harvested via centrifuge (12,000× g; 10 min) using a Beckman Coulter Microfuge 20 under 4°C. Degradation was determined in relation to a cell-free supernatant. The uninoculated sterile dye-containing
medium was used as control. The degradation ratio was calculated using the following equation:

\[
\text{Degradation\%} = \left( \frac{A_2 - A_1}{A_2} \right) \times 100\%
\]

\(A_1\) = Final absorbance of dye after microbe incubation; \(A_2\) = Initial absorbance (Control check). Absorbance was detected using a UV-Visible spectrophotometer at 585 nm (INESA 752N, China).

### 2.2. Dye Degradation Rate According to Different Parameters

The degradation rate of dyes can be influenced by the initial dye concentration, the pH of the treatment medium and the incubation temperature [7]. In this study, we chose single factor experiment, the initial dye concentrations tests were set at 50, 100, 200, 500 and 1000 mg∙L\(^{-1}\), using electronic micro balance (METTLER TOLEDO), the pH tests were containing in 5.5 - 8.0, adjusted by pH meter (METTLER TOLEDO), and the temperature tests were ranged from 15°C to 45°C with the blanking of 5°C, cultured in incubator shaker (Honour). As indicated above, excepted the variable of each group, the initial dye concentration, pH and temperature was kept at 50 mg∙L\(^{-1}\), 7.0 and 37°C respectively, and all the shake speed were at 120 rpm.

To explore the influence of carbon and nitrogen sources on aniline blue degradation, 0.5% of sucrose, glucose, mannitol, maltose, starch, peptone, yeast extract (YE), sodium carbonate, sodium lactate (SL) and SL + YE were loaded as carbon sources. Inorganic nitrogen (0.2% of (NH\(_4\))\(_2\)SO\(_4\), Urea, CH\(_3\)COONH\(_4\), NH\(_4\)NO\(_3\), and NaNO\(_3\)), organic nitrogen (0.2% of peptone, yeast extract) and sodium lactate (0.5%) were chosen as nitrogen sources. To examine the impact of salinity on degradation efficiency, a graded concentration of NaCl (0, 5, 10, 20, 40, 60, and 80 g/L) was added into the medium. We also considered it important to investigate the effects of several heavy metallic ions commonly found in dyeing wastewater on Lysinibacillus fusiformis N019a. The toxicity of heavy metals to microbes can be calculated using the following exponential function:

\[
y = ae^{-bx}
\]

\(x\) = The metallic ion concentration/mmol∙L\(^{-1}\); \(y\) = The biomass in the medium containing the metallic ions/mmol∙L\(^{-1}\); \(a\) = The biomass in the medium containing no metallic ion; \(b\) = The toxicity index. The \(b\) value is positively related to the toxicity.

### 2.3. Degradation Assay in Natural Sewage

As another part of this study, natural wastewater was collected from the Wuhan Printing and Dyeing Factory. The primary concentration of aniline blue was detected by spectrophotometry (585 nm) and 4 ml of Lysinibacillus fusiformis N019a (1.5 \(\times\) 10^8 CFU/mL) was inoculated in 100 ml natural sewage. The degradation ratio was detected by spectrophotometry (585 nm) every 4 h.
2.4. Phytotoxicity and Microbial Toxicity Assay

In order to assess the phytotoxicity and microbial toxicity of aniline blue and its degradation/degradation products, the length of plumule and germination of tobacco, corn and tomato was measured. 10 mL of aniline blue (50 mg/L) and fermentation supernatant (500 mg/L initial concentration and 24 h fermentation) were used to culture tobacco, corn and tomato with methylsulfonylmethane (MSM) as a control [44]. The toxicity to Bacillus amyloliquefaciens ZM9 was tested [45], with the mean inhibition zone (diameter in cm) treated by MSM, aniline blue and fermentation supernatant being recorded after 24 h of incubation at 30°C on an LB plate [44] [46].

2.5. Statistical Analysis

All of the experiments were performed independently in triplicate and the differences amongst the treatments were subjected to a one-way analysis of variance (ANOVA) using a Tukey Kramer multiple comparisons test.

3. Results and Discussion

3.1. Isolation and Identification of Microbes for Aniline Blue Degradation

As mentioned above, the wild-type strain Lysinibacillus fusiformis N019a was originally isolated from decomposed timber in a wood-working factory in China [7] [12]. The DNA sequence of the 16S RNA region of the microbe was blasted in NCBI and the resulting phylogenetic tree revealed that it was close to Lysinibacillus fusiformis strain NFS-STR-1 (MF079349.1), Lysinibacillus sp. B4 (KC310820.1) and Lysinibacillus fusiformis strain HBUM07011 (MF662437.1) (Figure 1). The microbe was therefore inferred to be Lysinibacillus fusiformis N019a and deposited in GenBank (accession number BankIt2112285 N019a MH327493).

Aniline blue can be decolored within 3 h when the strain is mature. When Lysinibacillus fusiformis N019a was cultured on the LB medium, an obvious transparent ring could be observed 8 h later. After a further 6 h, the aniline blue had been completely decolored (see Figure 2(a)). The degradation rate of aniline

![Figure 1](attachment://Figure_1.png)

**Figure 1.** Distance tree inferred from the alignment of N019a after an NCBI BLAST search (MEGA7).
blue initiated by *Lysinibacillus fusiformis* N019a was found to be higher than that reported for a number of other microbes in the literature [12] [39] [47].

About 3 h after inoculation of *Lysinibacillus fusiformis* N019 in a shake flask culture, the color of the aniline blue had started to discolor. 6 h later it was almost colorless (see Figure 2(b)) and after 24 h the fermentation culture had remained colorless. Comparing the degradation performance of *Lysinibacillus fusiformis* N019 for aniline blue against that of other microbes, it achieved degradation in a shorter period of time than *Shewanella oneidensis* MR-1 [12], *Streptomyces AG-56* [39], *Mucoromycotina fusiformis* HS-3 [48], *Bjerkandera fumosa* X X-3 and *Cerrena fusiformis* A-02 [47] [49].

UV-visible absorption spectra were used to analyze the degradation of aniline blue by *Lysinibacillus fusiformis* N019a. The absorption spectrum of aniline blue and its degradation products was measured across wavelengths ranging from 350 to 640 nm. Aniline blue solution has a characteristic absorption peak at 585 nm, but this absorption peak disappeared when it was treated with *Lysinibacillus fusiformis* N019a (Figure 3(a)). In previous investigations, disappearance of an absorption peak has indicated that the chemical structure of the triphenylmethane dye has changed [25] [50]. These results therefore suggested that the aniline blue had been degraded by *Lysinibacillus fusiformis* N019a.

The absorption spectrum of aniline blue was explored across the 24 h fermentation period. After just the initial 3 h, 80% of the aniline blue had been degraded and at 6 h this had risen to 85%. After 24 h 96.7% of the aniline blue had been degraded (see Figure 3(b)). All of this data indicates that the efficiency of aniline blue degradation induced by *Lysinibacillus fusiformis* N019a is higher than other microbes tested in the literature, such as *S. oneidensis* MR-1 (85.56% after 96 h) *Bjerkandera fumosa* (90% after 5 days) [12] [47], and *Mucoromycotina fusiformis* HS-3 (95% after more than 5 days) [48].

**Figure 2.** Schedule of aniline blue degradation induced by *Lysinibacillus fusiformis* N019a (a); Dynamic medium color change process after *Lysinibacillus fusiformis* N019 was inoculated (b).
Figure 3. Degradation assays using UV-V is spectral analysis (a) and absorption spectra (b). (a) UV-V is spectral analysis of aniline blue (black curve) and its degradation products after incubation for 24 h with *lysinibacillus* sp. N019a (red curve); (b) Degradation (%) of aniline blue by *lysinibacillus* sp. N019a over 24 h.

3.2. Dye Degradation Rate across Different Parameters

The degradation rate of dyes can be influenced by the initial dye concentration [50]. This phenomenon was also found to hold in this investigation. Aniline blue can be more rapidly decolored at lower concentrations than higher ones during the initial 6 h (see Figure 4(a)). After 6 h, 86.5% of aniline blue at a concentration of 50 mgL⁻¹ had been decolored. At 1000 mg∙L⁻¹, this figure had dropped to only 63.5%. However, after 12 h, 95.5%, 94.4%, 92.4%, 89.8% and 87.4% of the aniline blue had been decolored by *Lysinibacillus fusiformis* N019a at 50, 100, 200, 500 and 1000 mg∙L⁻¹, respectively. The overall tendency for degradation efficiency at different concentrations is for it to gradually decrease as the aniline blue concentration increases. In line with several previous investigations [7] [51], we find the implication of this to be that the initial concentration of triphenylmethane dyes and microbial degradation efficiency is negatively correlated [39].
Figure 4. Effects on the degradation efficiency of *lysinibacillus* sp. N019a for: different initial concentrations of aniline blue (a); pH values (b); temperatures (c); salinity (d); carbon sources (e); and nitrogen sources. All values represent the average of the results across three samples ± SD (standard deviation).

An optimum pH and temperature of the treatment medium is required to maximize the efficiency of dye degradation (Mishra and Maiti, 2018). *Lysinibacillus fusiformis* N019a possessed a degradation capacity at pH 6.0–8.0. Its maximum degradation efficiency (97.1%) was obtained at pH 8.0 after incubation for 24 h (see Figure 4(b)). The degradation activity of *Lysinibacillus fusiformis* N019a was significantly influenced by temperature and was positively correlated with temperatures from 15°C to 40°C. However, when the temperature was higher than 45°C, the degradation efficiency started to decline (Figure 4(c)). And the salinity tolerance of *Lysinibacillus fusiformis* N019a was higher than 40 g/L, although it dragged the growth of strain N019a at the front 12 h after inoculation, but it recovered after that, what can be seen clearly from Figure 4(d).

As mentioned in Section 2.2, a DAB medium to which we had added 0.5% sucrose, glucose, mannitol, maltose, starch, peptone, yeast extract (YE), sodium carbonate, sodium lactate (SL) and SL + YE was used to explore the influence of carbon sources on aniline blue degradation. The degradation rate of aniline blue induced by *Lysinibacillus fusiformis* N019a using yeast extract, peptone and so-
diurnium lactate as the unique carbon source reached 77.3%, 74.8% and 69.1%, respectively (see Figure 4(e)), while using a composite carbon source of sodium lactate + yeast extract (4:1) enabled the degradation rate to reach 80.2%. After comprehensive testing, sodium lactate + yeast extract (4:1) appears to be the optimal carbon source for Lysinibacillus fusiformis N019a. In addition, when inorganic nitrogen (0.2%), organic nitrogen (0.2%) and sodium lactate (0.5%) were selected as the nitrogen source, (NH₄)₂SO₄ and yeast extract produced the best degradation results (Figure 4(f)).

It was noticed that Lysinibacillus fusiformis N019a was capable of maintaining a high degradation efficiency of over 94% for aniline blue after 36 h of incubation when the added NaCl concentration did not exceed 40 g·L⁻¹. However, Lysinibacillus fusiformis N019a spent longer adapting to growth at above 40 g·L⁻¹ NaCl. Color reduction disappeared once the saline concentration reached 60 g·L⁻¹. As this figure is relatively high, it indicates that Lysinibacillus fusiformis N019a is able to tolerate a wide range of salt concentrations. This adaptability will clearly increase the feasibility of its practical application.

Some metal compounds in the environment do not have an adverse effect on microbes but, rather, are beneficial to them. However, when some metals, especially heavy metals, exceed a certain concentration, they have a toxic effect on microorganisms, inhibiting their growth and reproduction, damaging their respiration, making their cell morphology abnormal and even causing cell lysis. In that case, it was important to investigate the effects of several heavy metallic ions commonly found in dyeing wastewater on Lysinibacillus fusiformis N019a. AgSO₄, CdCl₂, K₃Cr₂O₇, and HgCl₂ had a significant toxic inhibition effect on the growth of Lysinibacillus fusiformis N019a, with a toxicity index ranging from 17.345 to 71.405 (see Table 1). The toxicity index of CuSO₄, MnSO₄, ZnSO₄, Pb(Ac)₂ was less, ranging from 0.094 to 2.591. This shows that Lysinibacillus fusiformis N019a has a strong resistance to Cu²⁺, Mn²⁺, Zn²⁺, Pb²⁺.

<table>
<thead>
<tr>
<th>Metallic ion</th>
<th>Biomass (OD600)</th>
<th>Metallic ion concentration (mmol/L)</th>
<th>Degradation (%)</th>
<th>Toxicity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>1.525</td>
<td>-</td>
<td>91.4%</td>
<td>-</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.115</td>
<td>1.00</td>
<td>6.2%</td>
<td>2.588</td>
</tr>
<tr>
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<td>0.04</td>
<td>3.7%</td>
<td>71.405</td>
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<tr>
<td>MnSO₄</td>
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</tr>
<tr>
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<td>46.648</td>
</tr>
<tr>
<td>K₃Cr₂O₇</td>
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<td>0.04</td>
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</tr>
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<tr>
<td>Pb(Ac)₂</td>
<td>1.389</td>
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</tr>
<tr>
<td>HgCl₂</td>
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<td>0.04</td>
<td>4.6%</td>
<td>71.216</td>
</tr>
</tbody>
</table>
3.3. Degradation Assay in Natural Sewage

For the degradation of dyes in wastewater, a large number of microorganisms have now been screened, with numerous lab studies [52]. However, the composition of natural sewage is very complex [53], so it is necessary to explore whether screened microorganisms perform so well when treating natural wastewater. The typical aniline blue content in natural wastewater is 398.5 ± 2.85 mg/L. 4 ml of Lysinibacillus fusiformis N019a. (1.5 × 10⁸ CFU/mL) was inoculated into 100 ml of natural sewage. After 16 h, 80.1% of the aniline blue had been removed (see Figure 5). These results suggest that Lysinibacillus fusiformis N019a performs well in natural wastewater.

3.4. Phytotoxicity and Microbial Toxicity Assay

The microbes used for dye degradation/degradation end up being disposed of in the environment and in bodies of water. In that case, it is important to ensure that the end-products of dye degradation/degradation processes are safe for the environment and their toxicity must be assessed [54].

In order to assess the phytotoxicity and microbial toxicity of aniline blue and its degradation/degradation products, the plumule length and germination of tobacco, corn and tomatoes were measured, with MSM being used as a control. The toxicity to Bacillus amyloliquefaciens ZM9 was also assessed and the mean inhibition zone (diameter in cm) was recorded after 24 h of incubation at 30˚C [44] [46]. The average germination of tobacco treated by MSM, aniline blue and fermentation supernatant was 89% ± 7.37%, 43% ± 1.25%, and 77% ± 0.94%, respectively (Figure 6(a)). The length (shoots + roots) of the tobacco treated by MSM, aniline blue and fermentation supernatant was 69.72 ± 5.91 mm, 58.09 ± 6.53 mm, and 62.60 ± 4.04 mm, respectively (Figure 6(b)). The germination and the length of the shoots and roots show that the degradation product has a low toxicity to plants. The diameter of the inhibition zone for Bacillus amyloliquefaciens ZM9 for MSM, aniline blue and fermentation supernatant was 0 mm,
Figure 6. Phytotoxicity of aniline blue and its degradation products. Germination (a) and the length of the shoots and roots (b) of tobacco, tomatoes and corn were measured.

8.95 ± 0.21 mm, 2.35 ± 0.19 mm, respectively. These results once again indicate that the product of aniline blue degradation does not have a particularly toxic nature. Previous investigations have shown that the product of some kinds of triphenylmethane dye degradation by microbes have almost no toxicity at all [44]. However, in this study, there was still less toxicity for the fermentation supernatant. This may imply that some of the aniline blue was not completely degraded. Indeed, our results have shown that 3.3% of the aniline blue was still not degraded after 24 h (see Figure 3).

4. Conclusion

A strain of microorganism, Lysinibacillus fusiformis N019a, was screened from the natural environment and its capacity for aniline blue degradation was analyzed using a UV-visible spectrophotometer. 96.7% of the aniline blue had been
degraded after 24 h. The efficiency of aniline blue degradation induced by *Lysinibacillus fusiformis* N019a is notably higher than that for some other microbes (see Figures 1-3). On the basis of previous investigations [6] [55], the disappearance of absorption peaks can be taken to demonstrate that the color removal of aniline blue by *Lysinibacillus fusiformis* N019a was largely attributable to biodegradation. *Lysinibacillus fusiformis* N019a was also found to effectively degrade aniline blue in natural sewage and it has a strong resistance to the heavy metallic ions Cu²⁺, Mn²⁺, Zn²⁺, and Pb²⁺ (see Figures 4-6 and Table 1). We were further able to confirm that the product of aniline blue degradation by *Lysinibacillus fusiformis* N019a has a low toxicity to plants and microbes (Figure 6).

**Acknowledgements**

This work was supported by the key technology projects of China National Tobacco Corporation (CNTC) under Contract No. 110201502014 and No. 110201502018, and the key technology projects of Hubei tobacco companies under Contract No. 027Y2014-013.

**Conflicts of Interest**

The authors declare no conflicts of interest regarding the publication of this paper.

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https://doi.org/10.1016/j.jenvman.2014.09.014


144-147.


