

Screening and Application of Phthalic Acid Degrading Bacteria

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Abstract

O-phthalic acid is a kind of important pollutant, which accumulates in the environment with the extensive use of plastics and other products. Meanwhile, phthalic acid is one of the high content of allelopathic autotoxic substances secreted by tobacco. The accumulation of phthalic acid in soil is an important cause of tobacco continuous cropping effect. In order to degrade phthalic acid accumulated in environment, the barrier effect of tobacco continuous cropping caused by phthalic acid accumulation in soil can be removed. A strain capable of degrading phthalic acid was isolated from sludge of sewage treatment plant and compared with 16 s DNA. The homology between this strain and *Enterobacter* sp. is 99%. The optimum growth conditions are as follows: pH7 at 30°C, 500 mg/L of o-phthalic acid, inoculation concentration $\geq 1.2\%$ and its highest degradation rate of o-phthalic acid is 74%. The results of pot experiment showed that the degradation efficiency of o-phthalic acid in soil was about 40%, which alleviated the inhibitory effect of o-phthalic acid accumulation on tobacco growth.

Keywords

O-Phthalic Acid, Allelopathic Autotoxic Substance, Tobacco

1. Introduction

O-phthalic acid is the main raw material for the production of phthalic acid ester plasticizer [1], phthalic acid and its product, phthalate (PAEs) [2], have penetrated into food, medicine, human body, food bottle packaging products, Water bodies and terrestrial ecosystems [3] have caused great harm to ecosystem and

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human health, and have been regarded as universal pollutants all over the world. Studies have shown that phthalic acid and its derivatives are also the main allelopathic autotoxic substances that cause continuous cropping barriers of tobacco, soybean, maize and other important economic plants [4], and are also important substances [5] [6] for synergistic soil-borne diseases and insect pests which caused serious economic losses [7]. The elimination of phthalic acid and its esters from phthalic acid derivatives to the environment, human beings and crops has attracted great attention in related research fields [8]. Methods of eliminating phthalic acid and its esters mainly contained physical [9], chemical [10] and biological methods [11]. At the same time, it is limited to the treatment of phthalic acid and its esters in industrial wastewater and has not been widely used [12]. However, biological methods, especially the microbial degradation of phthalic acid and its esters, have attracted much attention [13]. Phthalates are first hydrolyzed to phthalate monoesters and then hydrolyzed to phthalic acid in microorganisms. Finally it was degraded into organic acids to provide nutrients for microbes [14]. Therefore, microbial degradation of phthalic acid is characterized by high efficiency, low cost and environmental friendliness [15].

Screening or domesticating PAEs (phthalic acid esters) degradation strains from the environment is the most simple and economical way [16] [17]. Presently, the main high-efficient degrading bacteria are *Arthrobacter* sp., *Pseudomonas* sp. and *Pseudomonas* sp. etc. [18] [19]. All of these single bacteria have defects such as single substrate, incomplete degradation and so on [20]. Although the microbe consortia overcame the sole defect of substrate and the degradation efficiency were improved to a certain extent [21]. However, most of the studies focused on the degradation of phthalic acid esters, but underestimated the degradation of phthalic acid [22], probably due to the high content of phthalic acid in soil [23]. Additionally, most of the previous studies were limited to shaking flask experiment in laboratory, and there were few reports on whether biodegrading bacteria could still worked in soil environment. Moreover, microbes in soil environment are regarded as the largest biological diversity pool in nature [18], which contains more efficient microbes degraded phthalic acid and its esters.

Based on the above reasons, the biodegradation efficiency of phthalic acid in soil was tested by screening high efficiency phthalic acid degrading bacteria which could play an important role in soil environment, and the relationship between degradation bacteria and physiological index of tobacco was analyzed. It provides valuable information for the discussion of the mechanism of continuous cropping barrier and the prevention and control of soil-borne diseases in cash crops such as tobacco.

2. Materials and Methods

2.1. Screening and Identification of Bacteria

2.1.1. Sources and Medium of Bacteria

20 g of silt collected from the sewage treatment pool of Shahu Lake in Wuhan

City and the sewage outlet of Chemistry and Chemical Engineering College, Hubei University. The sludge was stored in the sterilized 250 mL triangle bottle and stored in -4°C refrigerator.

Basic salt medium containing (g/L): $(\text{NH}_4)_2\text{SO}_4$ 2.0, MgSO_4 0.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01, FeSO_4 0.001, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 1.5, KH_2PO_4 0.5, KH_2PO_4 1.5; Luria-Bertani (LB) medium containing (g/L): NaCl 10, yeast powder 5, peptone 10; Potato Dextrose Agar (PDA) medium containing (g/L): potato filtrate 200, glucose 20; Kos 1 medium containing (g/L): soluble starch 20, NaCl 0.5, KNO_3 1, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01; Beef extract peptone medium (BEPM) containing (g/L): beef extract 3, peptone 10, NaCl 5 g; Bengal red medium containing (g/L): peptone 5, glucose 10, KH_2PO_4 1, MgSO_4 0.5, Bengal red 0.03, chloramphenicol 0.1.

2.1.2. Enrichment and Screening of Phthalic Acid Degradation Strain

10 g silt was placed in the sterilizing triangle flask which contained 100 mL basic medium, and cultured in 30°C of 180 r/min orbital incubator overnight. And then 10 mL suspension was added to the 90 mL BEPM medium for enrichment, and the domestication was done by gradient pressure culture method. The concentration of phthalic acid in enrichment medium was gradually increased from 100 mg/L to 700 mg/L. A solid BEPM medium containing 500 mg/L phthalic acid was used to isolate the tolerant bacteria by dilution and coating plate method. The colony with good growth, clear colony and distinct morphology was selected, and a single colony was isolated by plate marking method. The isolated colony was phthalic acid tolerant bacteria.

2.1.3. Species Identification

A strain of bacterium that can specifically degrade phthalic acid was selected from the tolerant bacteria. Physiological and biochemical identification of bacteria referred to the “Berger’s systematic bacteriology manual” 9th edition. Molecular biological identification was carried out according to the following procedures: the strain was cultured on LB medium for 8 h, bacterium suspension was made by pick bacterium into 100 μL solution in 1.5 ml EP tube, and 12,000 r/min centrifuged for 2 min after 15 min of boiling water bath. The supernatant was isolated as a template for 16SDNA amplification and PCR amplification and the PCR reaction system was as follows: DNA template 5 μL , forward and reverse primer each 1 μL respectively, dNTP (10 mmol/l) 1 μL , $10\times$ Extaq buffer 5 μL , Extaq1 μL , supplemented with double distilled water to 50 μL . The primer sequence was: 27F: 5'-AGAGTTTCCTGCTCAG-3'; 1492R:

5'-GTTACCTTACGACTT-3'; Amplification procedure: predenaturation at 94°C for 3 min, 94°C denaturation for 1 min, 61°C annealing for 1 min, 72°C extension for 1 min, set 30 cycles and at the end of procedure set 72°C extension for 5 min, 4°C preservation for 30 min. 0.8% agarose gel electrophoresis to detect PCR products. The size of the amplified product was about 1450 bp, and sequenced by Wuhan Qingke Biological Company. The 16s DNA sequence results was blast in GenBank.

2.2. Culture Condition Optimization

The growth of *Enterobacter* sp. NO₃ was affected by different parameters and single factor experiments were designed. The single colony of *Enterobacter* sp. NO₃ was inoculated in LB medium and cultured overnight. After collected by refrigerated centrifuge at 5000 rpm for 2 min, the bacteria were resuspended in inorganic salt medium, and the value of OD600 was adjusted to 1, which was used as seed. Seed was inoculated in 50 mL basic salt medium containing 500 mg/L phthalic acid (except phthalic acid concentration test), and different culture temperatures (18°C, 30°C, 37°C, 45°C), pH (4, 5, 6, 7, 8, 9) and inoculation concentration (0.4%, 0.8%, 1.2%, 1.6% and 2.0%) were set respectively. After those parameters were optimized, different concentration of phthalic acid (100 - 1000 mg/L) experiment were set. OD600 was measured at 48 h after culture while the effect of inoculation concentration on growth was measured every 8 h. The experimental data were repeated 3 times and averaged.

2.3. Effect of Degradation Rate and Different Conditions on Degradation Efficiency

The effects of different conditions on the ability of *Enterobacter* sp. NO₃ to degrade phthalic acid were detected by liquid chromatography under the conditions mentioned in 2.2. The supernatant was gathered by 12,000 r/min centrifugation for 5 min. After the supernatant was extracted by the same amount of ethyl acetate, the extract was evaporated to crystallization by rotating evaporator, and then dissolved in aseptic water and filtered by 0.22 µm organic filter membrane. The content of phthalic acid was determined by high performance liquid chromatography (HPLC). The chromatographic conditions were as follows: Agilent ZORBAX SB-Aq (250 mm × 4.6 mm); mobile phase: 5 mmol/L H₂SO₄, flow rate: 500 µL/min, sample volume 20 µL.

2.4. Effect of Degrading Bacteria on the Growth of Tobacco Seedling

After transplanting tobacco seedlings, set experimental group 1 irrigated with 50 mL phthalic acid solution (2 g/L) and *Enterobacter* sp. NO₃ bacterial solution 5 mL (0.3×10^9 CFU/mL), set experimental group 2 with 50 mL phthalic acid solution (2 g/L) and water 5 mL, and set control group with 55 mL water. The plant height, stem diameter, fresh weight and dry weight of rhizome and leaf were measured after cultured in constant temperature and humidity greenhouse for four weeks. Each group has 10 pots of tobacco seedlings and the experimental data were repeated 3 times and averaged.

2.5. Detection of Degradation Effect of Degrading Bacteria in Soil

In order to study the degradation effect of *Enterobacter* sp. NO₃ on phthalic acid under the condition of soil source, the content of phthalic acid was determined by high performance liquid chromatography (HPLC) after filtration. The liquid chromatographic conditions are the same as 2.3.

2.6. Data Statistics and Analysis

Data processing and analysis using origin 8.5.

3. Results and Discussion

3.1. Screening and Identification of Strains

3.1.1. Main Physiological and Biochemical Characteristics of *Enterobacter* sp. NO₃

The colony of strain NO₃ present characteristics like milky white, translucent, round, smooth, regular in edge and flat in colony. Gram-stained bacteria were rod shaped and Gram-negative bacteria.

The physiological and biochemical characteristics of strain NO₃ are described in **Table 1**.

3.1.2. 16SDNA Identification Results

The DNA sequence of the 16S RNA region of the strain NO₃ was obtained and the length of the product was 1466 bp. The blasted results in NCBI and the distance tree revealed that it was close to *Enterobacter* sp. 3-1t (EU543690.1) and *Enterobacter* sp. E4M-P (GQ478269.1). The homology was 99% (**Figure 1**), the microbe was therefore inferred to be *Enterobacter* sp., and the strain was named *Enterobacter* sp. NO₃, deposited in GenBank (GenBank: MK128455.1).

Table 1. Main physiological and biochemical characteristics of strain NO₃.

Gram stain	–	Sucrose	–
Glucose	+	D-Fructose	+
D-Galactose	+	Trehalose	–
L-Arabinose	+	mannose	+
maltose	–	D-Xylose	–
D-Ribose	–	Rhamnose	–
Sorbitol	–	Sarcosine	+
Mannitol	–	Casein	+
Ethanol	–	Ammonia Production	+
D-Malate	+	Cellulose Decompose	+
Citraconate	+	Lecithin Enzyme	+
D-Alanine	+	Starch Hydrolysis	+
D-Tryptophan	+	Gelatine Liquefication	–
D-Tartrate	–	V-P Test	–
Glycolate	–	Denitrification	–
Pantothenate	–	Sarcosine	+

Note: +positive, –negative.

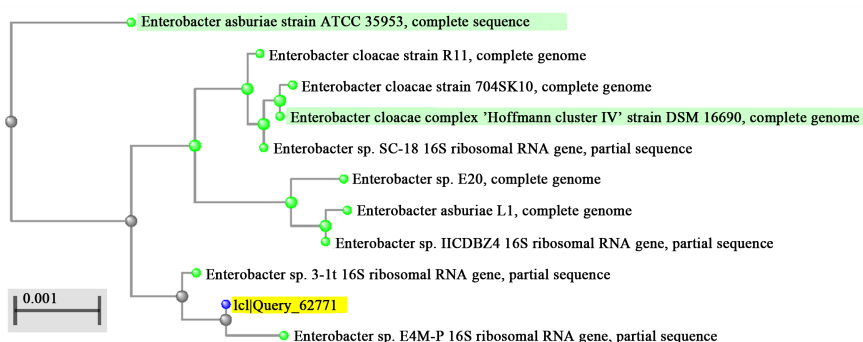


Figure 1. Phylogenetic tree.

3.2. Culture Condition Optimization

The common factors related to growth are pH, temperature, inoculums concentration and substrate concentration. The effect of cultured pH on the growth of *Enterobacter* sp. NO₃ was shown in **Figure 2(a)**. *Enterobacter* sp. NO₃ could tolerance pH 4.0 to 9.0 and its most optimum pH was 7.0. As **Figure 2(b)** had shown, when the concentration of phthalic acid was 500 mg/L, the growth of *Enterobacter* sp. NO₃ was the highest. **Figure 2(c)** represented the effect of culture temperature on the growth of *Enterobacter* sp. NO₃, its suitable temperature range was 30°C - 37°C, the growth of *Enterobacter* sp. NO₃ was inhibited while the temperature was below 30°C or above 37°C. The effect of inoculation concentration on the growth of the strain NO₃ also had been tested, the inoculation amount had no effect on the growth of *Enterobacter* sp. NO₃ when the inoculation concentration was greater than or equal to 1.2 which had been showed in **Figure 2(d)**. When the inoculation amount was less than 1.2, the growth was inhibited 72 hours before inoculation. Effects disappear after 72 hours.

3.3. Degradation Efficiency Factors of Phthalic Acid

The effect of cultured pH on the degradation efficiency of *Enterobacter* sp. NO₃ was shown in **Figure 3(a)**. When the medium pH was 7.0, the degradation efficiency was the highest, and the effect of initial phthalic acid concentration on the degradation efficiency of *Enterobacter* sp. NO₃ was shown in **Figure 3(b)**. When the concentration of phthalic acid is 500 mg/L, the degradation efficiency is the highest, and the effect of temperature on the degradation efficiency of *Enterobacter* sp. NO₃ is shown in **Figure 3(c)**. When the temperature is 30°C, the degradation efficiency of *Enterobacter* sp. NO₃ is the highest. Under the conditions of pH 7, culture temperature 30°C, initial concentration of phthalic acid 500 mg/L, inoculum = 1.2%, and shaking flask culture for 7 days, the maximum degradation rate of phthalic acid to phthalic acid by *Enterobacter* sp. NO₃ can be detected to be 74%.

3.4. Detection of Degradation Effect of Degrading Bacteria in Soil

The degradation rate of phthalic acid in soil was detected by liquid chromatography. The peak area of HPLC was 913487 and 3376 mAu*s respectively under

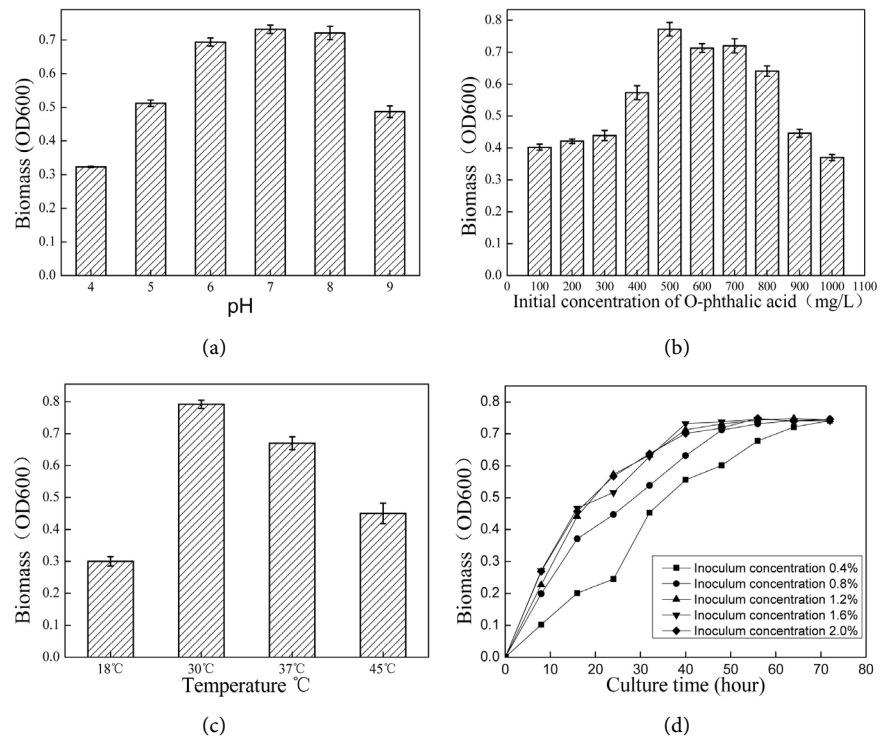


Figure 2. Effects of different culture conditions on *Enterobacter* sp. NO₃ growth: pH value (a); initial concentration of O-phthalic acid (b); temperatures (c); inoculums concentration (d).

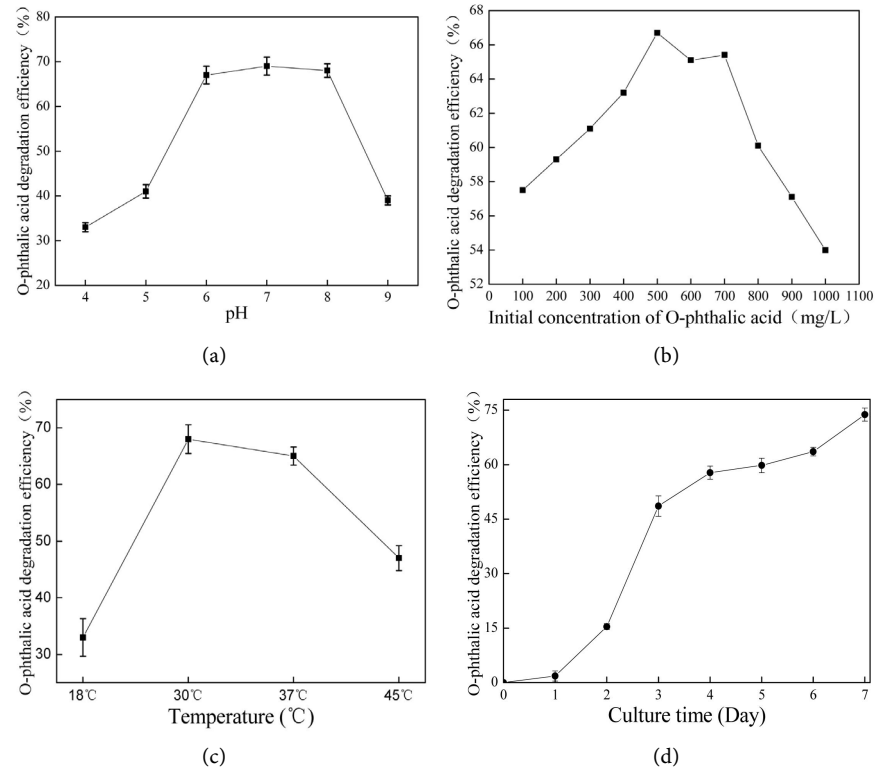


Figure 3. Effects of different culture conditions on the degradation efficiency of phthalic acid by *Enterobacter* sp. NO₃.

the condition of control, the peak area of the peak was 913487 and 3376 mAu*s, respectively (**Figure 4**), under the condition of phthalic acid and phthalic acid degrading bacteria, the degradation rate of phthalic acid in soil was determined by liquid chromatography. *Enterobacter* sp. NO₃ can still be degraded in soil *in situ*.

3.5. Effect of Degrading Bacteria on the Growth of Tobacco Seedling

Under the condition of Phthalic acid and phthalic acid degrading bacteria, there was significant difference in tobacco growth after 1 month of tobacco growth (**Figure 5**). The measured plant height values were 158.15 ± 64.91 109.67 ± 27.07 and 150.17 ± 76.50 mm, respectively. The diameter of tobacco stem was $9.01 \pm 1.54 \pm 0.59$ and 9.12 ± 2.28 mm, respectively. The mean fresh weight of leaf and rhizome of blank (CK) was 6.83 ± 1.02 9.5 ± 1.67 and 34.67 ± 4.51 g, respectively, and the mean fresh weight of roots and leaves of Phthalic acid was 7.33 ± 0.40 5.33 ± 0.87 and 28.33 ± 2.08 g respectively. The mean fresh weight of mycorrhizal stem and leaf for phthalic acid degradation was 15.67 ± 2.14 9.67 ± 1.35 and 35.83 ± 6.07 g respectively.

The average dry weight of root stem and leaf of blank (CK) were 4.64 ± 0.61 3.24 ± 1.44 and 12.59 ± 1.95 g, the fresh weight of roots and leaves of phthalic acid were 4.16 ± 0.31 2.27 ± 0.25 and 11.67 ± 0.58 g, respectively. The mean fresh weight of Phthalic acid degrading mycorrhizal stem and leaf was 7.08 ± 0.31 3.61 ± 0.97 and 13.69 ± 0.59 g (**Table 2**).

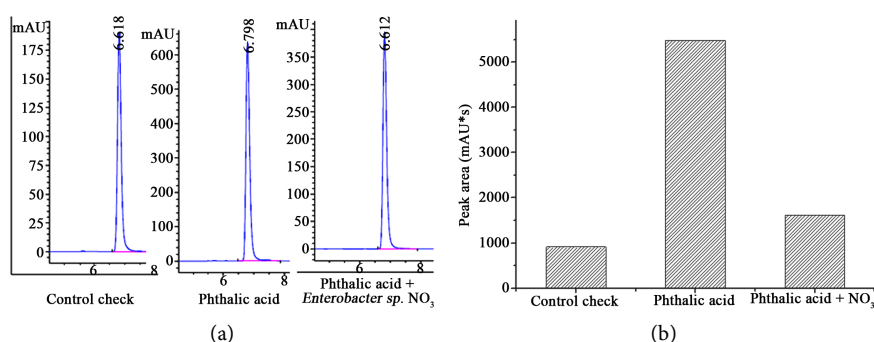


Figure 4. Degradation effect of phthalic acid in soil. (a) Peak time and retention time of high performance liquid phase; (b) Statistical results of peak area of degradation effect of phthalic acid.

Table 2. Physiological indexes of tobacco growth under different conditions (n = 90).

	Stem height ^a	Stem diameter ^a	Fresh weight [#]			Dry weight [#]		
Control check	158.15 ± 64.91	9.01 ± 1.54	6.83 ± 1.02^a	9.5 ± 1.67^b	34.67 ± 4.51^c	4.64 ± 0.61^a	3.24 ± 1.44^b	12.59 ± 1.95^c
O-phthalic acid	109.67 ± 27.07	8.50 ± 0.59	7.33 ± 0.40	5.33 ± 0.87	28.33 ± 2.08	4.16 ± 0.31	2.27 ± 0.25	11.67 ± 0.58
O-phthalic acid + <i>Enterobacter</i> sp. NO ₃	150.17 ± 76.50	9.12 ± 2.28	15.67 ± 2.14	9.67 ± 1.35	35.83 ± 6.07	7.08 ± 0.31	3.61 ± 0.97	13.69 ± 0.59

^ais the total number of tobacco seedlings sampled in the experiment; ^aunit: mm; [#]unit: g; ^{a,b,c}represents root, stem and leaf, respectively.

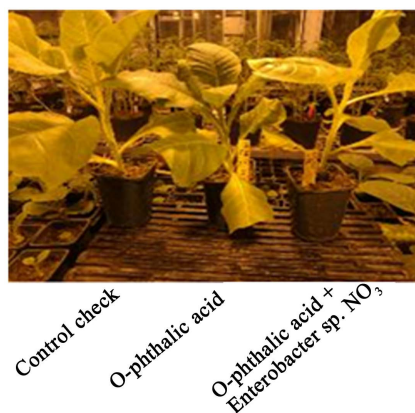


Figure 5. Phthalic acid and its degradation bacteria in pot culture.

4. Discussion

The pollution of phthalic acid and its derivatives to the environment has caused great attention at home and abroad [1] [2] [3], phthalic acid and its derivatives to tobacco, soybean. The effects of continuous cropping barriers and soil diseases on important cash crops such as maize have attracted much attention [4] [5] [6]. Using microbial degradation to eliminate the harm of phthalic acid and its derivatives is the most efficient, low-cost and environment-friendly approach in biological methods [15]. In this study, Phthalic acid degrading strains were screened from sludge of sewage treatment plant. The bacteria were identified as *Enterobacterium* by 16SDNA comparison analysis and named *Enterobacter* sp. NO₃ (Figure 1). The optimum growth temperature is 30°C and the initial concentration of pH = 7, the initial concentration of phthalic acid was 500 mg/L, inoculation dose was 1.2% (Figure 2).

The strain *Enterobacter* sp. NO₃ selected in this paper belongs to a new type of bacteria, which is similar to the previously studied strains such as (*Arthrobacter* sp.), pseudomonas (*Pseudomonas* sp.). The degradation efficiency of this strain was 74 under the condition of shaking flask in laboratory, which was higher than that of other single strains [18] [21] [22]. The degradation efficiency of the strain was related to the temperature, PH value and the concentration of phthalic acid. The degradation efficiency of the strain was the highest at 30°C, and the low concentration of phthalic acid promoted the degradation of the strain. The high concentration of phthalic acid could inhibit the degradation of the strain (Figure 3). The content of phthalic acid in soil environment is higher than that of phthalic acid derivative [23]. In this study, the degradation ability of phthalic acid was studied. The degradation rate of phthalic acid in soil was detected by liquid chromatography. Compared with blank control, the strain did not completely degrade phthalic acid, but compared with high concentration of phthalic acid in soil. The content of phthalic acid decreased by about 40% (Figure 4). The results showed that *Enterobacter* sp. NO₃ could still be degraded in soil *in situ*. The results of physiological index measurement of tobacco seedling growth showed that the average height of tobacco was 158.15 mm, 109.67 mm and

150.17 mm; under the condition of phthalic acid (*Enterobacter* sp. NO₃) and phthalic acid (*Enterobacter* sp. NO₃), respectively. The average wet weight and dry weight were 13.66 g and 20.39 g, 6.82 g, 6.03 g and 8.12 g, respectively.

It has been proved that phthalic acid has the effect of causing crop continuous cropping obstacle [7] [24] and synergistic effect of soil-borne diseases and insect pests [5] [6]. The results of our study show that phthalic acid inhibits tobacco growth. The inhibitory effect of phthalic acid on tobacco was obviously alleviated by adding degrading bacteria. In addition, the growth effect of phthalic acid degradation was not obvious, but the weight index analysis showed that the effect of phthalic acid degradation bacteria on tobacco leaf growth was obvious. The results provide new information for the prevention of tobacco continuous cropping barriers, but whether degradation of phthalic acid can alleviate the occurrence of soil-borne diseases needs further study.

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Conflicts of Interest

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

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