

RESEARCH ARTICLE

## Efficient Culture of *Rhodopseudomonas palustris* Using Landfill Leachate

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### Abstract

The efficient culture of *Rhodopseudomonas palustris* using landfill leachate was investigated. Total organic carbon (TOC) and total nitrogen (TN) in landfill leachate were 16.0 g/L and 1.1 g/L at pH 4.5, respectively, which was apparently reduced with the increase of pH. Compared with carbon or nitrogen source added, the addition of phosphorus could greatly promote the growth of *R. palustris* using landfill leachate as culture medium. Both the growth of *R. palustris* and the removals of TOC and TN were enhanced by increasing inoculation ratios (v/v) of *R. palustris* from 20% to 50% and the highest cell density of  $2.0 \times 10^9$  cell/mL was obtained. The viability and integrity of *R. palustris* was further detected with the fluorometry method of double staining and the highest proportion of 99.4% live cells was found at 72 h. This study is very important in the efficient utilization of landfill leachate to culture *R. palustris* as bacterial manure.

**Keywords:** *Rhodopseudomonas palustris*, Landfill leachate, Culture, Living cell.

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## INTRODUCTION

With the accelerating process of urbanization and the rapid improvement of urban people's living standards, large amounts of landfill leachate have been generated with the annual growth rate of 8% -10% and the million tons of wastewater has been discharged into rivers and oceans<sup>1</sup>. Landfill leachate contains large amounts of dissolved organic matter, inorganic macro components, including sulfate, chloride, and ammonia, toxic heavy metals, and toxic xenobiotic organic compounds<sup>2</sup>. Traditional waste disposal methods, including incineration, landfill and mechanical pulverization directly, bring serious consequences to the human ecological environment<sup>3</sup>. Merugu, R et al. thought purple non-sulphur bacteria could reduce chromium<sup>4</sup>. Kaitlyn D.Sniffen used an algae-based landfill leachate remediation system aims to remove nutrients from liquid waste by nitrogen assimilation into new algal biomass<sup>5</sup>. Therefore, the environmental friendly and efficient technology of high strength wastewater treatment is quite essential<sup>6</sup>. Compared with traditional methods, photosynthetic bacteria (PSB), with its high salt-tolerance and activity, has great potential in treating various high concentration organic wastewater. In addition to pollutant removal, the wastewater treatment with PSB can accumulate valuable biomass as bacterial manure<sup>7</sup>, food coloring agent, precursor of vitamin A in food and animal feed, <sup>2</sup>-carotene and astaxanthin in industry, and additives to porphyrin<sup>8,9</sup>. The extraction source of polyhydroxyalkanoate as biodegradable plastics from PSB was also reported<sup>10</sup>. Recently, increasing demands for products from "organic farming" have encouraged farmers to use bacterial manure instead of chemicals. PSB has been claimed to be essential because of its ability to fix N<sub>2</sub><sup>11</sup> and to produce plant growth promoter, such as 5-aminolevulinic acid (ALA), indole 3-acetic acid (IAA) (auxins) and cytokinins<sup>12,13</sup>. So the efficient utilization of landfill leachate to culture PSB has attracted great attention.

Rautter et al. proposed the common reaction formula of photosynthesis<sup>14</sup>, explained the biosynthesis phenomenon with biochemical uniformity, and laid a foundation for the research of PSB after its scientific classification and physiological research. Since 1960s, as a typical

bacterial strain of PSB, *Rhodospseudomonas palustris* has been used to treat dairy wastewater, soybean wastewater, food wastewater, fermented starch wastewater and domestic wastewater<sup>15,16,17</sup>. In the process of PSB wastewater treatment, the most important factor is the light-oxygen condition<sup>17,18,19</sup>. The light recipe including light intensity, light source and photoperiod could influence biomass and COD removal in the wastewater treatment<sup>7,20,21</sup>. Some studies have demonstrated that higher DO level achieved higher biodegradation efficiency and lower DO led to poorer bio-flocculating ability and less bound extracellular polymeric substances content of the activated sludge<sup>22,23,24</sup>. Although the treatments of wastewater with PSB were widely investigated, no information is provided on the resource utilization of landfill leachate to culture *R. palustris* effectively as bacterial manure.

The aim of this study is to reuse landfill leachate containing much amount nutrients of protein, sugar, oil and so on as medium to efficiently culture *R. palustris* for fixing nitrogen as bacterial manure. Both the treatment of landfill leachate and the production of *R. palustris* are simultaneously achieved.

## MATERIALS AND METHODS

### Materials

The bacterial strain of *Rhodospseudomonas palustris* (No. 1.2180) used in this experiment was bought from the China General Microbiological Culture Collection Center, which was routinely cultivated in our laboratory under outdoor sunlight at the temperature from 20°C to 40°C for 96 h as the seed<sup>25</sup>. The landfill leachate derived from the solid filtered liquid of kitchen waste, which was sent by Green Space Biotech Limited Company at Beijing, P.R.China, was used in this study.

### Methods

#### Detection of physicochemical indexes of landfill leachate

The contents of Cr, Cd, Cu, Ni, Mg, Zn and Pb in landfill leachate were tested by ICP optical emission spectrometer (715-ES, varian, United States) and Thermo Scientific (M6, Thermo, United States), respectively. Landfill leachate was diluted 200 times with purified water and filtered through a 0.2 μm membrane to measure TOC and TN on a TOC-V (CPH-CPN, Shimadzu, Japan).

### **Flocculation of TOC and TN in landfill leachate by increasing pH**

Initial pH 4.5 of landfill leachate was gradually increased to 7.0, 8.0, 9.0, 10.0 by directly adding 1mol/L sodium hydroxide, respectively, and after standing two hours, the supernatant of landfill leachate at pH 9 was used to culture *R. palustris*.

### **Effects of nutrients added on the growth of *Rhodopseudomonas palustris***

The supernatant of 1500 mL landfill leachate at pH 9 was added 3.0 g/L sodium acetate as carbon source, 1.0 g/L urea as nitrogen source, or 1.0 g/L disodium phosphate as phosphorus source, respectively, and then inoculated with 450 mL of *R. palustris* (v/v, 30%) prepared and initial pH was adjusted to 8.0, which was divided into 100 mL transparent plastic bottle to anaerobic culture *R. palustris* under sunlight outdoor at the temperature from 20°C to 40°C. The samples of culture solution were taken every day to measure optical density at wavelength of 500 nm (OD<sub>500nm</sub>) on a spectrophotometer (722s, Chain) to represent biomass of *R. palustris*, pH (PHS-25, Leici, Chain), TOC and TN. The average values of three samples with the standard deviation were indicated in figures.

### **Measurement of the cell density of *Rhodopseudomonas palustris* with flow cytometry**

A flow cytometer (Partec CY-S-3001, Germany) was used to measure the cell density of *R. palustris*. For a flow cytometer measuring, the gain numeric of handle system parameter was set as follows: FSC 200, SSC 240.

To measure cell density of *R. palustris*, the culture solution of 100 µL was taken and diluted with phosphate buffer solution (PBS; pH 7.0) to 1000 folds, and the sample of 800 µL was prepared in a 2 mL transparent test tubes for the detection of cell density with flow cytometer at the excitation and emission wavelengths of 488 nm at room temperature. Each sample was pumped at 2 ms<sup>-1</sup> for about 1.5 min.

### **Determination of cell viability of *Rhodopseudomonas palustris* by dual fluorescence assay**

A double-staining fluorescence procedure using fluorescein diacetate (FDA, Sigma–Aldrich) and propidium iodide (PI, Aladdin) was used to determine the cell viability of *R. palustris*. FDA labeled cells are considered as viable cells with

effective enzymatic activity, but PI labeled cells are considered as dead cells with compromised membranes. The fluorescence signals were detected in filter channel1 (FL1) and filter channel 2 (FL2), respectively<sup>26,27,28</sup>.

For flow cytometer measuring, the gain numeric of handle system parameter was set as follows: FSC 200, SSC240, FL1 300, FL2 330. 100 mL culture solution of *R. palustris* at 72 h was taken as fresh cells and 100 mL fresh cells was heat-killed in a water bath at 80°C for 30 min as dead cells. The mixtures of 100 mL fresh cells and 100 mL heat-killed cells was used as the control sample for analyses. Fresh cells were stained with FDA and heat-killed cells were stained with PI, and mixture cells were stained with both PI and FDA.

For analyzing the live cells of *R. palustris* cultured with landfill leachate, 1mL of sample was placed into a centrifuge tube and stained with 10 µL FDA working solution (5 mg/mL) and 30 µL PI working solution (1 mg/mL). The stained sample was then incubated in 30°C cupboard for 30 minutes in dark and then for the measurement according to the report<sup>28</sup>.

### **Statistical analysis**

Three repetitions of each experiment were performed and the average values were indicated in figures. Tukey's test was adopted to analyze the significance of data. The level of significance of different groups exceeded 95% (P < 0.05).

## **RESULTS AND DISCUSSION**

### **Characterization of landfill leachate**

The physiochemical properties of landfill leachate used in this experiment are shown in Table 1, which contained a high organic matter including TOC of 16.0 g/L and TN of 1.1 g/L, respectively. The content of NaCl was 15.2 g/L and the various metals such as Cd, Cr, Cu, Pb, Ni and Zn were also found in landfill leachate with low pH of 4.5.

Similar to the report by Qin et al.<sup>29</sup>, the landfill leachate from food processing as a high-strength wastewater was rich in organic contaminants, nitrogen, toxic metal and soluble salts with low pH (Table 1). The treatment of landfill leachate is challenging due to the high levels of contaminants including organics,

**Table 1.** Physiochemical characteristics of landfill leachate.

Parameter	Content
TOC (g/L)	1.6
TN (g/L)	1.1
NaCl (g/L)	15.2
Mg (g/L)	102
P (mg/L)	300
Cu (mg/L)	≤ 0.1
Pb (mg/L)	0.13
Ni (mg/L)	0.12
Zn (mg/L)	0.48
pH	4.5

ammonia, inorganic substances, toxic metals and toxic hydrocarbons (aromatic and phenolic compounds) together with the variability in its quantity and quality in both space and time<sup>30,31</sup>.

#### Effect of pH on the flocculation of organic compounds in landfill leachate

The changes of TOC and TN in landfill leachate as well as picture at different pH are shown in Figure 1. The original solution at pH 4.5 was very turbid with little sediment. With the increase of pH from 7 to 10 respectively, the larger amounts of precipitate was produced (Fig. 1a). TOC and TN in the supernatant of landfill leachate decreased gradually from 16.0 g/L and 1.1 g/L to 14.8 g/L and 0.9 g/L, respectively (Fig. 1b).

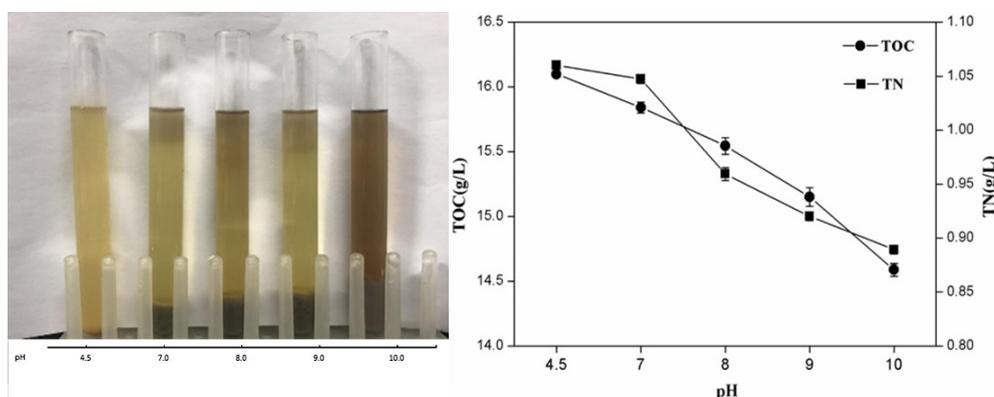
Generally the flocculation was achieved by adding flocculants such as ferric chloride (FeCl<sub>3</sub>) and polyaluminium chloride<sup>32</sup>. Better organic matter removal was observed when leachate was treated with FeCl<sub>3</sub><sup>33</sup>. Here we found that the organic compounds in landfill leachate can be

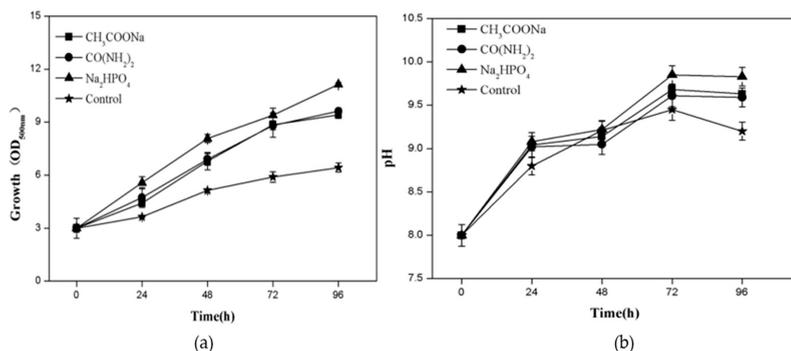
flocculated simply by increasing pH (Fig.1), which was not reported and probably attributed to the electrolytic rate of solution affected by different pH<sup>34</sup>. Furthermore the removal of TN from landfill leachate might be partly caused by the evaporation of ammonia gas produced by the increase of pH.

#### Effects of nutrients added on the growth of *Rhodospseudomonas palustris*

Figure 2 showed the growth of *R. palustris* and pH of culture solution by adding sodium acetate, urea and disodium phosphate, respectively. Compared with that of control, the growths of *R. palustris* were obviously promoted by adding carbon, nitrogen and especially phosphorus sources, respectively, and the highest OD500nm of 11.3 was obtained at 96 h (Fig. 2a). The results also indicated that pH were all gradually increased from 8.0 at 0 h to more than 9.0 at 96 h (Fig. 2b). Generally organic acids remained in landfill leachate could be used as carbon source by *R. palustris*, which might cause the increase of pH<sup>35</sup>.

Phosphorus as a nutrient element and stimulating factor of enzyme was found to play an important role in both hydrogen production and the growth of *R. palustris*<sup>36</sup>. Qin et al. also reported that PSB were not taking up the available carbon efficiently in phosphorus-limited condition<sup>29</sup>. Here we indicated that, compared with carbon and nitrogen sources, phosphorus was a most limiting factor in the growth of *R. palustris* using landfill leachate as culture medium (Fig.2a). Furthermore pH played an important role in the growth of *R. palustris*, which grew in the pH range from 6.5 to 10.0, and the appropriate range was from 8.0 to 10.0<sup>37</sup>. pH has an effect on the structure of the

**Fig. 1.** Removals of TOC and TN from landfill leachate by increasing pH.

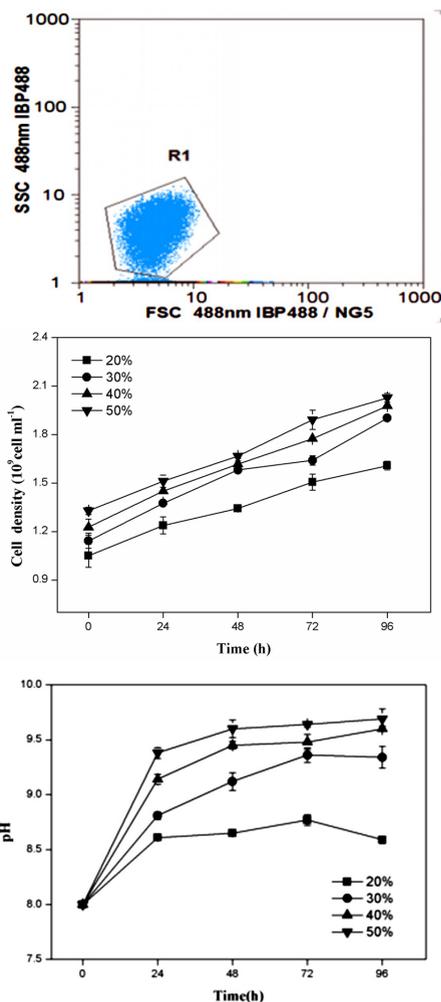


**Fig. 2.** Effects of CH<sub>3</sub>COONa, CO(NH<sub>2</sub>)<sub>2</sub> and Na<sub>2</sub>HPO<sub>4</sub> on the growth of *R. palustris* in landfill leachate, growth (a) and pH (b).

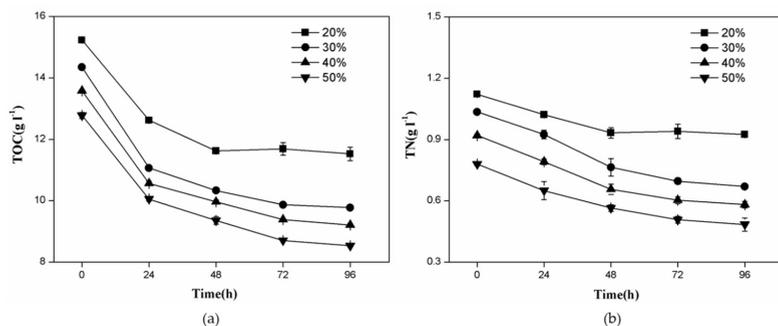
pigment-protein complex and the stability function of the entire cyclic structure. Low pH induced the deletion of B800 bacterial chlorophyll molecule in the LH2 complex of *Rhodobacter capsulatus*<sup>38</sup> and *Rhodospseudomonas acidophila*<sup>39</sup>. Here we found that the change of pH range from 8.0 to 10 was suitable to the growth of *R. palustris* using landfill leachate as culture medium (Fig.2b).

**Effects of inoculums on the growth of *Rhodospseudomonas palustris* in landfill leachate**

As shown in Fig. 3a, the cell scatter diagram of *R. palustris* (R1) could be clearly shown with flow cytometer. The cell densities of *R. palustris* with different inoculums from 20% to 50% (v/v) were all increased with time course in the period of 96 h and the highest cell density of 2.0×10<sup>9</sup> cell/mL was obtained with the inoculation proportion of 50% (Fig.3b). pH of culture solutions also raised to 9.0 or so with the different inoculums of *R. palustris* at 96 h (Fig. 3c).



**Fig. 3.** Effects of the different inoculums on the growth of *R. palustris* in landfill leachate, the cell scatter diagram of *R. palustris* (a), the cell densities of *R. palustris* (b) and the change of pH (c).



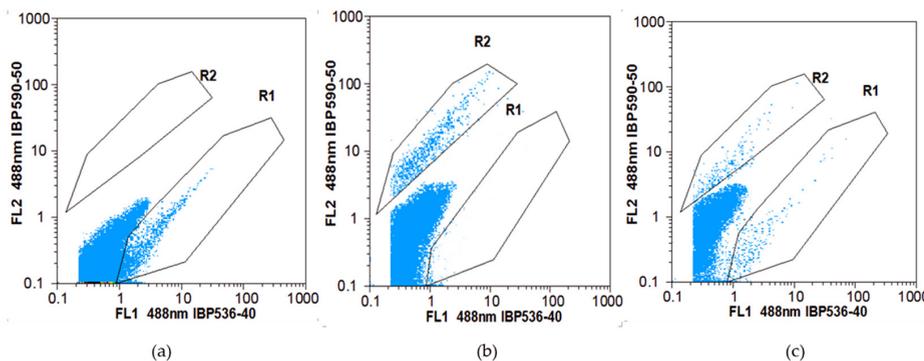
**Fig. 4.** Effects of the different inoculums of *R. palustris* on the removals of TOC (a) and TN (b) from landfill leachate. proportion of 10% (v/v), PSB were difficult to grow and form dominant bacteria, and the contaminating bacteria and odors are generated. When inoculum proportion was increased, both the growth of PSB and the removal of COD in the treatment of brewery improved<sup>40</sup>. Many studies concentrated on the removals of pollutants by PSB<sup>41,42</sup>, but less information focused on the growth of PSB as bacterial manure, which ignored the resource utilization of landfill leachate. **The viability and integrity of *Rhodopseudomonas palustris***

As shown in Figure 5, more than 99% of fresh cells (R1) displayed a green fluorescence with a corresponding fluorescence intensity (Fig.5a). In contrast, 98% of heat-treated cells (R2)

exhibited a red fluorescence intensity (Fig.5b). When the mixture of fresh and heat-killed cells was measured using FDA-PI double-fluorescent, the live cells (R1) were almost equivalent to the dead cells (R2) (Fig.5c).

**The live *R. palustris* grown on landfill leachate with time course**

Figure 6 indicated the total and live cell densities as well as the ratio of live cells using FDA-PI double-fluorescent staining in conjunction with a flow cytometer. The results showed that the live cell densities of *R. palustris* were almost same to total cell densities of *R. palustris* during the period of 96 h and the highest proportion of live cells of 99.4% was obtained at 72 h.

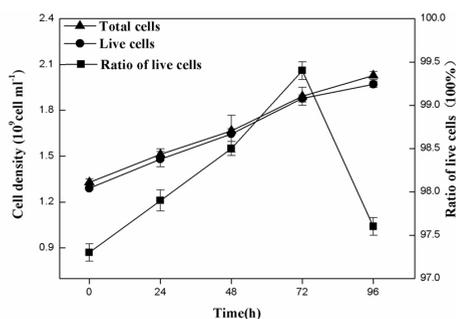


**Fig. 5.** Identification of live and dead cells of *R. palustris* with FDA-PI double-fluorescent staining. Dot plots representing carboxyfluorescein (R1) and propidium iodide fluorescent (R2) signals of *R. palustris*. Freshly harvested cells stained with FDA (a) and heat-killed cells were stained with PI(b). Multiparameter dot plot obtained after FDA-PI double staining of freshly harvested cells and heat-killed cells (c).

Many studies focused on the state of lactic acid bacteria with double-fluorescent staining in conjunction with a flow cytometer<sup>27,43,44</sup>. Here the viability and integrity of *R. palustris* grown on landfill leachate as culture medium were successfully assayed (Fig. 5), which was not previously reported and very important in the

culture of the live cells of *R. palustris* as bacterial manure.

Generally many studies paid attention on the removals of TOC and TN from landfill leachate, but less information concentrated on the growth and live cells of PSB. As a typical bacterial strain of PSB, *R. palustris* can be used in many areas



**Fig. 6.** The live *R. palustris* grown on landfill leachate with time course.

such as bacterial manure<sup>7</sup>, food coloring agent, precursor of vitamin A in food and animal feed,  $\beta$ -carotene and astaxanthin in industry, and additives to porphyrin<sup>8,9</sup>, so the efficient culture of *R. palustris* is very important in the resource utilization of organic waste water. Here both the treatment of landfill leachate and the growth of *R. palustris* were investigated together, which is benefit to culture *R. palustris* as bacterial manure with the efficient utilization of landfill leachate as culture medium.

## CONCLUSION

Alkaline condition could cause the flocculation and the removals of TOC and TN from landfill leachate. The growth of *R. palustris* in landfill leachate could be much improved by adding phosphorus. With the increase of inoculation ratio, both the growth of *R. palustris* and the removals of TOC and TN from landfill leachate were all enhanced. *R. palustris* maintained the good viability and integrity during the culture period of 96 h, which was detected with the method of FDA-PI double-fluorescent staining. This study revealed a biotechnology for the resource utilization of landfill leachate to culture *R. palustris* as bacterial manure.

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