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# Treatment of anaerobically digested swine wastewater by *Rhodobacter blasticus* and *Rhodobacter capsulatus*

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## HIGHLIGHTS

- *R. blasticus* or *R. capsulatus* or both were used for treatment of anaerobically digested swine wastewater.
- Treatment performance and biomass production were examined.
- The mixed-PSBs produced more biomass than any unitary strains at same COD uptake.
- The mixed-PSBs achieved high COD removal and cell yields.

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## ABSTRACT

Two strains of photosynthetic bacteria, *Rhodobacter blasticus* and *Rhodobacter capsulatus*, were used in this work to investigate the feasibility of using photosynthetic bacteria for the treatment of anaerobically digested swine wastewater. The effects of crucial factors which influence the pollutants removal efficiency were also examined. Results showed that anaerobically digested swine wastewater could be treated effectively by photosynthetic bacteria. The treatment efficiency was significantly higher by the mixed photosynthetic bacteria than that by any unitary bacterium. The optimal treatment condition by mixed bacteria was inoculation of 10.0% (v/v) of the two bacteria by 1:1, initial pH of 7.0 and initial chemical oxygen demand of 4800 mg $L^{-1}$. Under these conditions, the removal rate of chemical oxygen demand was 83.3%, which was 19.3% higher than when using *Rhodobacter blasticus* or 10.6% higher than when using *Rhodobacter capsulatus* separately. This mixed photosynthetic bacteria achieved high chemical oxygen demand removal and cell yields.



## 1. Introduction

China, one of the world's largest producers of pork, has approximately 48.7% of the world's pork eating population with breeding 977 million pigs in the year 2013 according to the statistic of Food and Agricultural Organization (FAO) (Luo et al., 2016). As a result, swine wastewater has become a significant agricultural pollutant, and the treatment of swine wastewater has already been an urgent problem for China. However, most of the swine industry is located in rural areas of China, where it is unfeasible to invest the high construction and operation costs to build sewage treatment plants (Zhang et al., 2016). Anaerobic digestion of livestock wastewater converts organic matter into biogas allowing the production of renewable energy (Sui et al., 2014), which has the advantages in simple process, low cost compared with traditional sewage treatment system, thus it has been vigorously promoted by the government. Nevertheless this technique produces large amounts of anaerobically digested liquid. The waste slurry left over from the biogas digester is not only rich in nutriments (Guo et al., 2013), inorganic salts, amino acids and B vitamins, but it also contains trace elements such as Cu, Fe, Zn (Kuo et al., 2012). All these materials in anaerobically digested swine wastewater would result in serious environment pollution such as eutrophication and nutrient

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waste if it has been discharged directly into river without any treatment (Anam et al., 2012).

Two wildly used approaches in treating anaerobically digested wastewater are (1) ecological processes, including oxidation pond, artificial wetland, soil infiltration (Zhao et al., 2014); and (2) advanced biochemical processes, such as sequencing batch reactors (SBRs) (Yan et al., 2007), oxidation ditch (Poach et al., 2007), single reactor system for high activity ammonium removal over nitrite (SHARON–ANAMMOX), and completely autotrophic nitrogen removal over nitrite (CANON) (Daverey et al., 2013). However, previous studies showed that because of the poor biodegradability and low COD/$NH_3$-N ratio in effluent, the efficiency of both methods was not satisfactory, and they also have the shortages of large area occupied, low treatment efficiency and sludge production (Zhao et al., 2014). Thus, the application of photosynthetic bacteria (PSB) in wastewater treatment has been concerned by more researchers in recent years, which not only costs less with a high efficiency, but also producing none activated sludge. PSB are a large class of microorganisms which can utilize different kinds of nutrients as carbon and energy source, and they can widely distribute in soil, paddy, swamp, lakes and ocean (Trüper and Imhoff, 1989). In 1970s', Japanese researchers found that PSB had the capability to purify polluted water (Kobayashi and Tchan, 1973). Since then, PSB have been used to treat various wastewaters such as skim latex wastewater, food processing wastewater, fish industry effluent, fermented starch wastewater, and sugar wastewater (Choorit et al., 2002; Kaewsuk et al., 2010; De Lima et al., 2011; Prachanurak et al., 2014; Zhou et al., 2014). These studies showed that PSB can efficaciously reduce the chemical oxygen demand (COD) of the wastewater which ranges from 560 to 9750 mg $L^{-1}$. Apart from the ability of degrading pollutants under a high COD load, PSB can also accumulate useful by-products such as single-cell protein, biopolymers, carotene, pantothenic acid, antimicrobial agents, and therapeutic compounds (Zhou et al., 2015a) and these by-products can be utilized in aquaculture and agriculture.

Several studies revealed that PSB has the potential to treat wastewater and recover biomass. It has the function to treat different kinds of wastewater, such as white spirit wastewater (Wang et al., 2016), sugar wastewater (Zhou et al., 2015b) citric acid wastewater (Zhou et al., 2016), municipal wastewater (Lee et al., 2015) and soybean wastewater (Wu et al., 2015). Physiologically, *Rhodobacter* species are among the most versatile species of the photosynthetic bacteria, which can perform a number of different growth modes (Garrity et al., 2006) and suitable for growing in various kinds of wastewater. However, relevant study about *Rhodobacter* species applied on digested wastewater treatment is scant.

In this work, two *Rhodobacter* strains of PSB, *Rhodobacter blasticus* and *Rhodobacter capsulatus*, were used to examine the feasibility of COD removal and biomass accumulation in anaerobically digested swine wastewater treatment, and some crucial factors were tested to examine their influences during the process. *Rhodobacter capsulatus* shows the major features generally considered to be characteristic of *Rhodobacter* species, which is hardy and stable to long-term storage under appropriate conditions (Weaver et al., 1975). Conversely, *Rhodobacter blasticus* differs from other species of the genus *Rhodobacter* by its replication mode (budding) and by the circular lamellar structure of its photosynthetic membranes (Garrity et al., 2006). Therefore, the application potential of *Rhodobacter blasticus* and *Rhodobacter capsulatus* in wastewater processing are well worth studying. Besides, whether the mixed-PSB of the two photosynthetic bacterium strains played a positive role in promoting treatment efficiency has been studied. Also, this study will contribute to broaden the application areas of PSB wastewater treatment technology.

## 2. Materials and methods

### 2.1. Microorganism strain and culture conditions

Two PSB strains, *Rhodobacter blasticus* (*R. blasticus* strain CGMCC 1.3365) and *Rhodobacter capsulatus* (*R. capsulatus* strain CGMCC 1.3366), were purchased from the China General Microbiological Culture Collection Center (CGMCC). They were applied for all the following experiments.

The pre-cultivation for strains was maintained in RCVBN medium (Weaver et al., 1975). Two loops from *R. blasticus* or *R. capsulatus* slant culture were used to inoculate 200 mL of the RCVBN medium in a 500-mL Erlenmeyer flask. The strains were cultivated at 30 °C and a light intensity of 3000 lx conditions for 5 days.

### 2.2. Piggery wastewater

The anaerobically digested swine wastewater was collected from a local piggery farm (26°36′95″ N, 112°05′08″ E) (Hunan Province, China) which used the anaerobic digestion tank for a typical mesophilic anaerobic digestion of organic substrates. Prior to experimentation the wastewater was centrifuged in 9000 rmp for 10 min to remove any visible particulate solids, and stored at 4 °C in order to avoid the variation of wastewater composition during the experiment period. Therefore, only the soluble fraction of the wastewater was used for the biodegradation test. The concentrations of chemical oxygen demand (COD), $NH^{4+}$-N and TP in the supernatant wastewater were 4792.5 ± 50, 913.2 ± 20, and 20.7 ± 0.3 mg $L^{-1}$, respectively, and the pH value was 7.9 ± 0.1.

### 2.3. Operation and evaluation

For all experiments, the bioreactors were the 500 mL conical flasks, which were sterilized at 121 °C for 30 min before experiments. During the experiment, 200 mL anaerobically digested swine wastewater was added to the bioreactor each time after sterilization by autoclave.

All the experiments were undertaken in a constant temperature (30 °C) illumination incubator, the parallel fluorescent bulbs positioned on the side of flasks acted as the light source, and the intensity of illumination at the surface of the bottles were about 3000 lx for wastewater treatment. These stock culture were inoculated to anaerobically digested swine wastewater at a volume ratio of 1:10 except for where it was noted.

For the feasibility study, the treatment efficiency of *R. blasticus*, *R. capsulatus* and the mixed-PSB were tested. The mixed-PSB was a stock culture by mixture of *R. blasticus* and *R. capsulatus* at a volume ratio of 1:1. Initial pH of 7.0, 10.0% (v/v) inoculum size was used in feasibility study according to previous literature (Hülsen et al., 2014; Zhou et al., 2015a).

For the optimization studies, five mixture proportions of *R. blasticus* and *R. capsulatus* were designed to explore the detailed impact of each strain. The total inoculation size was 10.0% (v/v) and the proportion of *R. blasticus* to *R. capsulatus* were 4:1, 2:1, 1:1, 1:2 and 1:4, respectively. Five levels of initial strains inoculation rates tested were 2.0%, 5.0%, 10.0% and 20.0% (v/v). Five levels of initial pH tested were 6.0, 7.0, 8.0, 9.0 and 10.0; 1000 mmol $L^{-1}$ NaOH or HCl was used to adjust the initial pH. Four levels of initial COD tested were 4800, 2400, 1200 and 600 mg $L^{-1}$.

All experiments were conducted in duplicates, and the reported results were the average values.

### 2.4. Analytical methods

Liquid samples of 10 mL were collected from bioreactors and were centrifuged at 9000 r/min for 10 min every 24 h. The collected PSB were used to measure the biomass (dry weight); the supernatant was used to test the COD and pH according to the national standard methods. The Dichromate Methods were put to measure chemical oxygen demand (COD) in the water samples by APHA standard methods (Eaton et al., 2005); pH were measured in a pH meter (PHS-3C). Light intensity was determined by TES-1330A digital light meter.

## 3. Results and discussion

### 3.1. Feasibility study

Prior to the exploring of wastewater treatment characteristic by photosynthetic bacteria, the stock culture of *R. blasticus*, *R. capsulatus* and the mixed-PSB were used to investigate the feasibility of anaerobically digested piggery wastewater treatment. In detail, the results could be seen in Fig. 1.

As the result of the observation, COD continuously decreased with the time moving forward, meanwhile, the bacteria proliferated in a fast rate until it reached an inflection point. Comparison test showed that the microbial community had superiority over the two strains for the degrading capacity and biomass growth rate. 83.3% of COD was removed at the 120th hour by mixed culture of *R. blasticus* and *R. capsulatus*, which was higher than that by *R. blasticus* (67.2%) and *R. capsulatus* (74.5%) separately. The maximal cell concentration of mixed-PSB can up to 852.3 mg $L^{-1}$, which increased by 29 times. Thus it can be seen that anaerobically digested swine wastewater was non-toxic and feasible for photosynthetic bacteria growth, and biotreatment of wastewater by co-treatment of *R. blasticus* and *R. capsulatus* was more efficient than treatment by unitary strains separately. For the treatment of anaerobic digested swine wastewater in previous studies, Yan et al. (2007) reported a full-scale SBR process and only obtained a removal of COD of 48.7%, the CANON reactor (Daverey et al., 2013) increased the processing efficiency of COD removal to 64.0%–83.0%, however, it used more than 250 days process time. Oxidation Pond is considered to be a low-cost and technically feasible process for treating anaerobic digested swine wastewater, but the deficiencies of large area occupied, overgrowth of algae and the seasonal change influence restricted the utilization of pond treatment system (Zhao et al., 2014). Compared with the general approached process, PSB process has the advantages of bioenergy and nutrient recovery, which is believed to be a promising option for the treatment of anaerobic digested swine wastewater.

In the lab-scale feasibility study, the anaerobic digested swine wastewater was centrifuged and sterilized to rule out other large particles and microorganism's interference with the experimental results, but the pre-treatment by autoclave is energy-intensive and complex for real operation. However, without sterilizing the system was vulnerable subject to contamination by algae and other bacteria, contributing to the main bottleneck of the amplification of cultivating photosynthetic bacteria in anaerobically digested swine wastewaters in larger scale in the future. To solve this problem, ensuring the photosynthetic microbes become the dominant microflora in the system is necessary, which can restrain other harmful bacterial breeding in process, and the initial inoculation capacity suitable to the PSB in non-sterilized wastewater is 20.0%–30.0% (v/v) (Tang et al., 2002). Besides, keeping the effluent at pH 7.0–8.0 can promote the continuing growing of PSB. The multiply of bacteria and the rising pH of system promote the sedimentation of PSB bacteria so that it can be recycled easily after treatment process. This PSB treatment process can achieve high chemical oxygen demand removal and cell yields with simple process, and useful materials such as single-cell protein, carotene, pantothenic acid, and therapeutic compounds can be accumulated in the process (Kuo et al., 2012), thus the excessive PSB biomass could be recycled as useful raw materials such as animal feed additive in agriculture industries, which was beneficial to the promotion of the economic interests.

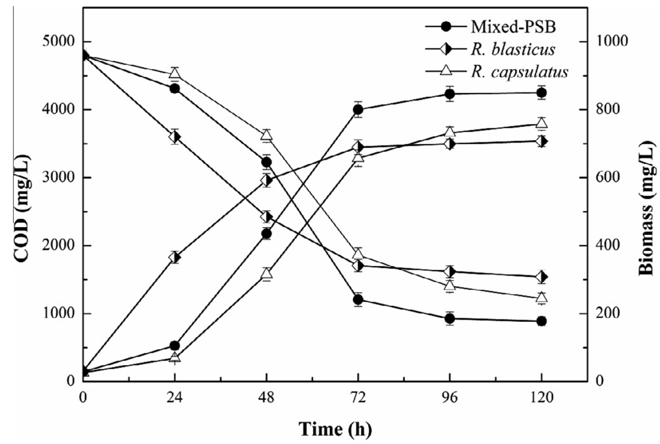


**Fig. 1.** COD removal and biomass increase of *R. blasticus*, *R. capsulatus* and mixed-PSB in anaerobically digested swine wastewater treatment.

### 3.2. Effects of mixture proportion of two strains on anaerobically digested swine wastewater treatment

In order to pursue the concrete quantitative contribution of these two PSB strains respectively on the degradation of COD in bioreatment, five mixture proportions of *R. blasticus* and *R. capsulatus* have been conducted, and the COD removal rate and biomass yields have been showed in Fig. 2. Biomass yields were defined as biomass-increase/COD-removal.

Note that at first 72 h, the higher proportion of *R. blasticus* in initial inoculum, the faster of removal rates, but ultimately the COD removal rates didn't appear to be much different by different mixture proportion of the two strains. This can be explained by that the growth rate of *R. blasticus* was faster than the *R. capsulatus* at the first 72 h, but after that, due to the nutritional stress, the *R. blasticus* population abandoned exponential growth and entered a non-growth state called stationary phase (Huisman et al., 1996). Compared with the *R. blasticus*, the adaptive phase of *R. capsulatus* was longer, at first 48 h the *R. capsulatus* grew slowly, and then the growth rate increased greatly. On the whole the mixture proportion had no significant changes in the final COD concentration. However, as it showed in Fig. 2b, the biomass yield of five proportions varied a lot and the proportions of 1:1 was higher than other groups, which presumably due to the preferable growth stimulatory between co-treatment of bacteria. The increase of biomass yield was very important since it meant that more biomass could be obtained with the same amount of wastewater COD (Zhou et al., 2016). So from the point of biomass yields, the proportion of *R. blasticus* to *R. capsulatus* of 1:1 was the optimal mixture proportions.

When the initial total cells which inoculated to anaerobically digested swine wastewater at a volume ratio of 1:10 was decreased to 1:20, the results showed the same trends (data not shown).

### 3.3. Effects of inoculation size of mixed-PSB on anaerobically digested swine wastewater treatment

Inoculation rates is one of the most important control factors of cells growth rate and COD removal processes. In a certain range, the increasing inoculation can improve the degradation efficiency; however, with more inoculation amount, biomass increased and specific growth rate decreased, too much inoculation may cause a lack of nutrients which affect the rate of cell growth, even lose

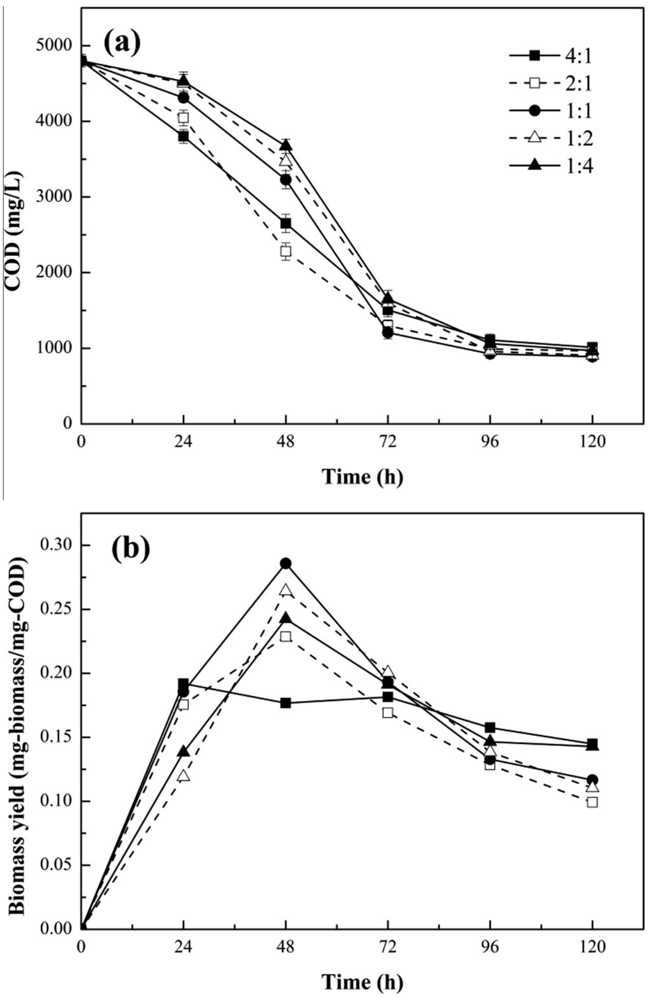


**Fig. 2.** Effects of different mixture proportion of *R. blasticus* to *R. capsulatus* on (a) COD removal and (b) biomass yields in anaerobically digested swine wastewater treatment.

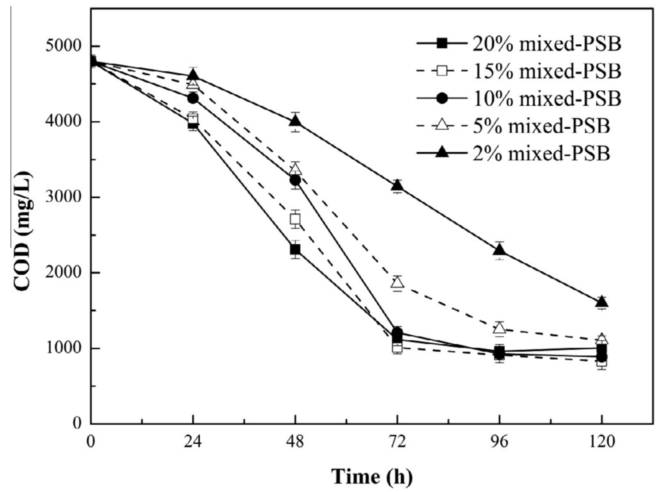


**Fig. 3.** Effects of different inoculation of mixed-PSB on COD removal in anaerobically digested swine wastewater treatment.

the ability of breeding and cause self-decomposition to release organic matter. Thus, it leads to the decline of the removal rate of organics. Therefore, appropriate inoculation quantity was needed for the removal and degradation.

A set of tests was performed at variable inoculation size from 2.0 to 20.0% and the effect of inoculation rates on COD removal in swine wastewater treatment by mixed-PSB has been showed in Fig. 3. When the inoculation was below 5.0%, the cells showed poor activity and low concentration, causing relatively low treatment efficiency. On the inoculation of 5.0%–15.0%, the increased inoculation promoted the efficiency of degradation. However, compared with the inoculation of 10.0% and 15.0%, the efficiency of degradation and biomass growth didn't increased exponentially. At inoculation of 20.0%, the COD removal reached the highest at the 72th hour and then dropped since in-sufficient food in the wastewater caused self-decomposition of bacteria and increase of COD. Although growing rapidly, the cells entered its stable phase and even decline phase in a short period of time. Thus, for the point of cost-effectiveness and degradation efficiency, the optimal inoculum concentration was 10.0%.

### *3.4. Effects of initial pH on anaerobically digested swine wastewater treatment by mixed-PSB*

The solution pH is considered to have a huge influence on microorganism, which changes the difference between the charge inside and outside cell, thus cause the changes of nutrimental absorption to promote or suppress cell growth (Fraser et al., 1999; Peng et al., 2014). The appropriate pH ranged from 5.5 to 9.4 for most PSB. Excessively high or low pH makes the catalytic action of enzyme decrease or disappear (Koku et al., 2002). Thus, the effects of different initial pH (6.0–10.0) on COD removal rate and biomass were examined to understand the effects of initial pH on anaerobically digested swine wastewater degradation with PSB.

The results showed that COD removal rate was higher than 80.0% when the initial pH was 7.0–9.0, but pH of 7.0 was optimal for PSB growth. It reconfirmed that a neutral pH usually required on PSB wastewater treatment and pre-adjustment of wastewater pH to approximately 7.0 was widely adopted (Honda et al., 2006; Nagadomi et al., 2000). Due to the multiplication of photosynthetic bacteria in treatment process, the pH value of effluent increased gradually until the PSB population entered stationary phase. At the condition of pH 7.0, the biomass of mixed-PSB in treatment process was increased by 7.97% and 15. 97% when compared with the process of pH 8.0 and pH 9.0 separately. Thus adjusting the initial pH of wastewater in process to keep the effluent in neural or mild alkali conditions can promote the PSB to grow continuingly. When the pH was above 10.0 or below 6.0 cell growth was inhibited. The cells were precipitated and died eventually. The final pH of bioreactors were on average 9.2 after 120 h treatment, and the increase was possible due to the removal of organic acids and phototrophic uptake of $CO_2$.

### *3.5. Effects of initial COD of mixed-PSB on anaerobically digested swine wastewater treatment*

The effects of initial COD in wastewater on organic matter degradation and microbial growth have been investigated (Wang et al., 2016; Zhou et al., 2016). The organic matter in wastewater provides essential nutrients to the growth of microorganism. However, too high or low concentration of the wastewater affects the osmotic pressure on microbial cells, which disadvantageous at nutrient absorption and bacterial growth and propagation.

In this study, the anaerobically digested piggery wastewater was diluted to 4800, 2400, 1200, and 600 mg $L^{-1}$, respectively. And the effects of initial COD on PSB wastewater treatment were shown in Fig. 4. The results showed that by diluting the anaerobically digested piggery wastewater, the COD degradation efficiency decreased with the dilution of anaerobically digested piggery

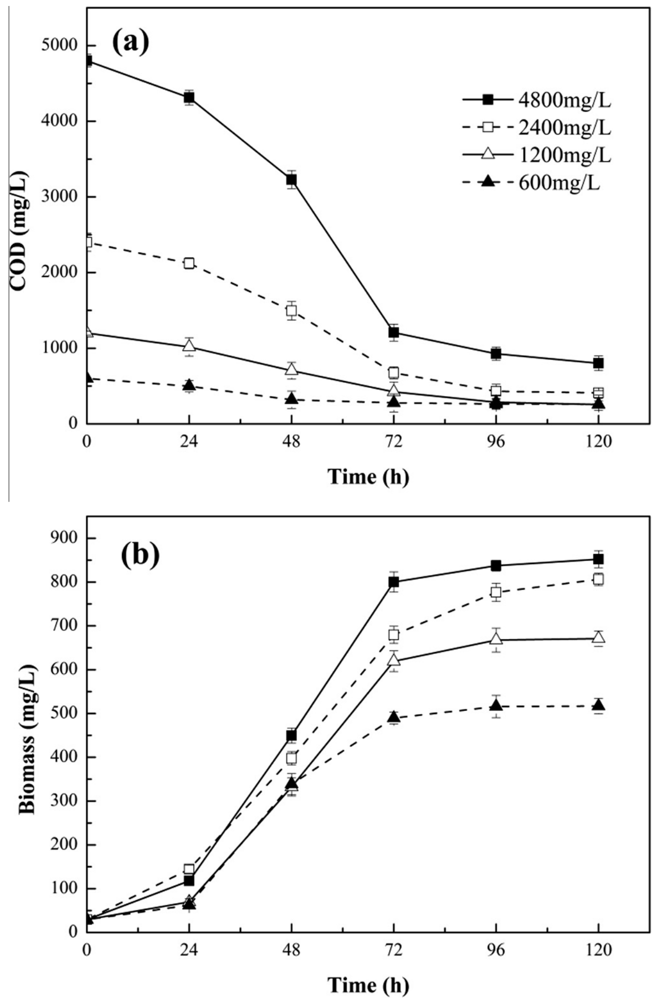


**Fig. 4.** Effects of initial COD on (a) COD removal and (b) biomass yields of mixed-PSB in anaerobically digested swine wastewater treatment.

wastewater. When the COD initial concentrations were 4800, 2400, 1200 and 600 mg $L^{-1}$, the COD degradation efficiency were 83.3%, 82.9%, 78.7% and 56.8% (Fig. 4a), respectively. By diluting the wastewater, the biomass was also decreased (Fig. 4b). This findings contrast with the research results by Kim et al., which observed phototrophic COD removal could be problematic without dilution in odorous swine wastewater treatment by PSB (Kim et al., 2004). This may because the initial COD concentration (10,000–20,000 mg $L^{-1}$) of odorous swine wastewater is far higher than the anaerobically digested swine wastewater (600–5000 mg $L^{-1}$) of this study. On the condition of high COD concentration, the dilution was necessary for bacterial growth, otherwise, the osmotic pressure and the concentration of toxic substances in wastewater was too high for the microorganism to survive. However, on the condition of low COD concentration, with the dilution of wastewater the organic and inorganic components and nutrients were not enough for microorganism to growth and proliferation, thus the degradation of wastewater became weak and ineffective.

## 4. Conclusions

Photosynthetic bacteria can assist with wastewater purification and useful biomass recovery, and the mixed culture of *Rhodobacter blasticus* and *Rhodobacter capsulatus* can promote degradation efficiency. On the condition of 1:1 mixture proportion of two bacterium, 10.0% (v/v) inoculation size, initial pH at 7.0 and initial chemical oxygen demand of 4800 mg $L^{-1}$, chemical oxygen demand removal rates increased to 83.3%, which is 19.3% and 10.6% higher than that by *Rhodobacter blasticus* and *Rhodobacter capsulatus* separately, the corresponding biomass was 852.3 mg $L^{-1}$. These results can serve as the foundation for further scale-up trials applying mixture of photosynthetic bacteria strains for nutrient removal and biomass accumulation in wastewater treatment field.

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