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Optimization of photosynthetic bacteria wastewater treatment and study of microbial species diversity

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ABSTRACT

The photosynthetic bacteria (PSB) wastewater treatment was optimized in terms of light and oxygen conditions, pH, temperature, initial PSB, and wastewater concentrations. The changes of microbial species were studied using PCR–DGGE technique. The results showed that in batch reactors the optimum conditions of PSB soybean wastewater treatment were natural light-micro oxygen, initial PSB concentration of 160 mg/L, initial wastewater concentration of 3,600 mg/L, pH of 8.0, and temperature of 30°C. In semi-continuous reactors, the results verified that under the optimum conditions PSB soybean wastewater treatment was effective and PSB biomass increase was good. PCR–DGGE analyses showed that the types of micro-organisms in the reactor gradually increased. Under the optimum conditions, *Lactococcus lactis subsp.* formed on the 7th day; *Veillonella sp.* and *Dysgonomonas sp.* formed on the 14th day; and iron-reducing bacterium and *Veillonella sp.* formed and *Lactococcus lactis subsp.* disappeared on the 21st day; *Janthinobacterium lividum* formed on the 28th day; and the community did not change from the 28th to 35th day; after that the system entered a stable situation. PSB were the dominate bacteria during the whole period.

Keywords: PSB; Wastewater treatment; Natural light-micro oxygen condition; Microbial species; PCR–DGGE

1. Introduction

Photosynthetic bacteria (PSB) are the earliest prokaryotic micro-organisms of initial photosynthesis system on earth [1]. They are widely spread in seas, rivers, lakes, and soil. Rhodospirillales can carry on photosynthesis in anaerobic conditions and do not release oxygen [2]. In dark-aerobic or light-anaerobic conditions PSB use organic material, such as sulfide, ammonia, and carbohydrates for biomass growth.

PSB can effectively treat high concentrated organic wastewater such as starch processing wastewater [3], soybean wastewater [4], beer wastewater [5], oil wastewater [6], industrial and domestic wastewater [7,8]. COD removal by PSB can reach 60–95%. Furthermore, the PSB biomass contains many nutrients and physiologically active substances [9,10], which can be used as the supplement in fertilizer [11], feed [12], and bait [13]. Therefore, PSB wastewater treatment can realize pollutant removal and useful biomass accumulation simultaneously.

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Most literatures reports used sterilized wastewater in PSB wastewater treatment studies in order to avoid the pollution of contaminating bacteria [14]. However, in actual wastewater treatment plants, it is infeasible to sterilize the wastewater. The raw wastewater and air might bring in contaminating bacteria. Results obtained with sterilized wastewater in laboratory cannot be directly applied to the real world.

Therefore, in this paper PSB together with contaminating bacteria were used to treat soybean wastewater in order to simulate the real-world wastewater treatment situation. The activated sludge was used to simulate contaminating bacteria. According to practical wastewater treatment, through a long running, PSB biomass might become less and less, and contaminating bacteria might become more and more. Then the value of recovery and utilization of PSB biomass would disappear. In order to keep PSB wastewater treatment effective and PSB dominate, conditions need to be provided to promote the PSB growth as well as to ensure the wastewater treatment efficiency. The factors influencing PSB growth included light-oxygen conditions [15], initial PSB concentration, initial wastewater concentration, pH [16], and temperature [17,18]. All these conditions were studied and the changes of microbial species under the optimum conditions were examined to ensure the effects of pollutant removal and with PSB growth in wastewater treatment process.

2. Material and methods

2.1. Material

PSB strain used was *Rhodospirillaceae*, *Rhodopseudomonas*, *Rhodopseudomonas sphaeroides*, and was obtained from China General Microbiological Culture Collection Center (strain number of 1.2183).

Diluted soybean milk was adopted to simulate soybean wastewater with COD of $8{,}500 \pm 500$ mg/L and pH of 6.2 ± 0.1. The characters of soybean wastewater were shown in Table 1. The wastewater was highly biodegradable and the BOD/COD value was higher than 0.9 during treatment. Since the measurement of BOD needs five days and is too long for experimental operation, COD was used to represent the wastewater concentration.

Table 1
Salt concentration and osmotic pressure in soybean wastewater

COD, mg/L	1,800	3,600	7,200	14,400
Salt concentration, mg/L	1.48	2.95	5.90	11.80
π (osmotic pressure), atm	0.06	0.12	0.24	0.49

Activated sludge was used to simulate the contaminating bacteria. Activated sludge was got from the secondary clarifier of A/O process in Harbin Taipin Wastewater Treatment Plant. The initial contaminating bacteria concentration was 16 mg/L.

2.2. Experimental setup

2.2.1. Optimization of PSB soybean wastewater treatment condition

Light-oxygen conditions, initial PSB concentration, initial wastewater concentration, pH, and temperature were optimized. Experiments were carried out in a batch reactor (3 L flasks). Each flask was filled with 2 L of artificial soybean wastewater. COD was measured every 12 h.

2.2.2. Diversity of microbial species

In order to observe the changes of microbial community in the long-term run, semi-continuous reactors were used to simulate the actual PSB wastewater treatment process. The bioreactors were 3 L flasks. Each flask was filled with 2 L of artificial soybean wastewater. Every day, 667 ml of treated wastewater was discharged and an equal volume of fresh wastewater was put in. Sampling started after seven days of domestication. The wastewater COD and the diversity of microbial species were measured every seven days.

2.3. Analysis methods

COD was measured by a COD detector (5B-1, Lianhua Com., China). Electric potential was measured by an electrophoresis instrument (DYY-2B, Shanghai Tianneng Technology Company, China). BOD was measured according to the APHA Standard Method.

Usually, the PSB biomass was measured by measuring the dry weight or the light transmission at 660 nm. However, in this paper, contaminating bacteria existed and contributed to the dry weight and light absorption. Therefore, the conventional methods were unsuitable. Since PSB contain CoQ10 and the content of CoQ10 is very stable in PSB while the contaminating bacteria contain no CoQ10, the concentration of CoQ10 can be used to represent the concentration of PSB in the reactor. High pressure liquid chromography (HPLC, Agilent 1200, Agilent Technologies, Inc, USA) was used to measure the CoQ10 concentration and thus to present the PSB biomass [19].

Amplifications of DNA were carried out by a thermocycler (TC020-24-230 V, Labnet International, Inc, American). Gel was isolated by a vertical electrophoresis instrument (Mini-PROTEAN 4, Bio-Rad, USA). The gel was photographed with UV transillumination and documented with GelDoc system (GelDoc-It/TS, Bio-Rad, USA).

PCR–DGGE was used to examine the changes of the microbial species during wastewater treatment. The details were as follows:

Genomic DNA was isolated from the 5 ml reactor sample using the method referenced in the "Short protocols in molecular biology" [20]. The product from DNA extraction was verified by electrophoresis in 0.8% agarose.

The primers 341f-GC (5′-CgC CCg CCg CgC gCg gCg ggC ggg gCg ggg gCA Cgg ggg gCC TAC ggg Agg CAg CAg-3′) were used to amplify the V3-16S ribosomal DNA (rDNA) region from the extracted DNA [21,22]. The portion of pufM gene was amplified using pufM primers 907r-GC (5′-GC-clamp-CCg TCA ATT CCT TTg AgT TT-3′). The interest of the pufM gene amplification was to easier identify the sequence.

Amplifications of V3-16S rDNA were carried out using a thermocycler [23]. The 25 μl templates contained 2.5 μl of 10 × PCR buffer (Promega, USA), 1 μl (each) primer (10 μmol/L), 2 μl $MgCl_2$ (25 mmol/L, Promega, USA), 0.5 μl DNA templates, and 0.2 μl of Taq DNA polymerase (5 U/μl, Promega, USA). After an initial denaturation at 95℃ for 5 min, 30 cycles of touchdown PCR were carried out (denaturation at 94℃ for 30 s, annealing temperature decreased from 58 to 53℃ in 30 cycles, and extension at 72℃ for 30 s), followed by 30 cycles of regular PCR.

Composite PCR products were loaded into a polyacrylamide gel (10% acrylamide-bisacrylamide (37.5:1, W/V). The gels were subjected to a constant voltage of 100 V at 60℃ for 10 h. The gel was then visualized by silver staining [23], photographed with UV transillumination and documented with GelDoc system.

The selected bands were excised from the gel and eluted in 20 μl tissue culture water at 4℃ overnight. Three microliters of the eluted DNA was re-amplified by PCR following the program described above [23]. Each reaction mixture was also subjected to DGGE analysis to confirm the melting behavior of the band recovered. The cloning and sequencing steps followed the conventional methods [24]. Subsequently, idiographic sequences were sent to Shanghai Yungjun Bio-technology Inc. for classification and sequencing. Finally, clone sequences were manually aligned with GenBank software program [25].

3. Results and discussion

Blank experiments using sterilized wastewater without PSB strain or activated sludge were performed and the results showed that COD was not degraded after 96 h.

3.1. Optimization of PSB soybean wastewater treatment conditions

3.1.1. Light-oxygen conditions

Fig. 1 shows that the optimum light-oxygen condition was natural light-micro oxygen. Fig. 1(a)

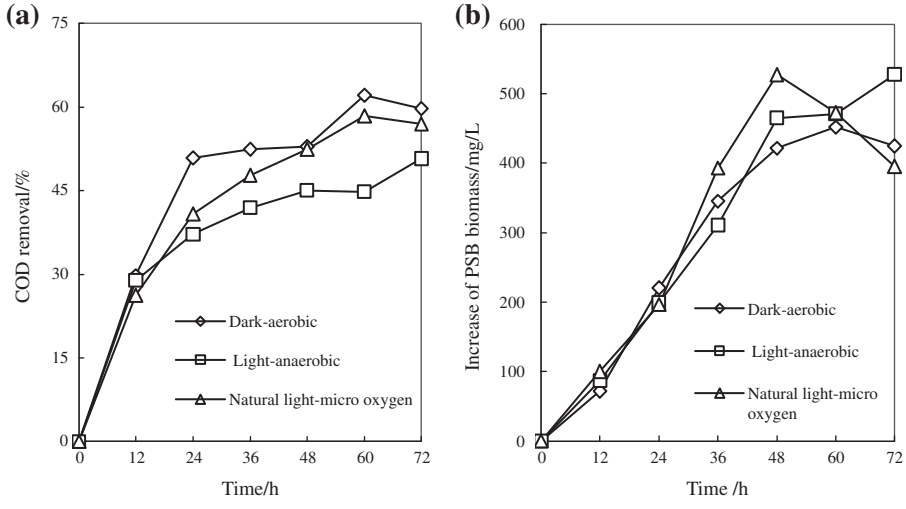

Fig. 1. Effect of light-oxygen conditions on COD removal and PSB biomass, soybean wastewater concentration 3,600 mg/L, PSB 360 mg/L.

shows that under different light-oxygen conditions, PSB could treat wastewater effectively. COD removal was nearly the same and COD reached 50.71–59.72%. Fig. 1(b) shows that under different light-oxygen conditions, PSB biomass was nearly the same, and PSB biomass could reach 624.97–687.38 mg/L. Lu also reported that under different light-oxygen conditions, PSB could treat wastewater effectively [26]. The test result of Ryo Honda [27] cleared that feeding in the morning is the optimum feed-timing control from the aspects of growth of purple non-sulfur bacteria and single-cell protein production. Dissolved organic carbon removal could reach 94% independent of the timing control. In this paper, only the treatment time was considered, and operation timing of feeding and withdrawal of PSB was unconsidered. More research in this aspect may be needed in the future. In practical scale operation, the energy consumption was very high at 24 h lighting. Previous research revealed that, if we light for 12 h by natural sunlight and 12 h by lamp, the energy consumption decreased, but PSB biomass production and COD removal all decreased by 60 mg/L and about 5% separately. Based on an overall consideration of energy consumption, PSB biomass production and COD removal, the natural light-micro oxygen was determined to be the optimum light-oxygen condition. The research of Izu et al. [28], indicated that maximum PnSB ratio (up to 80%) was obtained both at non-aeration condition and at constant ORPs less than −200 mV. At aeration, PnSB was less competitive to chemoheterotrophs than Rb spheroids. In previous research, we got the same result. It was a disadvantage of PSB competitive to contaminants if we supply oxygen. Since no artificial light or oxygen was needed under the natural light-micro oxygen condition, the energy consumption was the lowest. Therefore, the natural light-micro oxygen was determined to be the optimum light-oxygen condition in terms of energy cost.

3.1.2. Initial PSB concentration

Fig. 2 shows that the optimum initial PSB concentration was 160 mg/L. Fig. 2(a) shows that when the initial PSB concentration was 160–4,000 mg/L, PSB could treat soybean wastewater effectively, and COD removal could reach 90.85–94.70%. When the initial PSB concentration was 32 mg/L, COD removal was the lowest (44.66%). Fig. 2(b) shows that when the initial PSB concentration was 160 mg/L, the increase of PSB biomass was the highest (354.23 mg/L). When PSB was 32 and 800 mg/L, PSB biomass concentration remain unchanged, and PSB biomass concentration increased 6.56–73.52 mg/L. When PSB was 4,000 mg/L, PSB biomass concentration decreased continuously. After 72 h, the increase of PSB biomass was 160 mg/L > 800 mg/L > 32 mg/L > 4,000 mg/L. So the optimum initial PSB concentration was 160 mg/L.

3.1.3. Initial wastewater concentration

Fig. 3 shows that the optimum initial wastewater concentration was 3,600 mg/L. Fig. 3(a) shows that when initial soybean wastewater was 3,600–14,400 mg/L PSB could treat wastewater effectively, and COD removal could reach 79.43–94.70%. When soybean wastewater was 1,800 mg/L, PSB could not treat wastewater effectively, and COD removal could reach 37.81%. Fig. 3(b) shows that when soybean

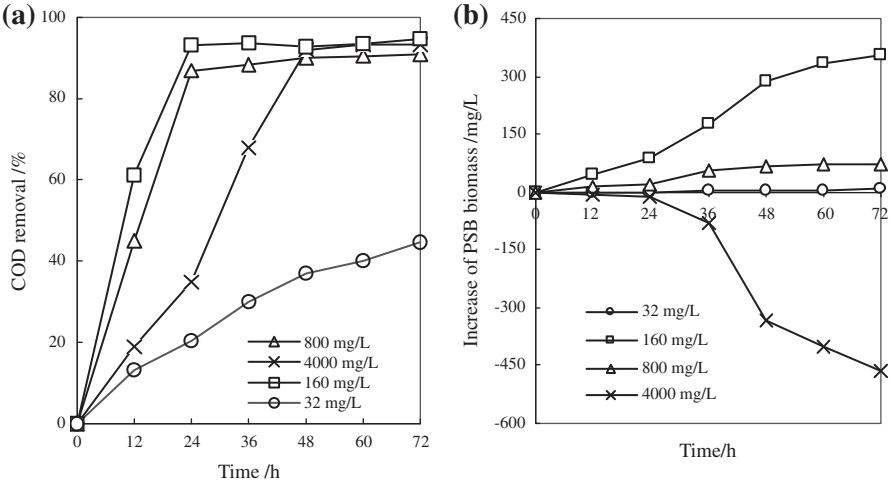

Fig. 2. Effect of initial PSB concentration on COD removal and PSB biomass, soybean wastewater concentration 7,200 mg/L, natural light-micro oxygen condition.

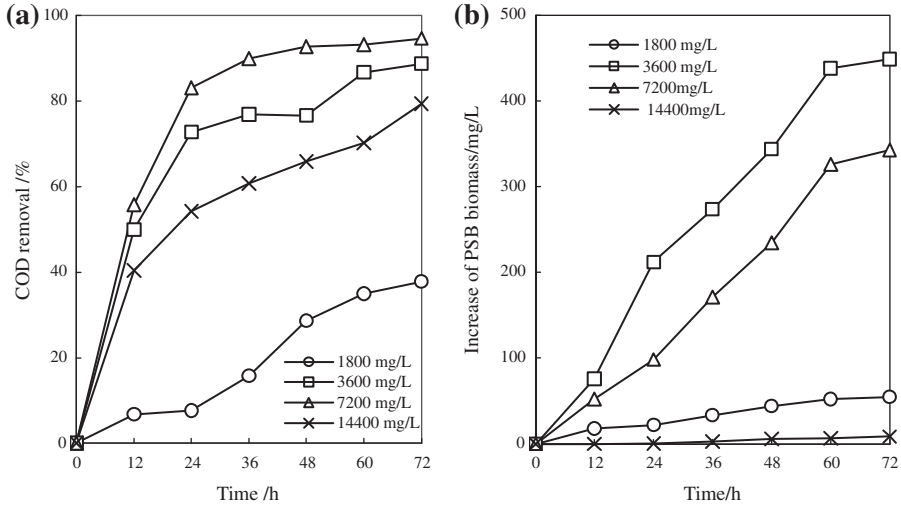

Fig. 3. Effect of initial wastewater concentration on COD removal and PSB biomass, initial PSB 160 mg/L, natural light-micro oxygen condition.

wastewater was 3,600, 7,200, and 14,400 mg/L PSB biomass concentration increased. When wastewater was 3,600 mg/L the increase of PSB biomass was the highest (437.83 mg/L). If wastewater concentration was low, it will lead to nutritional deficiencies for PSB growth. So when the initial soybean wastewater COD was 1,800 mg/L PSB biomass concentration remain unchanged. If wastewater concentration was high, it would raise the osmotic pressure and cause PSB cell water lose. So when the initial soybean wastewater COD was 7,200–14,400 mg/L, the PSB biomass concentration also increased little. So the optimum initial wastewater concentration was 3,600 mg/L.

3.1.4. Initial pH

Fig. 4 shows that COD removal and PSB biomass was the highest when the initial wastewater pH was 8.0. After 72 h, COD removal was 86.48% (pH of 8.0) >80.15% (pH of 7.0))>74.91% (pH of 6.0)>71.61% (pH of 9.0)>36.22% (pH of 10.0)>25.03% (pH of 5.0). After 72 h, PSB biomass was 674.67 mg/L (pH of 8.0) >437.72 mg/L (pH of 6.0)>253.19 mg/L (pH of 9.0) >162.88 mg/L (pH of 7.0)>77.09 mg/L (pH of 10.0) >53.42 mg/L (pH of 5.0). So the optimum pH was 8.0.

3.1.5. Temperature

Fig. 5 shows that under different temperatures, COD removal and PSB biomass was the highest at 30℃. After 72 h, COD removal was 79.23% (30℃) >65.82% (20℃)>43.61% (10℃)>31.49% (40℃). PSB biomass was 569.48 mg/L (30℃)>431.84 mg/L (20℃) >243.37 mg/L (10℃)>184.62 mg/L (40℃). The studies of Sasaki et al [29] showed that the optimal temperatures for growth and COD removal were 35℃ for *R. gelatinosa* and 25℃ for *R. gelatinosa* A1. Previous research indicated that the optimum temperature of PSB under artificial culture was 25–35℃. The test results of ours in the same scope. So in this paper, the optimum temperature was 30℃.

In summary, the optimum conditions of PSB soybean wastewater treatment were natural light-micro oxygen condition, initial PSB of 160 mg/L, initial wastewater concentration of 3,600 mg/L, pH of 8.0, and temperature of 30℃.

3.2. Changes of microbial species during 35 day treatment

In order to examine the changes of the microbial species in the PSB wastewater treatment system in long term, semi-continuous operation was adopted. The experiments lasted two months, in which the first seven days were for bacteria domestication. The changes of diversity of microbial species were examined along the treatment process.

3.2.1. PSB wastewater treatment effect and biomass under the optimum conditions

Fig. 6 confirms that in semi-continuous reactor under optimum condition PSB could treat soybean wastewater effectively, and PSB biomass increased. Fig. 6(a) shows that under the optimum condition PSB could treat wastewater effectively, and COD removal could reach 68.6%. Fig. 6(b) shows that under the optimum conditions PSB biomass could reach 352.90 mg/L. Contaminating bacteria could reach

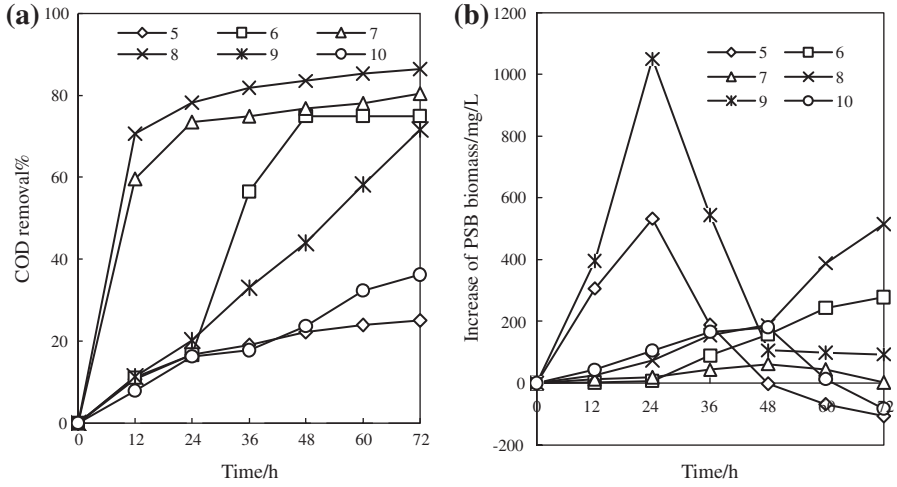

Fig. 4. Effect of initial pH on COD removal and PSB biomass, soybean wastewater 3,600 mg/L, initial PSB 160 mg/L, natural light-micro oxygen condition.

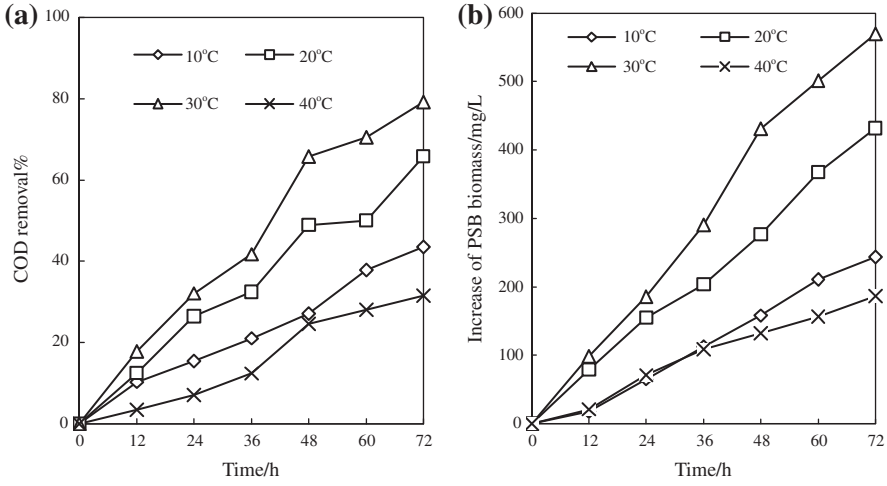

Fig. 5. Effect of temperature on COD removal and PSB biomass, soybean wastewater 3,600 mg/L, initial PSB 160 mg/L, natural light-micro oxygen condition, pH of 8.0.

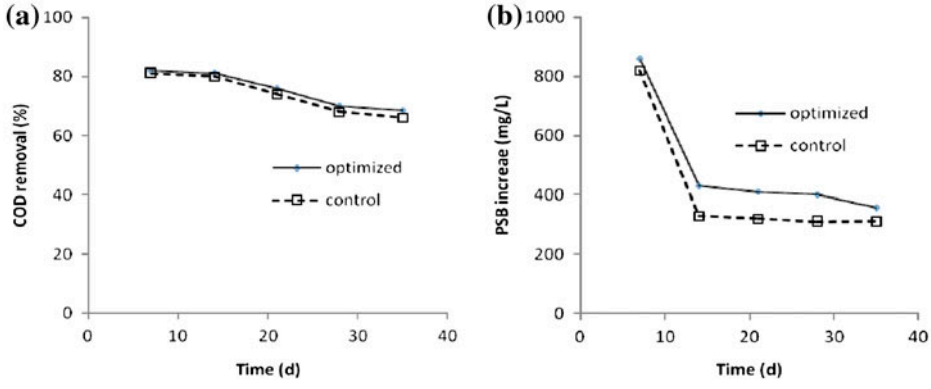

Fig. 6. PSB wastewater treatment effect and biomass in semi-continuous reactor. Optimized refers to the optimized conditions: natural light-micro oxygen condition, initial PSB of 160 mg/L, initial wastewater concentration of 3,600 mg/L, pH of 8.0, and temperature of 30˚C; control refers to the non-optimal conditions: dark-aerobic, initial PSB of 360 mg/L, initial wastewater concentration of 3,600 mg/L, pH, and temperature not adjusted.

133.86 mg/L. The percentage of PSB to total bacterial in the reactor was 72.5%. Compared with contaminating bacteria PSB was the dominant bacteria. PSB biomass has the value of recovery and utilization. In a semi-continuous reactor under optimum condition not only could keep wastewater treatment effective and but also could keep PSB competitive advantage. In the semi-continuous reactors, PSB could treat soybean wastewater effectively and PSB biomass increased. The wastewater COD removal efficiency and the PSB biomass accumulation were similar with results found in the batch reactors. The COD change stabilized after seven days but the microbial community kept change till 28 days.

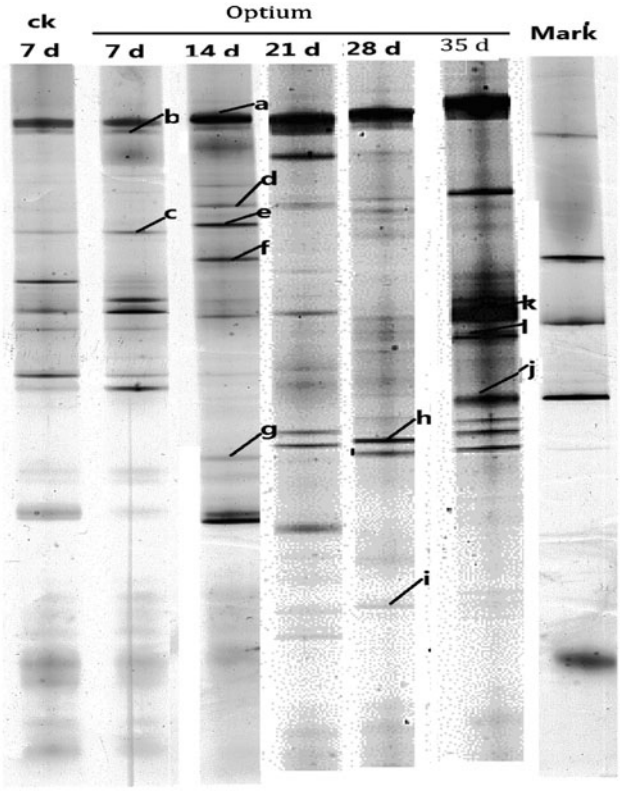

Fig. 7. Change of DGGE picture with time in PSB soybean wastewater treatment system (ck lane is the diversity of microbial species in the non-optimized system).

3.2.2. Diversity of microbial species under the optimum conditions

Fig. 7 and Table 2 show a large increase in micro-organism species with the passage of time. The types of micro-organisms present gradually increased with the passage of time. Under the optimum conditions, compared with control, the microbial species changed effectively. The DGGE bank of k was PSB, under the optimum conditions, compared with control, the DGGE bank of k still exists, and the colors of bank k darken, the line grew thicker eventually through 35 day operation. These results indicated that PSB was the dominate bacteria and has advantage in competition. Under control condition, *Porphyromonadaceae bacterium*, *Iron-reducing bacterium*, *Clostridiaceae bacterium*, and PSB were included on the 7th day. Under the optimum conditions, similar results were found, *Lactococcus lactis subsp*. formed on the 7th day. *Lactococcus lactis subsp*., *Veillonella sp*., and *Dysgonomonas sp.* formed on the 14th day. *Iron-reducing bacterium* and *Veillonella sp.* formed and *Lactococcus lactis subsp.* disappeared on the 21st day. *Janthinobacterium lividum* formed on the 28th day. After 28th day, this system entered a stable situation. The community did not change from 28th day to 35th day (Fig. 6) or later (data not shown). These result indicated that the opti-

Table 2
Component of DGGE bands

DGGE bands	Strain	Similarity (%)	Sequence IDs
a	*Porphyromonadaceae bacterium*	93	>gi|281494255|gb|GU247216.1|
b	*Iron-reducing bacterium*	98	>gi|209865496|gb|FJ269054.1|
c	*Clostridium sp.*	96	>gi|327359410|gb|JF346753.1|
d	Fluviicola taffensis	97	>gi|62184633|gb|AF493694.2|
e	*Parabacteroides sp.*	99	>gi|305387456|gb|HQ020488.1|
f	*Lactococcus lactis subsp.*	99	>gi|148357812|gb|EF589778.1|
g	*Clostridiaceae bacterium*	98	>gi|166063924|dbj|AB298726.2|
h	*Iron-reducing bacterium*	99	>gi|209865496|gb|FJ269054.1|
i	*Veillonella sp.*	99	>gi|227353242|gb|FJ374768.1|
j	*Dechloromonas sp.*	98	>gi|148748886|gb|EF632559.1|
k	Rhodobacter sphaeroides strain Z08 *(PSB)*	95	>gi|294821794|gb|GU990615.1|
l	*Janthinobacterium lividum*	96	>gi|325464740|gb|JF262574.1|

mum conditions not only could supply suitable growth condition to PSB but also to contaminants bacteria. The optimum conditions contribute to the form of new bacteria, but not all the new formed bacteria could suit the optimum conditions, some kinds of new formed bacteria extinguished by the pass of time. During the whole period, PSB were still existed. Combining with the results of Fig. 6 PSB was the dominate bacteria.

4. Conclusion

From above studies, following conclusions may be drawn:

(1) With the existence of contaminating bacteria, PSB could effectively treat the soybean wastewater, increased the biomass, and kept dominating in the system.
(2) The optimum conditions of PSB soybean wastewater treatment was natural light-micro oxygen condition, initial PSB of 160 mg/L, initial wastewater COD of 3,600 mg/L, pH 8.0, and 30˚C.
(3) In semi-continuous reactor under the optimum conditions, PSB could treat soybean wastewater effectively and PSB biomass increased. The types of micro-organisms increased with the passage of time. The community did not change from 28th day to 35th day. Twenty-eight days later, this system enters a stable situation. During the reactor stable performance period, PSB were still the dominate bacteria.

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