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ORIGINAL PAPER

Growth of *Chlorella pyrenoidosa* in wastewater from cassava ethanol fermentation

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Abstract In a cycle tubular photobioreactor, *Chlorella pyrenoidosa* was cultured in undiluted wastewater from ethanol fermentation using cassava powder as raw material. The results showed that the optimum cultivation conditions were initial pH of 6.0, temperature at 27°C, continuous illumination at 3,000 lux, and cycle speed of 110 ml min^{-1}. Under these optimum conditions, after the logarithmic phase of batch cultivation with wastewater of pH 6.0, the reactor could be continuously operated with natural pH wastewater (3.8) as feed solution. By a dilution ratio of 0.17 day^{-1}, it could be operated stably for over 30 days in continuous cultivation. pH, removal rate of chemical oxygen demand, and biomass (cell dry weight) concentration ranged from 6.22 to 6.47, 72.21 to 76.32% and 3.55 to 3.73 g l^{-1}, respectively. After treatment, the wastewater could be used again in the process of ethanol fermentation.

Keywords Bio-treatment · Cassava · *Chlorella pyrenoidosa* · Ethanol · Tubular photobioreactor · Wastewater

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Introduction

Cassava is a major and widely planted agricultural product in southern China. Due to its high starch content and low cost, it becomes an important raw material of fuel ethanol production. About 8–10 ton wastewater is discharged when 1 ton of anhydrous alcohol is produced. The wastewater containing organic and acidic substances is harmful to the environment. Because of its poor nutrient content, low BOD_5/COD_{cr} ratio, and low pH (Hu et al. 1998), the productivity was very low when *Candida* or methane-producing bacteria were used to produce single cell protein or methane using the wastewater as material. The high production cost limits the application of these technologies.

With microalgae and macrophytes, a two-step sequential treatment process was used to treat the recalcitrant wastewater from ethanol and citric acid production. The wastewater was diluted 10 times with wash water from the production line (Valderrama et al. 2002). Microalgae or cyanobacteria have also been cultured in many other kinds of wastewater for algal production and to treat the waste water, for example *Spirulina maxima* and *Chlorella pyrenoidosa* in swine wastewater (Canizares-Villanueva et al. 1995) and olive mill wastewater (Villasclaras et al. 1996), respectively. The wastewater must be pretreated with percolation, dilution, pH regulation, and sterilization. This process consumes a large quantity of fresh water for dilution.

In our laboratory, we have selected and acclimatized the microalga *C. pyrenoidosa* which has been previously cultured in undiluted wastewater from cassava ethanol production (WCEP). In this article, a simplified cycle tubular photobioreactor on an experimental scale is reported, aimed at producing *C. pyrenoidosa* with WCEP which would be treated economically at same time. The operating conditions and process of the photobioreactor were analyzed and

discussed. The results indicate that the process was successful. This method of bio-treating wastewater with poor nutrient content could reduce dilution, save freshwater and be applied to production. If it was combined with the process of ethanol production, almost zero discharge of wastewater would be realized.

Materials and methods

Maintenance and pre-culture of microalgae

C. pyrenoidosa, FACHB-9 was purchased from Institute of Hydrobiology, the Chinese Academy of Sciences, Wuhan, China. After it had been acclimatized, it was maintained on WCEP of pH 6.0.

The inocula were performed under sterile conditions. Aliquots of 20 ml were transferred to 250-ml conical flasks containing with 100 ml of WCEP of pH 6.0 and cultured at temperature (25°C) under continuous illumination (2,000 lux) and artificial intermittent shaking (four times in a day) for 5 days.

WCEP preparation

In a 25-l fermenter, ethanol fermentation was carried out in the medium consisted of cassava powder and water at the ratio of 1:3 (g:g). After 60-h fermentation, the ethanol content, reducing sugars, total sugars, and pH of culture liquid were analyzed. Ethanol was removed by distillation. As a pre-treatment, the effluent obtained was centrifuged at 2,000*g* for 10 min to decrease the concentration of total solid substance (TSS). The supernatant was designated as wastewater from cassava ethanol production (WCEP). The COD, BOD, TSS, and pH of WCEP were 9.32×10^4 mg l^{-1}, 4.61×10^4 mg l^{-1}, 670 mg l^{-1}, and 3.80, respectively. WCEP was kept at −10°C for future use.

Cycle tubular photobioreactor and culture method

A schematic diagram (Fig. 1) shows a 200-ml laboratory scale cycle tubular photobioreactor which was comprised of: a cycle glass tubular, fluorescent lamp, and two constant pumps, designed according to methods described by Watanabe and Saiki (1997), Travieso et al. (2001), Scragg et al. (2002), and Walter et al. (2003). This design has some advantages, such as a large surface-to-volume ratio, easy control of temperature, and occupies a small ground area. The reactor was set in a simple greenhouse with an air-conditioner, filled with hypochlorous acid for 1 day, flowed and washed clearly with sterile water for use. Batch, fed-batch, and continuous operation were used to culture the microalgae, according to each case condition.

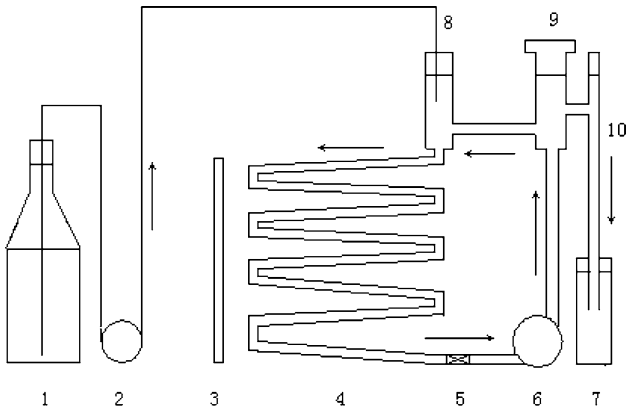

Fig. 1 Schematic diagram of the cycle tubular photobioreactor. (1) Feed solution or microalgal seeds, (2) feed solution pump, (3) fluorescent lamp, (4) photostage, (5) outlet, (6) cycle pump, (7) sample collector, (8) inlet, (9) degasser, (10) flooding outlet

Analytical determinations

The algal cells were washed with 0.5 M HCl to remove the precipitated salts and other inorganic substances, rinsed with distilled water, dried at 105°C for 24 h and weighed. When the volume of sample was so small in batch operation or the cell concentration was so low that cell weight could not be accurately determined, it was estimated by measuring the optical density at 680 nm (Spectrophotometer U3000, Japan). In the latter case, the O.D. readings were converted to cell dry concentration using predetermined calibration curves. COD, BOD, TSS, and organic acid were analyzed according to standard methods (APHA et al. 1992). Ethanol content was determined by distillation. pH was measured by Mettler Toledo Electrochemical analytical meter Model Seven Multi. Microscopy was performed using an Olympus Microscope Model CX31. Light intensity was measured by DT-1301 Light meter, CEM Company limited (Hong Kong). The quantity of organic acid was confirmed by external standard method with Agilent, HP-1100, US. COD removal rate (CODrr), productivity value (*P*) (different operation with different formula), and conversion yield of COD into algal cell weight ($Y_{B/COD}$) were calculated using the following formulae:

$$\text{CODrr} = (\text{COD}_{\text{inital}} - \text{COD}_{\text{final}})/\text{COD}_{\text{inital}} \times 100\%$$

P_{batch} = cell weight/culture time (used in batch operation, culture time = 6)

$P_{\text{fed-batch}}$ = cell weight × partition (used in fed-batch operation)

$P_{\text{continuous}}$ = cell weight × D (used in continuous operation)

$Y_{B/COD}$ = cell weight/($\text{COD}_{\text{inital}} - \text{COD}_{\text{final}}$)

All data were the average of the triplicates for sample.

Results and discussion

Effect of cultivation conditions on *C. pyrenoidosa*

Cultivation conditions have an important effect on the growth of microalgae (Pirt et al. 1983; Shi et al. 2000; Walter et al. 2003) and the effects of different cultivation conditions vary. Thus, it was necessary to study the optimum conditions for growth of *C. pyrenoidosa* in WCEP. The main factors to impact on microalgal growth included pH, temperature, light intensity, and mixture of culture liquid.

Initial pH

The pH in the medium could affect the efficiency of carbon usage, absorbability of ions, recycling, and toxicity of metabolites in the growth of microalgae (Borowitzka and Borowitzka 1988). The optimum growth pH was different between different algae. Figure 2A illustrated that *C. pyrenoidosa* could grow at initial pH values between 5.0 and 7.5, but pH 6.0 was optimum.

Temperature

The temperature is also an important factor which could affect algal metabolism (Goldman and Carpenter 1974). According to the optimum growth temperature, *Chlorella* could be divided into two kinds of strains: low temperature (25–30°C) and high temperature (35–40°C) strains (Sorokin 1959). Generally, the low temperature strains are chosen to be cultured in industry to save energy costs (Martinez and Orus 1991; Ogbonna et al. 1997). Figure 2B shows that the optimum culture temperature was 27°C. By the way, the tubular photobioreactor could operate well in a simple greenhouse to save heating and artificial illumination costs.

Light intensity

There existed an optimum light intensity which was called "light saturation point" in the growth of each microalga (Gong and Chen 1997). The overfull light intensity could not be used by algae and would increase the production cost. Deficient light intensity limited microalgal growth (Myers 1953). The result was that light saturation point was 3,000 lux (Fig. 2C).

Cycle speed

Flowing cultivation was an important condition to be used in order to maximize biomass. Good mixing can increase photosynthetic efficiency, prevent agglomeration and sinking of microalgae, and reduce the concentration gradients of substrates and metabolites (Borowitzka and Borowitzka 1988). The result (Fig. 2D) further proved the thesis. If the cycle speed was too low to mix the culture liquid well, the algae could not grow well. On the other hand, a cycle speed so fast that the shear stress of the pump on the algae was increased also led to poor growth. The optimum cycle speed was 110 ml min^{-1} (Fig. 2D).

In conclusion, the results mentioned above showed that the optimum cultivation conditions were initial pH of 6.0, temperature at 27°C, illumination at 3,000 lux, and cycle speed of 110 ml min^{-1}.

Batch operation in photobioreactor

Under above optimum conditions, batch operation was operated with undiluted WCEP in which the pH was regulated with 2 M NaOH, 3 ml culture liquid was sampled from outlet (Fig. 1-(5)). Cell weight, COD, the content of organic acids, and pH were examined every morning at the same time. The growth of *C. pyrenoidosa* in batch operation is shown in Fig. 3. The logarithmic phase was from 1 to 4 days.

The cell weight achieved the maximum (3.64 g l^{-1}) at the 6th day. pH rose from 6.00 to 8.52. CODrr, *P*, and $Y_{B/COD}$ were 71.21%, 0.61 g (day × l)$^{-1}$, and 0.05 g g^{-1}, respectively (Table 1). The removal rate of lactic acid, acetic acid, citric acid, and succin acid were 96.42%, 100%, 95.89%, and 39.44%, respectively (Table 2).

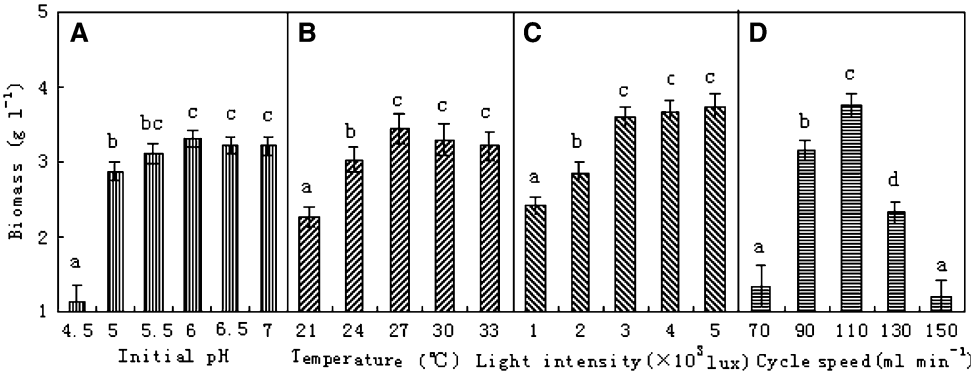

Fig. 2 Effect of cultivation conditions on *C. pyrenoidosa* (A: initial pH, B: temperature, C: light intensity, D: cycle speed). In each subfigure, columns denoted by a different lower case letter differ significantly with *t*-test at $P \leq 0.05$. Bars represent standard deviation

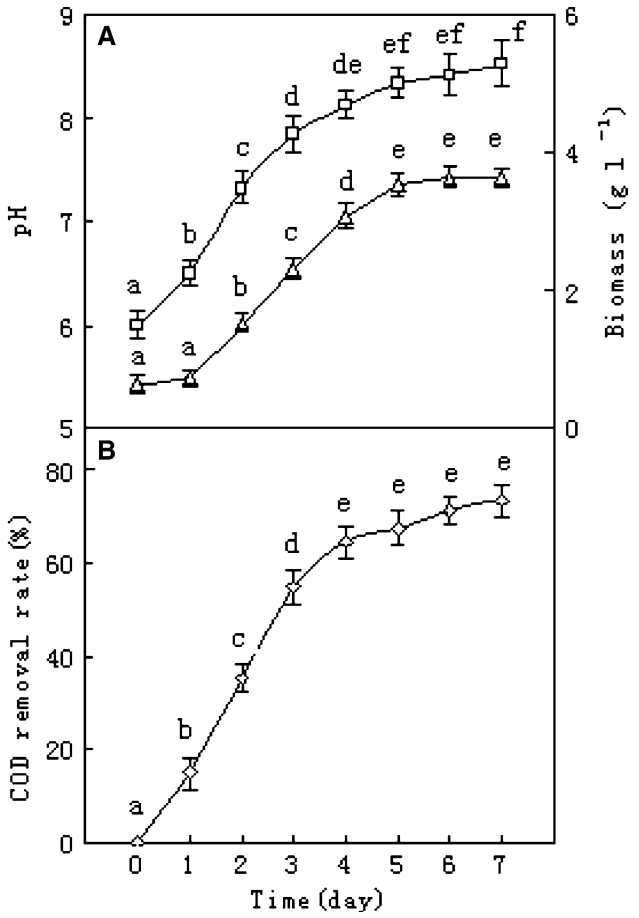

Fig. 3 The growth of *C. pyrenoidosa* in batch operation (□:pH, Δ:Biomass, ◇:COD removal rate). Points on curves denoted by a different lower case letter differ significantly with *t*-test at $P \leq 0.05$. Bars represent standard deviation

As a culture grew, the pH of the medium changed greatly when either nitrate or ammonium was used. In particular, the rapid utilization of ammonium ion by an alga caused a severe drop in pH (Shi et al. 2000). However, in this study, the pattern of pH, cell weight, and CODrr showed the COD was bioconverted by algae. Furthermore, the pH rise in medium was very significant. This phenomena suggested that fed-batch and continuous operation might be operated with WCEP of unadjusted pH (3.8) as feed solution.

Fed-batch operation with unadjusted pH (3.8) WCEP as the feed solution

First, the photobioreactor was operated as batch reactor with undiluted WCEP (pH 6.0) as medium. The growth of *C. pyrenoidosa* was very similar to that shown in Fig. 3. Second, from the 5th day of batch operation, fed-batch operation was operated by a partition of 20% (V/V) every day. Briefly, 40 ml culture liquid was removed, and

Table 1 Comparison of results of different operation

Operation	D (day^{-1})	pH				CODrr (%)		Biomass ($g\ l^{-1}$)		P [$g\ (day \times l)^{-1}$]		$Y_{B/COD}$ ($g\ g^{-1}$)		Time (day)	Stability
		Initial	Final	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max		
Batch	–	6.00 ± 0.14	8.52 ± 0.22	6.00 ± 0.14	8.52 ± 0.22	–	71.21 ± 3.12	–	3.64 ± 0.14	–	0.61 ± 0.02	–	0.05 ± 0.01	6	–
Fed-batch	0.20	8.12 ± 0.16	5.54 ± 0.12	5.54 ± 0.12	8.12 ± 0.16	68.46 ± 3.51	77.53 ± 3.88	2.91 ± 0.13	3.57 ± 0.16	0.58 ± 0.03	0.71 ± 0.03	0.05 ± 0.01	0.06 ± 0.01	6	Unsteady
Continuous															
I	0.20	8.23 ± 0.15	6.84 ± 0.14	6.84 ± 0.14	8.23 ± 0.15	70.14 ± 3.49	76.12 ± 3.23	3.44 ± 0.11	3.72 ± 0.17	0.69 ± 0.02	0.74 ± 0.03	0.05 ± 0.01	0.06 ± 0.01	12	Unsteady
II	0.17	6.84 ± 0.14	6.47 ± 0.16	6.47 ± 0.16	6.84 ± 0.14	74.13 ± 3.21	76.32 ± 2.30	3.58 ± 0.16	3.62 ± 0.12	0.61 ± 0.03	0.62 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	6	Unsteady
III	0.17	6.47 ± 0.16	6.34 ± 0.15	6.22 ± 0.16	6.47 ± 0.16	72.21 ± 3.76	76.32 ± 2.30	3.55 ± 0.19	3.73 ± 0.14	0.60 ± 0.03	0.63 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	30	Steady
IV	0.20	6.34 ± 0.15	4.81 ± 0.20	4.81 ± 0.20	6.34 ± 0.15	59.30 ± 4.50	76.13 ± 2.89	3.08 ± 0.12	3.64 ± 0.09	0.62 ± 0.02	0.73 ± 0.02	0.05 ± 0.01	0.07 ± 0.02	6	Unsteady

Mean and standard deviation of three replicate are shown. D, CODrr, Biomass, P and $Y_{B/COD}$ represent dilution ratio, cell dry weight, productivity value, and conversion yield of COD into algal biomass, respectively. The batch operation medium consisted of undiluted WCEP. Initial pH was regulated to 6.0 with 2 mol l^{-1} NaOH. The feed solution of fed-batch and continuous operation only consisted of undiluted WCEP of nature pH (3.8)

Table 2 The removal rate of organic acids

Organic acids	Initial content (g l^{-1})	Final content (g l^{-1})	Removal rate (%)
Lactic acid	4.96 ± 0.22	0.18 ± 0.05	96.42 ± 3.53
Acetic acid	1.03 ± 0.12	0.00 ± 0.00	100.00 ± 0.00
Citric acid	1.65 ± 0.18	0.07 ± 0.02	95.89 ± 4.12
Succin acid	1.81 ± 0.16	1.1 ± 0.02	39.44 ± 3.45

Mean and standard deviation of three replicates are shown

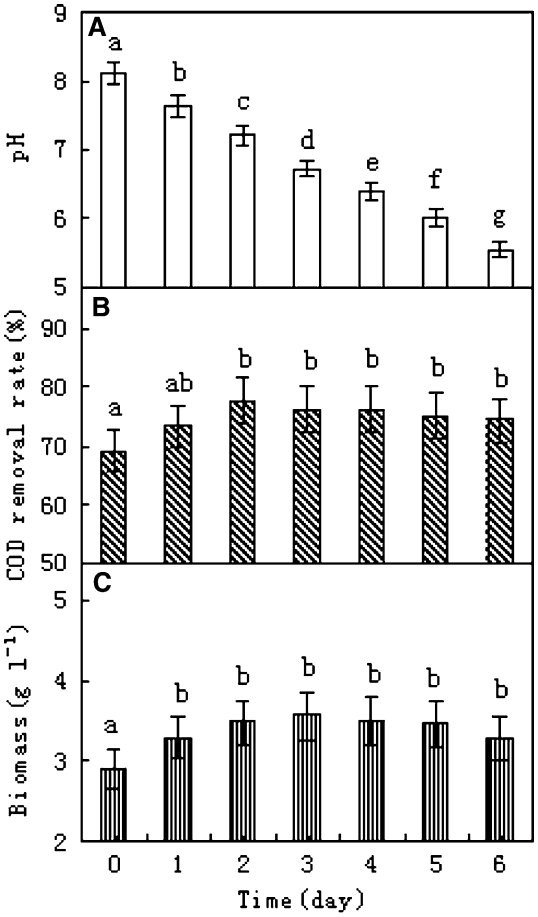

Fig. 4 The result of fed-batch operation (A: pH, B: COD removal rate, C: Biomass). In each subfigure, columns denoted by a different lower case letter differ significantly with *t*-test at $P \leq 0.05$. Bars represent standard deviation

simultaneously the same volume of undiluted and sterile WCEP of natural pH (3.8) was added to the reactor daily. Cell weight, pH, and COD of samples were measured. Figure 4 shows that the bioreactor could be operated for 6 days at this partition.

C. pyrenoidosa could utilize the substances in WCEP and this resulted in a significant rise in pH (Fig. 3). However, Fig. 4 shows that fed-batch operation with natural pH (3.8) WCEP at this partition (20% day^{-1}) resulted in the newly added acidic material not being fully bio-converted by the microalgae in a day. Systematically, the pH decreased day by day. At the 6th day, the pH decreased to 5.54 (Table 1). If natural pH WCEP was pumped in, the initial pH would decrease to below 5.0 and the microalgae could not be cultured (Fig. 2A). Therefore, the whole culture period consisted of two steps: 5 days batch operation and 6 days fed-batch operation by a partition of 20% day^{-1}. During the latter step, the pH decreased from 8.12 to 5.54. Maximum values of P and $Y_{B/COD}$ were 0.71 g $(day \times l)^{-1}$ and 0.06 g g^{-1}, respectively.

Fed-batch operation saved the time for washing of the system and preparation of microalgal seeds, so it was easier to operate and more economical than batch operation.

Continuous operation with natural pH (3.8) WCEP as the feed solution

In the first step, the photobioreactor was operated as batch reactor for 5 days, as described previously.

In the second step (Fig. 5-I) and fourth step (Fig. 5-IV), the dilution ratio (D) was 0.20 day^{-1}, and 40 ml culture liquid was collected at the same time every day from the sample collector (Fig. 1-(7)). The second step was from the 5th day of batch operation, the cycle bioreactor was operated in continuous cultivation with natural pH (3.8) WCEP as the feed solution for 12 days. The pH decreased slowly day by day over the period. Maximum values of P and $Y_{B/COD}$ were 0.74 g $(day \times l)^{-1}$ and 0.06 g g^{-1}, respectively. The fourth step was started at day 48. The pH decreased to below 5.0 at day 54. This step remained for only 6 days. The results show that continuous operation (D = 0.2 day^{-1}) could last more than 12 days, and was easier to operate than fed-batch operation (partition of 20% day^{-1}). However, at this dilution ratio, the reactor could not be operated stably.

In the third step, starting at day 12, the reactor was operated at a dilution ratio of 0.17 day^{-1}, in continuous operation with the same feed solution. About 34 ml culture liquid was gathered every day. After a regulated period (Fig. 5-II), the reactor could be operated for over 30 days stably (Fig. 5-III), keeping almost the same pH, same CODrr, and same biomass. They ranged from 6.22 to 6.47, 72.21 to 76.32%, and 3.55 to 3.73 g l^{-1}, respectively (Table 1). Continuous operation (D = 0.17 day^{-1}) which could omit the time for addition of medium and discharge of culture liquid, preparation of microalgal inoculum, washing of the system, and other non-productive processes, was the most stable, most economical, and easiest process

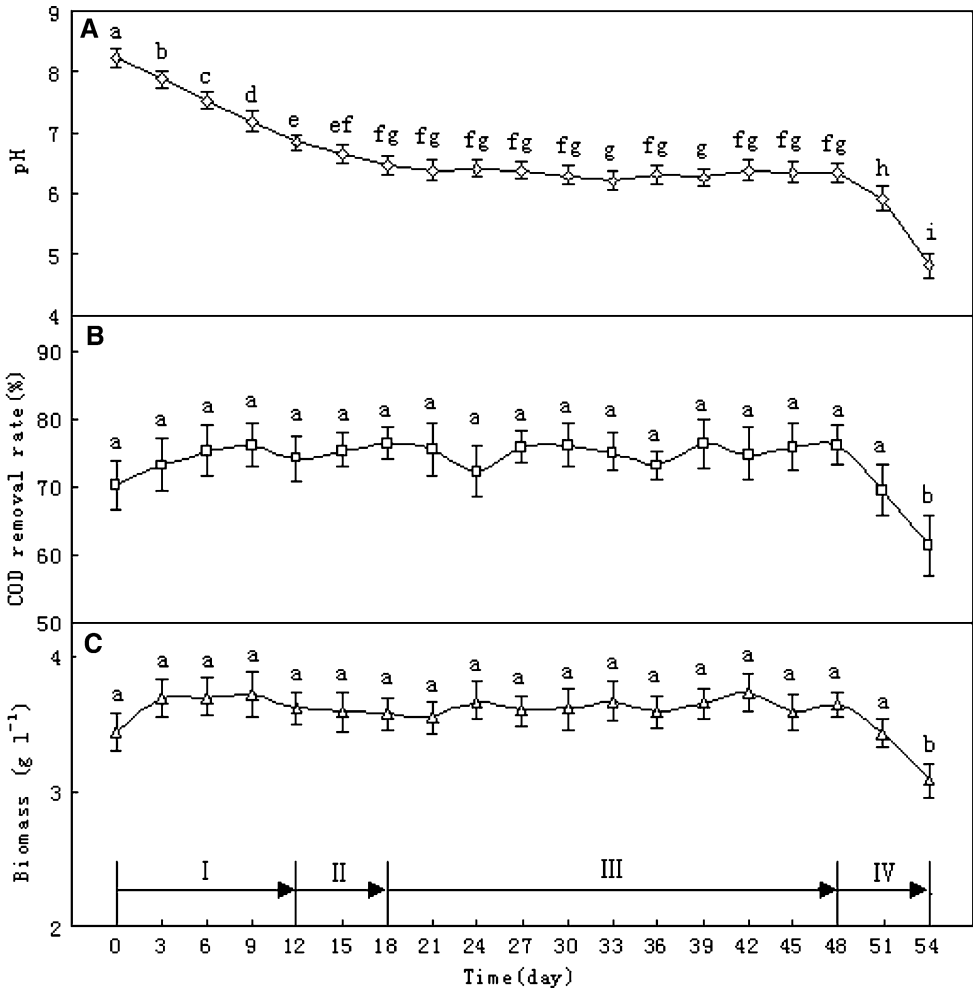

Fig. 5 The result of continuous operation (A: pH, B: COD removal rate, C: Biomass). I, II, III, and IV represent $D = 0.20\ day^{-1}$(unsteady), $D = 0.17\ day^{-1}$(unsteady), $D = 0.17\ day^{-1}$(steady), and $D = 0.20\ day^{-1}$(unsteady), respectively. In each subfigure, points denoted by a different lower case letter differ significantly with *t*-test at $P \leq 0.05$. Bars represent standard deviation

to operate. After treatment, algae could be harvested. The pH of the wastewater was adjusted. A drop in COD which ranged from 2.59 to 2.21 $\times$ 10^4 mg l^{-1} with an average of 2.31 $\times$ 10^4 mg l^{-1} was achieved.

Treated water was recycled in ethanol fermentation

According to our preciously study (Zhang and Fan 1989; Ding et al. 2003), most of the lees filtrate could be recycled in the process of ethanol production, after the pH of wastewater had been adjusted with NaOH. The discharged wastewater could be reduced greatly. However, the wastewater could not be ignored and must be treated. The treated water could be recycled in the ethanol fermentation with 500-ml flasks, and then newly produced WCEP could be bio-treated with the process again. The result (Table 3) showed that the treated water had no negative effect in the process of ethanol production through four times recycle.

Table 3 The result of treated water was recycled in ethanol fermentation

Recycle time	Ethanol content (%V)
0	11.4 ± 0.9
1	12.2 ± 0.8
2	10.9 ± 0.9
3	12.5 ± 1.0
4	11.7 ± 0.7

Mean and standard deviation of three replicates are shown

Conclusion

It was very economic that the wastewater from cassava ethanol fermentation could be treated by microalgae in order to produce microalgal biomass, regulate pH, and make a drop of COD. After harvesting the algae, although some part of COD remained in the treated water, it was so low (2.31 $\times$ 10^4 mg l^{-1}) that it had no negative effect in the process of ethanol production. The character of our technology for wastewater biotreatment were that WCEP was not diluted and directly used by the microalgae, and the treated wastewater could be recycled in the process of ethanol production entirely and directly.

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