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Biological treatment of the refractory gardenia-yellow-manufacturing wastewater and microbial community shift in A/O reactors

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ABSTRACT

Treatment of wastewater from natural-pigment-manufacturing process is a challenge due to its complex composition and recalcitrance. This study investigated the use of biological method to degrade high-concentration gardenia-yellow-manufacturing wastewater (GYMW) in a laboratory and then applied the anaerobic-aerobic (A/O) process in pilot- and full-scale plants. The maximum anaerobic volume load reached 6 kg chemical oxygen demand (COD) m^{-3} d in laboratory and 5.5 kg COD m^{-3} d in full-scale reactor, while the COD removal efficiency was consistently above 90%. Single-factor control experiments were conducted in sequencing batch reactors to examine the factors that have an influence on the anaerobic treatment, which is responsible for the majority of COD removal. After adjusting the operating temperature, pH, and influent COD, the effluent COD further decreased by 300 mg L^{-1}. In addition, high-performance liquid chromatography, which was used to analyze the main contaminants in GYMW, confirmed that the toxic geniposide was considerably reduced. The high-throughput sequencing analysis illustrated that, under the selective pressure from GYMW, the microbial community structure can degrade biorefractory pollutants enriched in the A/O reactors and contributed to the satisfactory performance of GYMW treatment.

Keywords: Gardenia-yellow-pigment-manufacturing wastewater; Anaerobic treatment; HPLC; High-throughput sequencing

1. Introduction

The public concern about the food safety has led to the use of natural pigments as food additives. However, the natural-pigment-manufacturing process produces a large volume of wastewater containing coarse pigment, lactic acid, cellulose, hemicellulose, lignin, gums, and other recalcitrant contaminants even after the recovery of useful substances. Henan Luohe Zhongda Natural Food Additive Co., Ltd., China uses ethanol to extract gardenia yellow pigment from gardenia fruit and separates the product using active carbon or macroporous adsorbent [1]. The possible substances present in gardenia-yellow-manufacturing wastewater (GYMW) include geniposide, crocin, ethanol, and chlorogenic acid (Fig. 1) [2–5]. Developing a cost-effective treatment to meet the discharge standard for this high-strength refractory wastewater is a great challenge.

Biological methods, especially the anaerobic-aerobic (A/O) process, are widely used in the industrial and municipal wastewater treatment in combination with physical and/or chemical methods. In particular,

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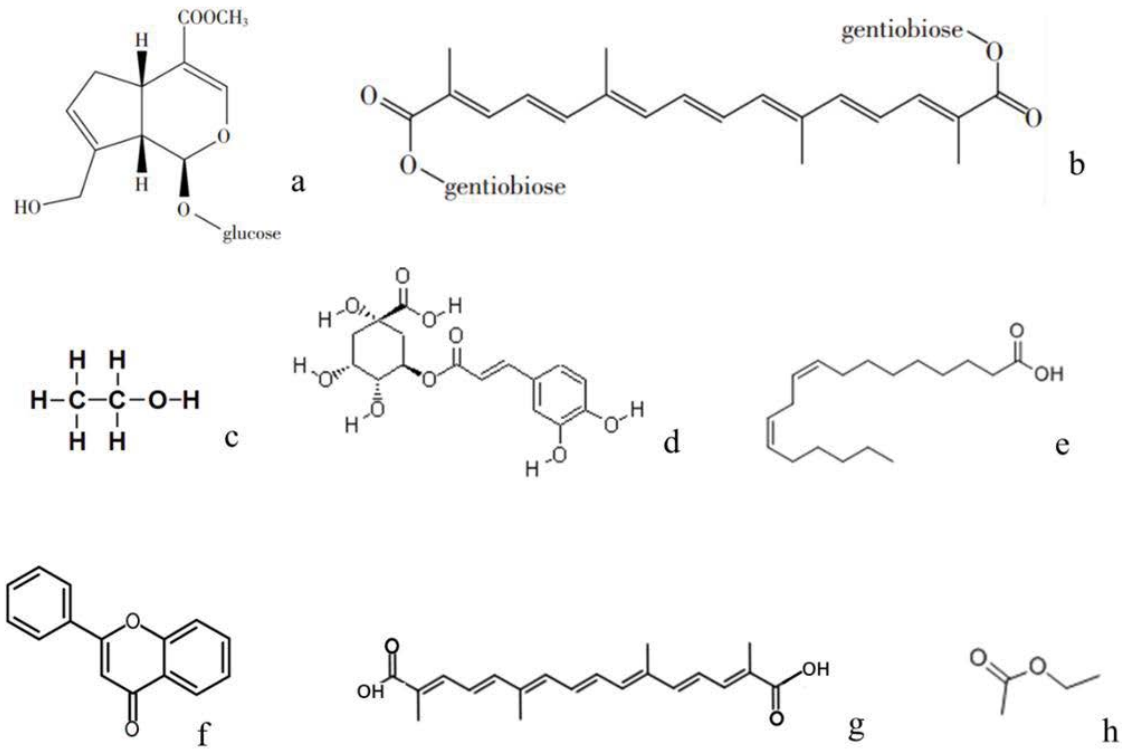

Fig. 1. Some substances may be held in GYMW (a) Geniposide, (b) Crocin, (c) Ethanol, (d) Chlorogenic acid, (e) Linoleic acid, (f) Flavone, (g) Crocetin acid, and (h) Ethyl acetate.

anaerobic digestion has its advantages such as low costs, small footprint, less residual sludge production, and energy recovery potential. However, the poor performance at low temperatures [6,7] and the narrow optimum pH range [8] limit its application.

Previous studies on the treatment of natural-pigment-manufacturing wastewater have mainly focused on the recovery of useful substances [1,9,10] and the engineering commissioning [11]. Some studies used advanced oxidation as pretreatment to enhance the biodegradability of GYMW [10], though this technology is expensive. The plant-scale application of GYMW biological treatment is rarely reported. This study investigated the effectiveness of A/O method to biodegrade GYMW in laboratory and the operational factors that have an influence on the performance of anaerobic treatment using single-factor control experiments. Then, this technology was applied in pilot- and full-scale plants. The findings from this study could provide the fundamental knowledge required for the treatment of wastewater generated during the natural-pigment-manufacturing process.

2. Materials and methods

2.1. Characteristics of the wastewater

The wastewater used in this study was sourced from the Regulation Pool in Henan Luohe Zhongda Natural Food Additive Co., Ltd, and the wastewater characteristics are listed in Table 1. The index values exhibited minor fluctuations due to unstable manufacturing process.

Table 1
Characteristics of wastewater used in this study

COD (mg L^{-1})	6,000–11,000
pH	4.6–5.1
VFA (mmol L^{-1})	33–39
TP (mg L^{-1})	16–22
TN (mg L^{-1})	91–101
Ammonia nitrogen (mg L^{-1})	22–31
Oxidation-reduction potential (mV)	–211 to –229

2.2. Laboratory-scale test for A/O technique

Fig. 2 illustrates the laboratory-scale continuous A/O reactors. A 3.5-L anaerobic reactor was operated at 33°C–35°C, and the reaction temperature was maintained using a rubber muff in a hot water bath. Volcanic rocks (diameter of 5–10 mm) were used to fill the packed layer. The reactor was not completely enclosed to allow the release of generated gas. The anaerobic granular sludge sourced from a flour mill in Henan was seeded at the beginning of the experiment (on the 0th day), and the mixed liquor suspended solid (MLSS) concentration was approximately 36,000 mg L^{-1}. The chemical oxygen demand (COD) loading rate of the anaerobic reactor was 1 kg COD m^{-3} d^{-1} at the beginning and then gradually rose to the maximum of 6 kg COD m^{-3} d^{-1}. Meanwhile, the pH of the influent was maintained between 6.3 and 6.5.

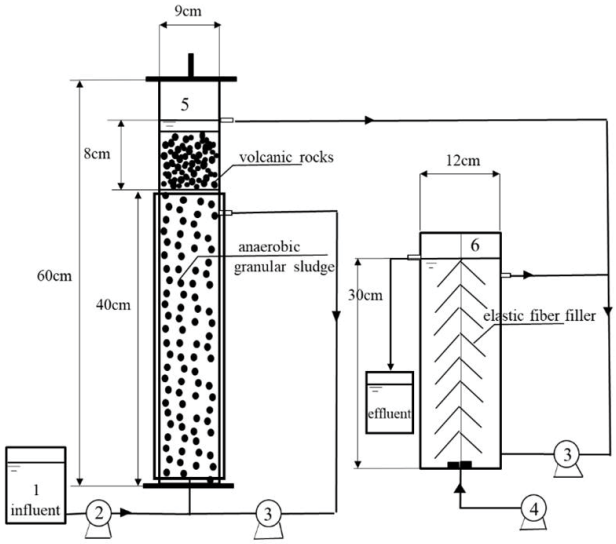

Fig. 2. Scheme of the experimental system. (1) influent tank, (2) peristaltic pump of the feeding media, (3) return peristaltic pump, (4) air pump, (5) anaerobic reactor, and (6) aerobic reactor.

The aerobic reactor was made of a 12 cm^3 × 12 cm^3 × 35 cm^3 cuboid plexiglass column with built-in elastic fiber filler. The seeded activated sludge was taken from the anoxic section of the anaerobic-anoxic-oxic process in Wulongkou wastewater plant, Zhengzhou, Henan province, China, with initial MLSS concentration of approximately 4,360 mg L^{-1}. The aerobic reactor was placed in series with the anaerobic reactor. The aerobic reactor was operated at the room temperature, and its pH was maintained between 7 and 8.

2.3. Single-factor control experiments

Single-factor control experiments were conducted to investigate the impacts of environmental factors on anaerobic digestion. All experiments were completed in 1,500-mL sealed sequencing batch reactors with a working volume of 800 mL. Water samples were collected at regular time intervals (11–13 h). Wastewater collected from the regulation pool was diluted to the required influent concentrations for the experiments. The inoculation sludge was the same as that used in continuous anaerobic reactor. During the sludge acclimation process, the influent COD, MLSS, pH, and the operating temperature were maintained at 4,000 mg L^{-1}, 26,500 mg L^{-1}, 7.5 ± 0.05, and 35°C, respectively. The acclimation process was continued until the COD reached the stable minimum between 1,100 and 1,200 mg L^{-1} for four consecutive cycles of operation. Then, single-factor control experiments for temperature, pH, and influent COD were conducted. All experiments were performed in triplicates.

2.4. Application of A/O technique in pilot- and full-scale plants

Continuous A/O pilot-scale (231 L) and full-scale reactors (590 m^3) were constructed in Luohe plant based on the results of laboratory-scale experiments. The influent flow rates were maintained between 3 and 9 L h^{-1}, while the upflow velocity was maintained at 1 m h^{-1} in the pilot-scale reactor. In contrast, the influent flow rates were maintained between 3 and 12 L h^{-1}, whereas the upflow velocities were between 0.09 and 0.3 m h^{-1} in the full-scale plant. The specific influent loading rates varied under different conditions.

2.5. Analysis

The effluent samples were centrifuged at 7,000 rpm for 5 min (TG16-WS, China). Then, COD concentrations, volatile fatty acids (VFA), and MLSS were measured using the potassium dichromate method (HCA-102, China), the distillation method, and the gravimetric method, respectively. pH and oxidation-reduction potential were measured using the electrometric technique, whereas ammonia nitrogen, total nitrogen (TN), and total phosphorus (TP) were measured using colorimetric technique after the preliminary distillation and the persulfate digestion (Persee TU-1900, China). The methods used in this study were consistent with the Standard Methods for the Examination of Water and Wastewater (APHA).

Water samples were filtered through 0.22 μm polyvinylidene fluoride membranes for particulate removal and sterilization before the analysis. The UV-Vis spectrum was obtained using UV-vis spectrophotometer at 190–900 nm. The chromatographic analyses were carried out by a high-performance liquid chromatography (HPLC) system (P230 II, Dalian Elite, China) with an Agilent 5 TC-C18 (5 μm, 250 mm× 4.6 mm) column to separate the organic matters. A 20 μL of 10 times diluted water sample was injected at a flow rate of 1 mL min^{-1}. The method of the gradient programmer was as follows: water (solvent A) and acetonitrile (solvent B) were used for this pump at a volume ratio of 85:15, the wavelength for detection was 200 nm, and the total run time was 15 min.

The sludge samples were taken on Day 0 and Day 50 from the laboratory-scale A/O reactors and the DNA extraction, polymerase chain reaction (PCR) amplification, and high-throughput sequencing were performed as described by D. Wan et al. [12]. The primers used for PCR of the Miseq sequencing platform and the specific sequences are listed in Table 2.

3. Results and discussion

3.1. Performance of biological treatment of GYMW in laboratory experiments

A/O was the preferred method for the biodegradation of high-strength GYMW. The influent COD increased from 6,400 to 9,900 mg L^{-1} during 50 d of operation. In general, the overall COD removal efficiency was over 90%, and 80% of COD was removed during the anaerobic process (Fig. 3(a)).

The anaerobic volume load (AnVL) increased from 1 to 6 kg COD m^{-3} d^{-1} as the anaerobic hydraulic retention time (HRT) dropped from 88 to 22 h (Fig. 3(b)). The effluent COD for the largest AnVL increased by 67% compared with the previous stage (AnVL = 5 kg COD m^{-3} d^{-1}). Therefore, the anaerobic HRT was not further reduced after 40 d of operation. Although the maximum aerobic volume load was only 0.1 kg COD m^{-3} d^{-1}, the effluent COD increased from 200 to 700 mg L^{-1} when the aerobic HRT decreased from 126.7 to 31.7 h.

Table 2
Primers for PCR amplification

	Primers	The primer sequences
Bacterial community	341F	CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG
	805R	GACTGGAGTTCCTTGGCACCCGAGAATTCCA GACTACHVGGGTATCTAATCC
Archaeal community	1st-340F	CCCTAYGGGGYGCASCAG
	1st-1000R	GGCCATGCACYWCYTCTC
	349F	CCCTACACGACGCTCTTCCGATCTN (barcode) GYGCASCAGKCGMGAAW
	806R	GACTGGAGTTCCTTGGCACCCGAGAATTCCA GGACTACVSGGGTATCTAAT

The TN removal efficiency continued to increase. On the 50th day, the effluent TN concentration decreased to 22 mg L^{-1} and the TN removal efficiency was 62% higher than that at the beginning of the operation (Fig. 3(c)).

3.2. Single-factor control experiments

The laboratory-scale test showed that the COD removal primarily occurred in the anaerobic phase. Further, the refractory organic matters in GYMW could affect the performance of the aerobic reactor. These outcomes and the benefits of the anaerobic process mentioned above emphasized the importance of improving the anaerobic conditions. Therefore, a series of single-factor control experiments were conducted to investigate the factors that could potentially influence the anaerobic digestion.

3.2.1. Temperature

The effluent COD dropped to the lowest value of 1,100–1,200 mg L^{-1} for all tested temperatures (15°C–40°C), though their removal efficiencies were different (Fig. 4(a)). The changes in temperature between 25°C and 40°C did not show any significant effect on pollutant removal. Nonetheless, the reaction period was significantly prolonged when the temperature continued to decrease from 25°C to 15°C. The VFA degradation also exhibited a similar trend (Fig. 4(b)), resulting in a low gas production at low temperatures (15°C–25°C).

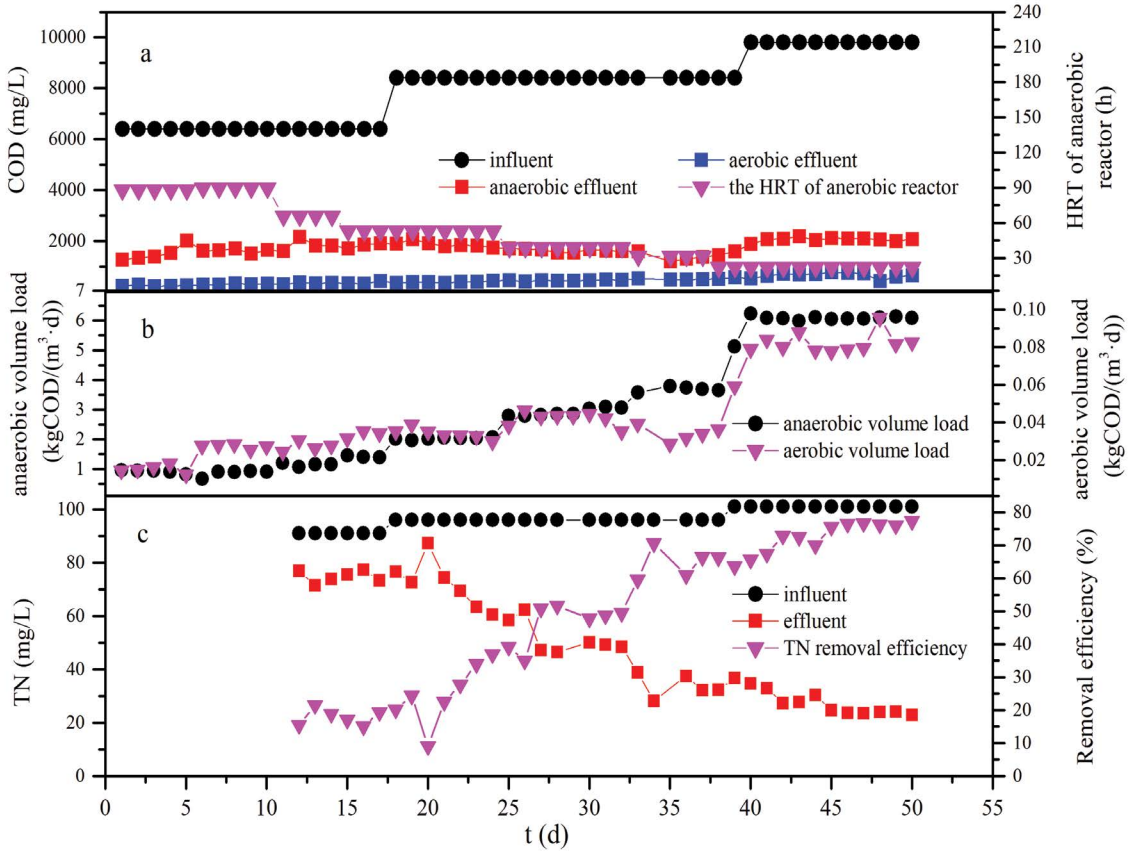

Fig. 3. (a) The COD concentration in influent and effluent under different HRTs, (b) the corresponding reactor volume load, and (c) TN removal of the anaerobic reactor. (The average MLSS was 36,000 mg/L and the temperature was 31°C–33°C).

Three models were used to describe the anaerobic COD degradation under different temperatures (Table 3):

$$\text{zero-order kinetics}: C = C_0 - K_0 \times t \quad (1)$$

$$\text{frist-order kinetics}: C = C_0 \times \exp\left(-K_1 \times t\right) \quad (2)$$

$$\text{second-order kinetics}: C = \frac{1}{\left(\frac{1}{C_0} + K_2 \times t\right)} \quad (3)$$

where C_0 is the initial COD (mg L^{-1}) and C is the COD (mg L^{-1}) at time t (h). K_0 is the zero-order rate constant (mg L^{-1} h^{-1}), K_1 is the first-order rate constant (h^{-1}), and K_2 is the second-order rate constant (L mg^{-1} h^{-1}).

According to the correlation coefficient, the first- (R > 92%) and the second-order kinetic models (R > 93%) fitted the experimental data better. In this study, the first-order kinetic model was discussed. The degradation curves at 25°C, 30°C, 35°C, and 40°C were similar, and the K_1 values were around 0.017 h^{-1}. However, these results contradict the findings of most studies suggesting that the K_1 values increase with increased temperature (up to about 40°C) since the methanogenic bacterial activity peaks at temperatures between 30°C and 37°C [13]. However, some other studies have reported results that are consistent with this study; for example, Dague pointed out that excess biomass can eliminate the impact of temperature reduction from 35°C to 25°C [14]. Further, Bowen et al. [15] found that the temperature effect is minimal for complex wastewater composition.

At temperatures below 25°C, the reaction rate decreased simultaneously with temperature. K_1 value at 15°C was 0.00629 h^{-1}, which was almost 2.5 times lower than that at 25°C. These observations can be explained from different perspectives. First, as the temperature decreases, the solubilities of substances are reduced [16]. In this study, the concentration of suspended solids at 15°C (pH 7.5) was 971 mg L^{-1}, which is double the solubility at 40°C. Further, the hydrolysis rate of suspended solids is lower than that of liquids, and the insoluble material wrapped on anaerobic granular sludge can impede the mass transfer efficiency [17,18]. Second, the microbial hydrolysis mainly relies on the extracellular enzymes. Low temperatures tend to impair the secretion of extracellular enzymes and the hydrolytic enzyme activity, resulting in reduced hydrolytic efficiency [19,20]. Furthermore, low temperature can inhibit the activity of anaerobic microorganisms, especially the methanogens [16,21], leading to the accumulation of VFA that may lead to the acidification of the system and consequent inhibition of microbial activities [22].

3.2.2. pH

The COD removal efficiency peaked (>70%) at pH 7.5 and 8.5 (Fig. 4(c)), which was slightly higher than the values reported in most studies [8,23]. The COD removal efficiency was considerably low at about 12.5% and 2% when pH was between 6.5 and 5.5 because methanogens are extremely sensitive to acidic environments [24]. In addition, the specific wastewater used in this study rapidly acidify at pH below 7.0 and lead to the system collapse. In contrast, the granular sludge began to disintegrate at higher pH of 9.5, resulting in significant odor (data not recorded).

3.2.3. Influent COD

Fig. 4(d) shows the COD removal at different influent CODs (3,000–6,000 mg L^{-1}). The removal efficiency reached the maximum of 76.79% when the influent COD was 4,000 mg L^{-1}. Due to the existence of refractory COD (about 1,000 mg L^{-1}), lower influent concentration did not result in higher COD removal. In contrast, excessive influent concentration also impeded the COD removal. The COD removal efficiency dropped to 58% when the initial COD was 6,000 mg L^{-1} because it had been beyond the microbial degradation capacity [25].

3.2.4. Adjustment to the anaerobic reaction condition

Based on the experimental results discussed in Sections 3.2.1 to 3.2.3, the anaerobic reaction temperature, pH, and influent COD were adjusted to 35°C, 8.5, and 4,000 mg L^{-1}, respectively. The change in COD was observed for 72 h, and the performance was compared with that under the original condition (Table 4). Additionally, K_1 increased from 0.01746 to 0.02203 h^{-1}, suggesting that the reaction rate improved (p < 0.01). These results presented important references for the design of pilot- and full-scale anaerobic reactors.

3.3. Analysis of geniposide removal in GYMW

Since geniposide is a highly toxic by-product of gardenia yellow [26], the geniposide removal was monitored throughout

Table 3
Kinetic parameters calculated from the COD removal

Model	Zero-order kinetics			First-order kinetics			Second-order kinetics			Effluent
Parameters	K_0 (mg L^{-1} h^{-1})	R	$t_{1/2}$ (h)	K_1 (h^{-1})	R	$t_{1/2}$ (h)	K_2 (L mg^{-1} h^{-1})	R	$t_{1/2}$ (h)	COD (mg L^{-1})
15°C	14.2401	0.9656	140.45	0.00629	0.993	110.2	2.64E-06	0.981	94.55	1,293
20°C	19.2836	0.8474	103.72	0.00919	0.9842	75.42	2.92E-06	0.9705	85.56	1,368
25°C	22.351	0.8264	89.48	0.01451	0.9288	47.77	6.68E-06	0.977	37.42	1,125
30°C	42.7285	0.8571	46.81	0.01746	0.9699	39.7	6.02E-06	0.9398	41.52	1,355
35°C	35.9968	0.9072	55.56	0.0177	0.976	39.16	8.33E-06	0.9953	30.03	1,219
40°C	43.1132	0.93	46.39	0.0178	0.9849	38.94	7.12E-06	0.9921	35.11	1,310

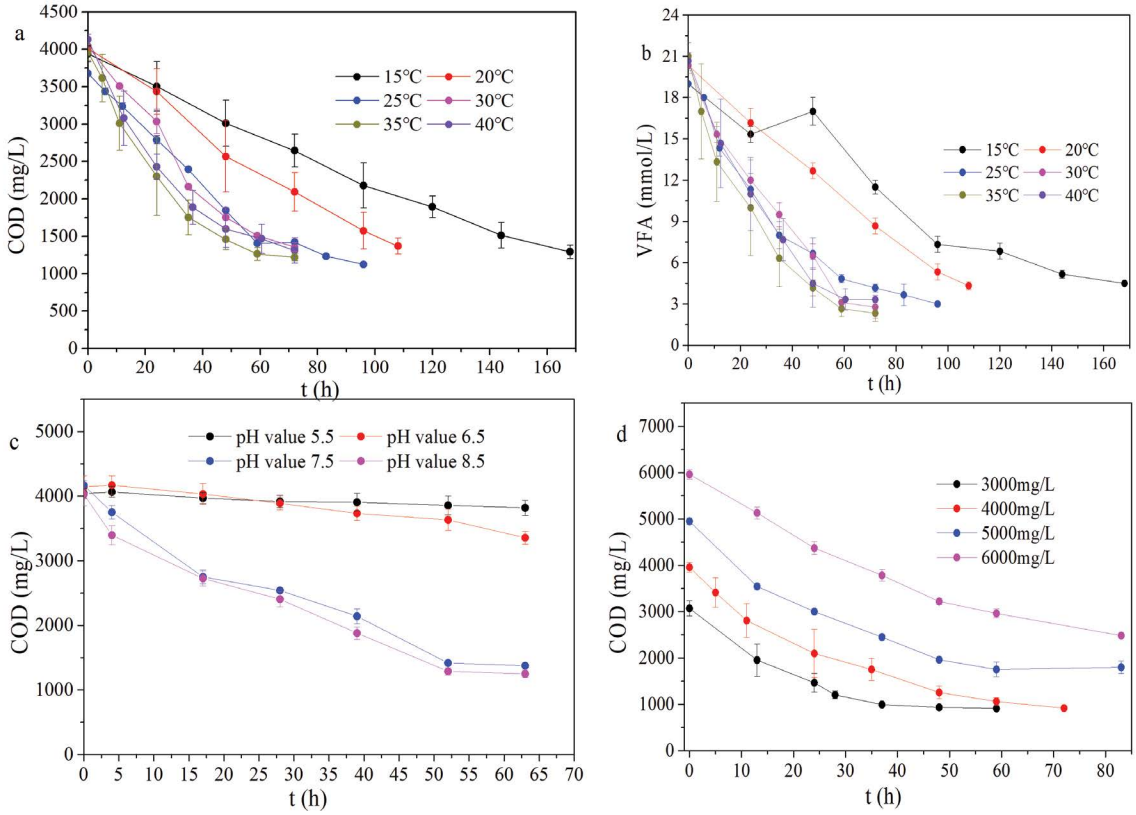

Fig. 4. The performance of anaerobic process at different operating conditions. (a) The removal of COD, (b) the removal of VFA at different temperatures (the initial COD concentration was 4,000 mg/L, pH value was 7.5 ± 0.05), (c) the COD degradation with time at pH value 5.5, 6.5, 7.5, and 8.5. (The initial COD concentration was 4,000 mg/L and the temperature was 35°C), and (d) the removal of COD at different initial COD concentrations (pH value was 8.5 ± 0.05, the temperature was 35°C), MLSS for all sequencing batches was about 26,500 mg/L.

Table 4
The performance of anaerobic reactor before and after adjustment

Model	First-order kinetics			Effluent
parameters	K_1 (h^{-1})	R	$t_{1/2}$ (h)	COD (mg L^{-1})
Before	0.01746	0.9699	39.7	1,219
After	0.02203	0.98141	31.46	919

the A/O treatment. All water samples (influent, anaerobic effluent, and aerobic effluent) exhibited high absorbance at approximately 200 nm in UV-Vis spectra scan for the aromatic compounds they contained, e.g., geniposide (Fig. 5(a)). Therefore, the subsequent HPLC was performed at the detection wavelength of 200 nm. As shown in Fig. 5(b), the primary composition of organic substances in the wastewater was not changed much, although its peaks were significantly lower after biodegradation. In addition, their retention times were much the same as the geniposide standard. The HPLC results illustrated that A/O process can significantly remove geniposide and reduce the toxicity of GYMW.

3.4. High-throughput sequencing and microbial community analysis

The sludge samples were collected on the 0th day (An_0 – the inoculation anaerobic sludge; A_0 – the inoculation aerobic sludge) and the 50th day (An_{50} and A_{50}). The extracted DNA was analyzed by 16S ribosome deoxyribonucleic acid (rDNA) high-throughput sequencing, and the effective DNA sequence readings were clustered into operational taxonomic units by setting a 0.03 distance limit using the MOTHUR program. The Shannon diversity index and the species richness estimator of Chao1 and ACE indices were also generated by the MOTHUR

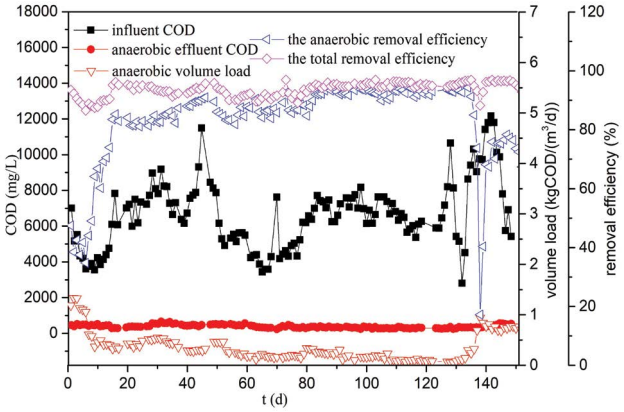

Fig. 5. (a) UV analysis of 50 times diluted g water samples and (b) showed the chromatograms from the HPLC analysis of the 10 times diluted water samples and 60 mg/L Geniposide standard.

Table 5
Summary of pyrosequencing data of the sludge

	Bacterial community				Archaeal community	
	An_0	An_{50}	A_0	A_{50}	An_0	An_{50}
Sequence numbers	71,094	70,412	63,238	69,937	37,094	37,809
Coverage	0.94	0.95	0.94	0.95	0.97	0.96
Operational taxonomic unit numbers	7,009	5,295	5,829	5,019	1,293	1,557
ACE index	34,491.42	44,134.66	37,823.80	51,797.38	27,426.92	39,335.01
Chao1 index	23,608.2	24,925.33	21,727.90	27,330.85	9,378.3	14,545.94
Shannon index	6.71	5.6	6.66	5.82	2.73	2.77
Simpson	5.30E-03	0.01	3.8E-03	9.3E-03	0.14	0.15

program. The sequencing results for the bacterial and archaeal communities are summarized in Table 5. Their coverages exceeded 94%, suggesting that the majority of microbial communities were detected successfully. The Chao1 and ACE indices are positively related to the microbial richness. The greater An50 and A50 values indicate that the microbial species were more abundant after 50 d of acclimation. The Shannon index is positively related to the diversity of microbial communities, whereas the Simpson index is the opposite. Although the archaea indices did not show a significant change, the bacterial diversity of both anaerobic and aerobic reactors declined due to the selective pressure produced by the refractory GYMW.

Sequences from sludge samples were analyzed at different taxonomic categories to distinguish the relationship between the microbial communities and their functions. At the phylum level, the main bacterial community structures in A/O reactors and the respective inoculated sludge were generally similar (Fig. 6(a)). The dominant phyla in the anaerobic reactor were *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, and *Chlorobi*, and dominant phyla in the aerobic reactor were *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Chloroflexi*. These observations are consistent with Hwang et al. [27], who reported that *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and Bacteriodetes were predominant groups for natural dye treatment. In addition, *Proteobacteria* was the most common phylum in activated sludge. *Chloroflexi* were reported to be responsible for degrading soluble microbial products including carbohydrates and cellular materials [28]. *Firmicutes* were related with acidogenesis and acetogenesis and often appeared in reactors of refractory wastewater treatment [29,30]. This could be the reason for the substantial increase in An0 from 5.54% to 23.17% on the 50th day. *Planctomycetes*

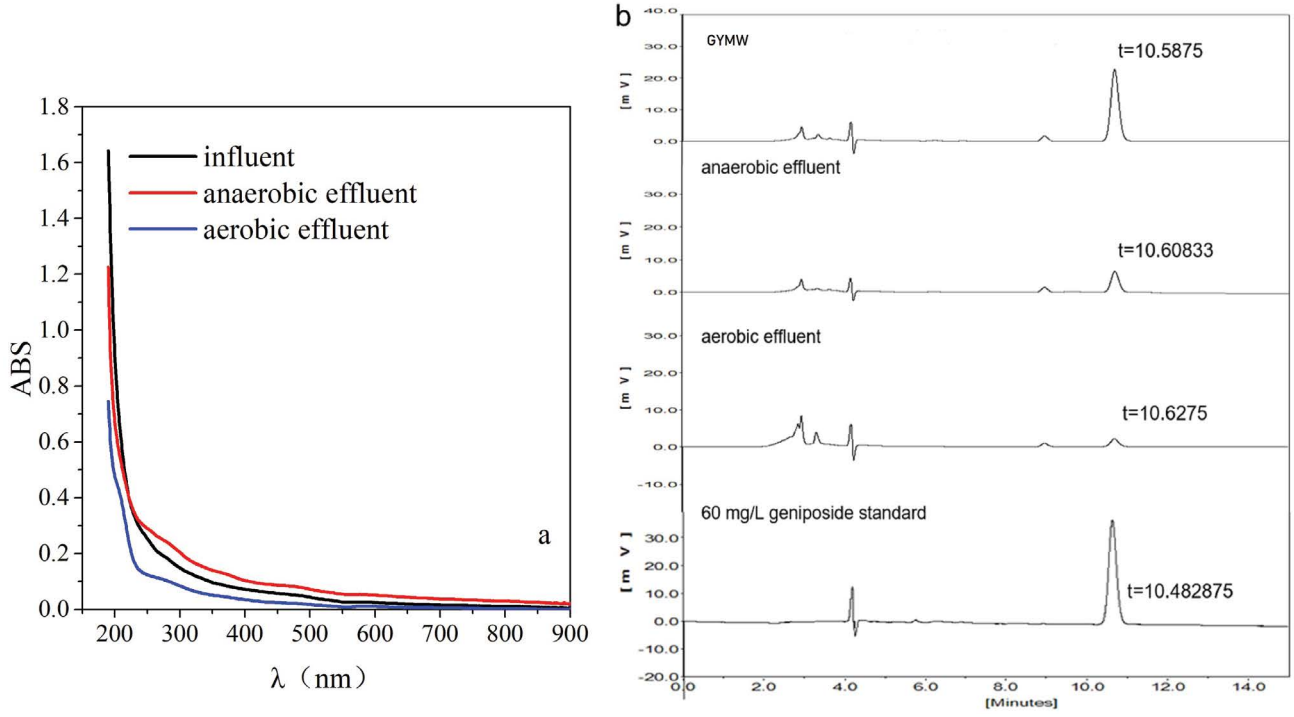

Fig. 6. 16S rDNA for classification of the bacterial communities at (a) phylum, (b) genus level, and (c) archaea communities at genus levels. The relative abundance less than 1.5% genus in all the samples were defined as "others".

Table 6
Representative operation data of pilot-scale anaerobic reactor

t (d)	Influent COD (mg L^{-1})	Effluent COD (mg L^{-1})	HRT (h)	*T* (°C)	Volume load (kg COD m^{-3} d)
8	2,388.8	227	27.84	31	2.11
9	5,804	1,086.4	61.68	32	1.16
14	11,936	4,688	84.96	29	2.05
32	6,314.88	2,388.32	50.16	28.5	1.88
34	11,982.08	2,469.28	86.64	29.5	2.97
37	11,982.08	1,807.72	69.12	29.5	3.53
39	6,356.8	1,748.12	119.04	28.5	0.93
40	5,854.73	2,224.32	62.4	30.5	1.4
41	5,854.73	2,041.61	46.08	28	1.99
48	9,407.04	3,333.79	55.92	29.5	2.61
56	9,706.18	3,160.61	39.6	29.5	3.98
64	7,791.04	2,453.78	51.6	31	2.48

and *Verrucomicrobia* decreased after the anaerobic operation. *Planctomycetes*, which produce acids from sugars, were eradicated as the sugar content of GYMW was significantly lower than that of wastewater from the starch plant [31]. Meanwhile, the alkaline environment in the anaerobic reactor had potentially led to the reduction of *Verrucomicrobia* since they only survive in an acidic and high-temperature environment [32]. On the contrary, the archaeal community showed no changes at the phylum level (data not shown).

In contrast, the composition of the anaerobic and the aerobic microorganisms varied substantially at the genus level. The anaerobic bacterial groups that were affected by the acclimation process included *Syntrophomonas* (from 0.19% to 9.76%), *Comamonas* (from 0.29% to 4.71%), *Pseudomonas* (from 0.01% to 4.71%), *Chlorobium* (from 0.69% to 4.44%), *Hydrogenophaga* (from 0.03% to 3.2%), *Tissierella* (from 0% to 2.48%), *Sphingobium* (from 0.08% to 2.34%), *Acinetobacter* (from 0% to 2.13%), *Diaphorobacter* (from 0% to 1.95%), and *Smithella* (from 6.45% to 0.61%) (Fig. 6(b)). *Syntrophomonas* is an anaerobic non-phototrophic bacterium that can beta-oxidize saturated fatty acids [33] present in GYMW [34].

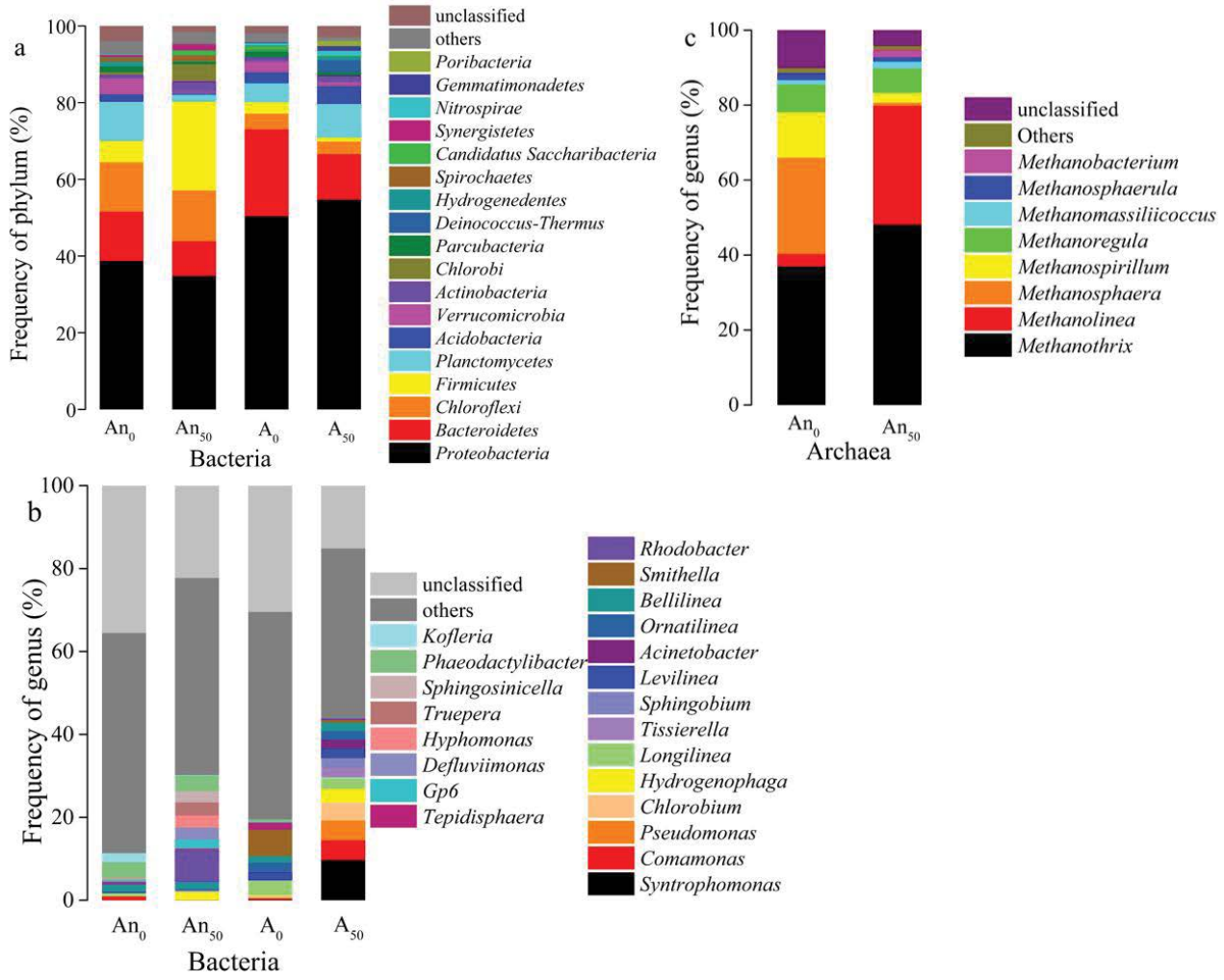

Fig. 7. COD variation in influent, reactor volume load and COD removal efficiency in the engineering application (the average MLSS was 15,000 mg/L and the temperature was 31°C–33°C).

Comamonas was reported to be involved in the degradation of refractory pollutants [35], whereas *Pseudomonas* is known for breaking down polycyclic aromatic hydrocarbons and fatty acids in wastewater [36]. *Chlorobium* is a green sulfur eubacterium that helps the metabolism of sulfur and nitrogen [37], while *Hydrogenophaga* is often found in textile wastewater and can degrade complex contaminants [38]. These characteristics of the diverse microbial environment favored the high COD removal efficiency in the anaerobic reactors used in this study. Meanwhile, the abundance of propionate oxidizing bacterium *Smithella* was reduced [39] because propionic acid is an important intermediate for starch anaerobic degradation, but not in GYMW. For the aerobic reactor, the bacterial groups changed included *Rhodobacter* (from 0.06% to 7.6%), *Truepera* (from 0% to 3.14%), *Kofleria* (from 2.15% to 0.14%), and *Nannocystis* (from 1.17% to 0.01%). *Rhodobacter* is typically a photosynthetic bacterium that helps in the removal of nitrogen and phosphorus [40] and *Truepera* favors desulfurization and denitrification. *Comamonas*, *Pseudomonas*, *Acinetobacter*, *Rhodobacter*, *Truepera*, and *Diaphorobacter* also involve in the processes of the nitrogen cycle. These bacteria could have contributed to the substantial TN removal during the later stages of operation [36].

Archaea can produce methane from acetic acid, methanol, formate, propionic acid, hydrogen, and formic acid. The different methanogenic substrates in starch plant wastewater and in GYMW caused methanogenic groups shift from An0 to An50: *Methanolinea* (from 3.24% to 31.65%), *Methanosphaera* (from 26.75% to 0.86%), and *Methanospirillum* (from 12.12% to 2.16%) (Fig. 6(c)).

3.3. Biological treatment for GYMW in pilot- and full-scale plants

Pilot- and full-scale reactors were constructed based on the results of the laboratory experiments. Representative data for the pilot-scale anaerobic reactors including before and after load undulations and the stationary period are presented in Table 6. After the start-up phase, the influent COD fluctuated between 6,000 and 11,000 mg L^{-1} due to the unstable production of the plant. The effluent COD was generally higher than 2,000 mg L^{-1} during lifting load or influent COD. The maximum AnVL was only 3.98 kg COD m^{-3} d in the pilot-scale reactor and was difficult to maintain.

In the full-scale A/O reactors, the first 50 d were the start-up commissioning period. The influent COD of the anaerobic reactor during this period was between 3,000 and 12,000 mg L^{-1} and the influent loads were between 1.5 and 4.5 kg COD m^{-3} d^{-1} (Fig. 7). The effluent COD and VFA concentration at the end of the start-up period were less than 700 mg L^{-1} and 3 mmol L^{-1}, respectively. At this point, the commissioning period was over and the stable operation was initiated. Between Day 50 and Day 133 of the operation, the anaerobic removal efficiency was above 90% and aerobic effluent COD remained below 500 mg L^{-1}. In addition, the maximum AnVL reached 5.5 kg COD m^{-3} d^{-1}. These results are better than those previously reported during the commissioning of plant-scale pigment wastewater treatment [11,41–43].

4. Conclusions

Biological treatment of the refractory GYMW showed good performance in the laboratory-, pilot-, and full-scale A/O reactors with overall COD removal efficiency reaching 95%, even with the AnVL of up to 6 and 5.5 kg COD m^{-3} d^{-1} in the laboratory- and full-scale plants, respectively. The first-order kinetic model well described the anaerobic degradation of GYMW. Temperature, pH, and influent COD had influences on the removal rate and should be considered in anaerobic reactor design. After adjusting the conditions, the effluent COD further decreased by 300 mg L^{-1}. Furthermore, HPLC analysis confirmed that the biological treatment significantly reduced geniposide, which is the main toxic pollutant in GYMW. The high-throughput sequencing results indicated that microbial communities with the ability to reduce recalcitrant pollutants and/or nitrogen removal were enriched in the A/O reactors.

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