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Development and application of mixed cultures capable for decolorating and mineralizing azo dyes with an anaerobic-aerobic circle method

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ABSTRACT

An anoxic-aerobic circle method was proposed to develop mixed cultures which could decolorate and mineralize azo dyes efficiently. With accumulation and acclimation processes, mixed cultures which completely degraded azo dyes in the medium containing 5 g/L yeast extract at pH 6–7 under anoxic-aerobic circumstance were obtained. Azoreductase, NADH-DCIP reductase, laccase, tyrodinase and lignin peroxidase were involved in the degradation reaction. The acclimated mixed cultures could decolorate a variety of water soluble dyes and completely mineralize aromatic amines without trichlorobenzo[d]thiazole structure. High-throughput 16S rDNA sequencing analysis revealed that members belonging to *Aridibacter*, *Comamonas*, *Gemmatimonas* and *Nannocystis* were enriched in anoxic environment which may be responsible for decoloration. The abundance of *Brevundimonas*, *Petrimonas* and *Chryseobacterium* genera increased under aerobic circumstance implying their important roles in mineralization stage. Process performances of the mixed cultures were studied using a 30 L SBR equipped with a stirrer and a covering. SBR was operated under anoxic-aerobic condition via hydraulic retention time shortened strategy and the azo dye removal rates could surpassed 90% with an initial concentration of 60 mg/L in 24 h. It is suggest that the mixed cultures demonstrate great adaptability to various types of azo dyes and can be used in biological treatment of azo dye wastewater.

Keywords: Azo dyes; Decoloration; Mineralization; Mixed cultures

1. Introduction

Dye, one of the most widely synthesized organic chemicals, is commonly detected in industrial wastewater, causing huge damages to the environment. Dyeing wastewater, which contains complicated constituent, deep chrominance, high concentration of toxic and non-biodegradable substances is hard to treat [1]. Azo dyes are the largest group of synthetic dyes and characterized with the presence of azo bonds (-N=N-) [2]. They can be divided into monoazo dyes, diazo dyes and polyazo dyes based on the number of azo bonds. The -N=N- linkage is easily to be broken down, however the resulted products (aromatic amines) are toxic, mutagenic and carcinogenic [3]. Thus, completely removing azo dyes from environment is of significant importance.

Currently, various methods involving physical, chemical and biological technologies are employed for the azo dye wastewater treatment. Physico-chemical methods are effective on decolorating dye wastewater, however high operational cost and generation of toxic byproducts hinder their application [4–6]. While, biological treatment is proved to be convenient, cost effective and environmentally friendly [7]. It is an economical and effective method to dispose recalcitrant organic pollutants. The conventional biological process includes aerobic, anaerobic and anaerobic-aerobic technolo-

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gies, among which, anaerobic-aerobic process is widely used in azo dyeing wastewater treatment [8,9].

Biotreatment of azo dyes begins with cleavage of -N=N- bonds and corresponding aromatic amines are generated. The reactions are mainly happened under anaerobic circumstances and result in the decoloration of wastewater. Some anaerobic bacteria are effective on decolorating azo dyes. However, the produced aromatic amines are more toxic and difficult to be further degraded under anaerobic condition [10]. Degradation of aromatic amines are usually happened in aerobic environment and aerobic bacteria are involved. The structures of aromatic amines are complex and toxic. Aerobic bacteria are limited to remove these recalcitrant organics [11]. In recent years, several researchers had selected strains with both decoloration and degradation activities to azo dyes, such as *Candida oleophila* [12], *Alternaria alternata* [13], *Penicillium ochrochloron* [14], *Brevibacterium sp*. [15], and *Pseudomonas aeruginosa* [16]. Most of the studies were focused on the degradation characteristics, azo reductase and degradation mechanism of individual species.

However, single strains demonstrated limited completely degradation ability in many cases for the reason that the catabolic pathway of aromatic amines is lack. Mineralization of azo dyes needs the synthrophic and catabolic interactions of different individual species [17] and pure cultures cannot be applied for the treatment of real wastewater since it is impossible to maintain them. Thus treatment of azo dye with mixed cultures were more effective than that of pure strains [18].

There are two problems need to be concerned when mixed cultures are developed. First, the strains must be capable of decolorating azo dyes under anoxic circumstance and mineralizing aromatic amines in aerobic environment. Second, due to the complex and multiple structures of azo dyes, the mixed cultures must demonstrate adaptability to a broad spectrum of azo dye [19,20].

In this study, in order to enrich mixed culture which could decolrize and mineralize various types of azo dyes, a new anoxic-aerobic circle method was proposed. Then the biotransformation process was scaled up in a 30.0 L sequencing batch reactor (SBR) with shortened hydraulic retention time (HRT) strategy to provide a useful guidance for the application of mixed cultures.

2. Materials and methods

2.1. Materials

The azo dyes of industrial grade were procured from Jinsui Biological Technology Co. Ltd. (Shanghai, China). Other chemicals used in this study were analytical grade and purchased from Sinopharm chemical Regent Co., Ltd.

2.2. The enrichment medium

The mixed cultures were enriched in the medium containing 20 mg/L Acid orange 7 (AO7), 20 mg/L Congo Red (CR), 20 mg/L Methyl Orange (MO), 20 mg/L Amino Black (AB) (Fig. S1), 5.0 g/L glucose, 5.0 g/L yeast extract, 0.2 g/L $MgSO_4$ 0.4 g/L KH_2PO_4, 0.1 g/L K_2HPO_4.

2.3. Anaerobic-aerobic circle enrichment

2.3.1. Enrichment process

The mixed cultures were enriched from anaerobic and aerobic sludge in Shaoxing wastewater treatment plant, which treat was water containing more than 90% of dyeing and printing wastewater. 5 mL of the anaerobic sludge was inoculated into a 500 mL flask containing 200 mL enrichment medium and incubated at 30ºC under static condition until the color was faded. Then 5 mL of aerobic sludge was inoculated into the faded solution and shaken at 30ºC at 150 rpm. Every 12 h, 5 mL of the mixture was taken out, centrifuged at 8000 rpm for 10 min and the suspension was scanned with UV-Vis spectrophotometer from 200–800 nm. The aerobic experiment was stopped when the peak assigned to aromatic amines at 257 nm was disappeared in UV-Vis spectrum. Then 5 mL of mixed cultures with 5 mL of aerobic sludge were transferred to 200 mL fresh enrichment medium and repeated the static and shake experiments for four times (Fig. 1a).

2.3.2. Acclimation process

Repeated transfers were carried out with fresh media containing different carbon source (no carbon, glucose, sucrose, starch), different nitrogen source (no nitrogen, peptone, yeast exact, urea and NH_4Cl) and at different pH (5–9). The microbial sample with the highest azo dye removal efficiency was used as the acclimated mixed cultures. At last, the influence of initial azo dye concentration (20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, 100 mg/L each) was studied (Fig. 1b).

2.4. Enzyme assays

Reduction enzymes (NADH-DCIP reductase, azoreductase) and oxidization enzymes (laccase, tyrosinase, lignin peroxidase) produced by the mixed cultures in decoloration and mineralization process were measured to reveal the degradation mechanism. The mixture was collected every 12 h and sonificated for 10 min (pulse 5 s, interval 5 s) at 4ºC. The solution was centrifuged at 10000 rpm for 5 min and raw enzyme was obtained.

The azoreductase activity was measured based on reduction of methyl orange, 0.1 mL of the raw enzyme was incubated with 2 mL of 25 mg/L methyl orange in 50 mM potassium phosphate buffer (pH 7.4) at 30ºC. The residual methyl orange was determined at 618 nm and calculated with the standard curve. One unit of azoreductase (U) was defined as the amount of enzyme that decolorate 1 μM of dye min^{-1}. NADH-DCIP reductase activity was detected in 5.0 mL potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mL raw enzyme, 100 μM DCIP and 1.142 mM NADH at 30ºC. The residual DCIP was measured at 590 nm with an extinction coeffcient of 19 mM^{-1} cm^{-1}. One unit of NADH-DCIP reductase activity was defined as the amount of enzyme that degrade 1 ug of DCIP per minute. Laccase or tyrosinase activity was determined in 5 mL acetate buffer (0.1 M, pH 4.9) with 0.1 mL of raw enzyme and 10% ABTS (or 0.01% catechol) at 30ºC, the increased optical density was determined at 420 nm. Lignin peroxidase activity was measured based on formation of propanaldehyde. 0.1

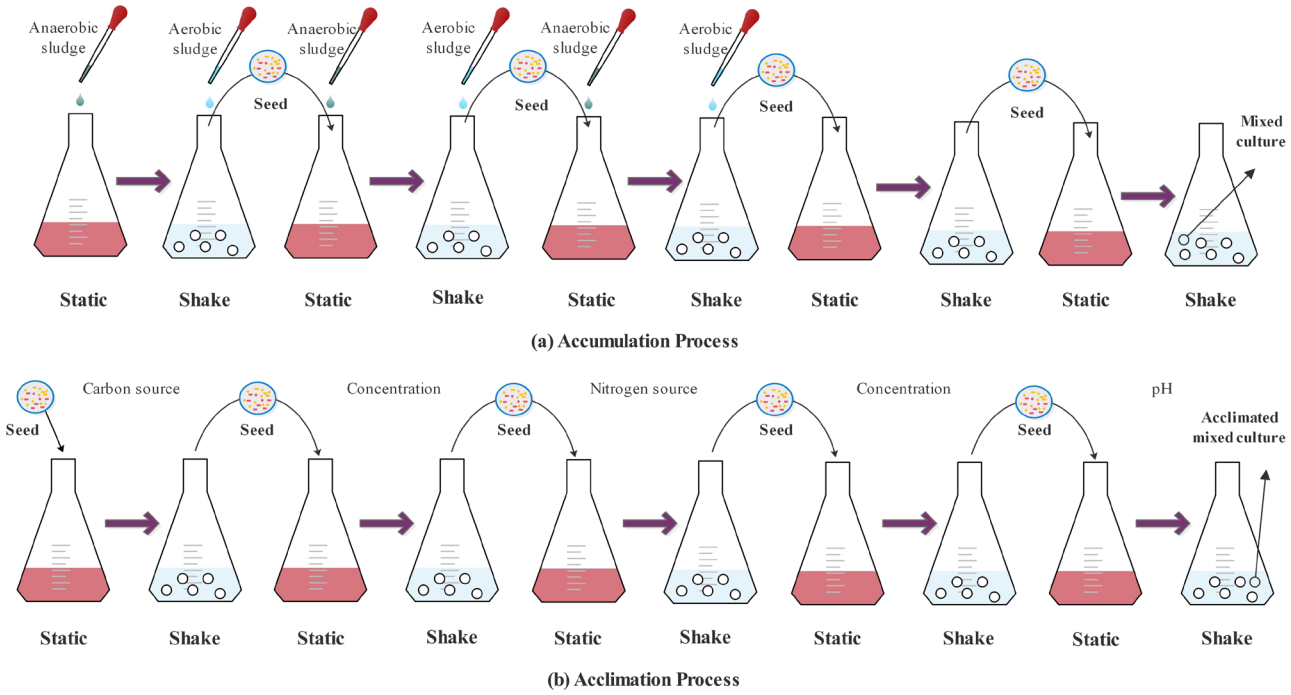

Fig. 1. The procedure of anoxic-aerobic circle accumulation process (a) and acclimation process (b).

mL raw enzyme was incubated with 100 mM n-propanol, 250 mM tartaric acid and 10 mM H_2O_2 The produced propanaldehyde was measured at 300 nm. One unit of enzyme activity can be defined as the amount of enzyme which can result in the increase of 1.0 unit absorbance per min.

2.5. Broad-spectrum azo dye degradation ability experiment

Broad-spectrum azo dye degradation ability experiment was carried out in 500 mL flasks with 200 mL medium containing 60 mg/L azo dye and 5 g/L yeast extract at pH 6–7. Eight kinds of azo dyes with different molecular structure and functional groups were selected for the test (Table S1). They were: Reactive dyes (Reactive Black KN-B and Reactive Red X-3B), Disperse dyes (Disperse Red GS 200 and Disperse orange 25), Direct dyes (Direct Blue 15 and Direct Dark brown M) and Acid dyes (Acid Light Yellow and AO7). 5% of the mixed cultures was inoculated to the medium and the flask was stack at 30°C for 24 h and then shaken at 150 rpm at 30°C for another 24 h. Every 12 h, 5 mL of the solution was taken out, centrifuged and scanned with UV-Vis spectrophotometer.

2.6. Microbial community analysis

For Illumina Miseq analysis, mixed cultures sample was centrifuged at 10000 rpm at 4°C and the pellets were collected. DNA was extracted with Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacture's instruction. The V3 and V4 variable regions of 16S rDNA gene were PCR-amplified targeting with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′) in Geneamp R 9700 PCR System (ABI company, USA) [21,22]. Illumina Miseq analysis was performed on the platform in Sangon Biotech (Shanghai, China) Co., Ltd. The raw reads were submitted to the NCBI Sequence Read Archive and accession number of SRP119807 was obtained.

2.7. Bioreactor

SBR (Φ15 × 43 cm) was constructed with plexiglass (Fig. 2). Initially, SBR was operated with wastewater containing four azo dyes (60 mg/L each) and 5 g/L yeast exact at an anoxic HRT of 24 h (agitation with covering) and an aerobic HRT of 24 h (aeration and agitation without covering). After 20 d, both of the anoxic and aerobic HRTs were maintained at 18 h. After obtaining stabilized azo dye removal rate, the anoxic and aerobic HRTs were shorten to 12 h. UV-Vis (620 nm, 485 nm, 257 nm) was employed to characterize the -N=N- azo bond and aromatic rings in influent and effluent.

3. Results and discussion

3.1. Effects of different physico-chemical parameters

With the anoxic-aerobic circle method, mixed cultures which could decolorate azo dyes under anoxic circumstance and mineralize aromatic amines in aerobic environment was developed. The effects of carbon source, nitrogen source, pH and dye concentration were evaluated and shown in Fig. 3. The mixed cultures could completely decolorate and mineralize azo dyes without any carbon source. While supplementary of glucose, sucrose, starch or sodium acetate led to decreases of both decoloration and

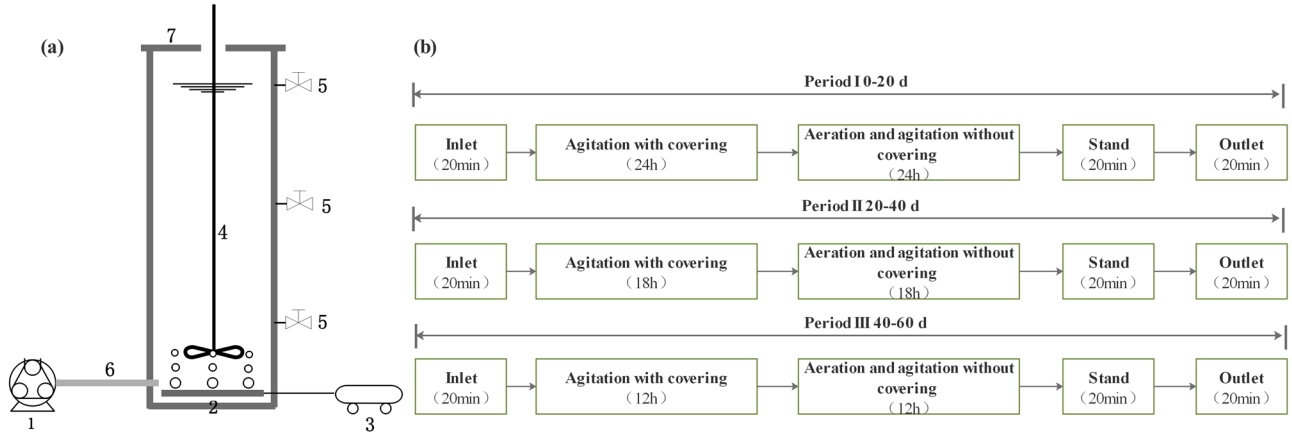

Fig. 2. Schematic structure (a) and operation method of SBR. 1: Peristaltic pump; 2: Aeration Heater; 3: Air pump; 4: Stirrer; 5: Outlet; 6: Inlet; 7: Covering.

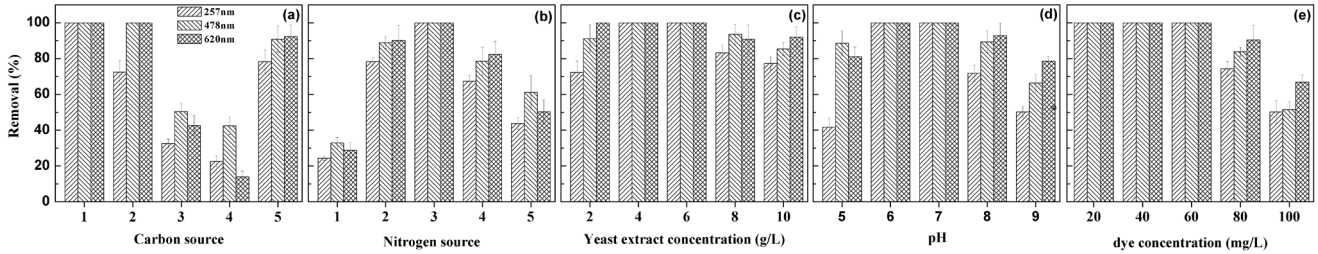

Fig. 3. (a) Effect of different carbon sources (5 g/L) on mixed azo dyes (20 mg/L each) degradation with 5 g/L yeast extract. (b) Effect of different nitrogen sources (5 g/L) on mixed azo dyes degradation without carbon source. (c) Degradation of azo dyes by mixed cultures at increasing concentration of yeast extract. (d) Azo dyes degradation with mixed cultures at different initial azo dye concentration.

mineralization efficiency (Fig. 3a). Most of the previous studies showed that azo dyes were too recalcitrant to be utilized as carbon sources for microorganisms. They were usually removed in co-metabolism situations [19]. While, some single strains or mixed cultures, have demonstrated the ability in utilizing azo dyes as carbon source [20,23,24]. Hence, development of microorganisms which could utilize azo dyes as a carbon source was of significant importance because it might provide an economical and efficient alternative for treatment of dye wastewater in biological way [25,26]. Compared with other carbon source, azo dyes are hardly to be degraded and utilized. It was also reported that although microbes grew better in the presence of carbon source than in the absence of carbon sources. Microbes utilized other carbon source for growth, resulting in lower degradation efficiency of azo dyes.

The mixed cultures were able to reduce 32.9% of -N=N- double bond at 478 nm and 28.8% of -N=N- double bond at 620 nm, and only remove 24.3% of aromatic amines without any nitrogen source (Fig. 3b). Whereas on supplementing yeast extract in the medium, the removal rates were increased to 100%. Providing other kinds of nitrogen sources such as peptone, urea, NH_4Cl could increase the removal efficiency, but could not remove azo dyes from the medium completely. In accordance with previous studies, yeast extract was the best nitrogen source for azo dye biodegradation [27].

The effect of pH is shown in Fig. 3c. Optimal azo dye removal efficiency was obtained when pH was 6.0 and 7.0. In addition, the influence of dye concentration was estimated. It is obvious that the mixed cultures exhibited excellent degradation ability when azo dyes concentrations were less than 60 mg/L (Fig. 3d). Therefore, it was suggested that the optimal conditions for azo dye removal were as follows: no carbon source, 4 g/L yeast extract, pH 6.0–7.0 and 60 mg/L azo dyes.

3.2. Biodegradation pathway

Decoloration and mineralization pattern of four azo dyes by mixed cultures under static (0–24 h) and shaken (24–48 h) conditions was estimated using UV-Vis (Fig. S2). As shown in Fig. S3. In the UV-Vis spectrum of AO7, the peaks at 485 nm, 250 nm and 309 nm were assigned to -N=N-, benzene and naphthalene structures. In the spectrum of CR, peak at 496 nm assigned to -N=N- azo bond and peaks at 342 nm and 248 nm corresponded to naphthalene and benzene structure were identified. Fig. S3(c) exhibits that MO solution generated two absorption peaks at 462 and 284 nm, which were corresponded to -N=N- double bond and benzene, respectively. The spectrum of AB showed peaks at 252 nm and 321 nm due to benzene and naphthalene ring in UV region and a maximum absor-

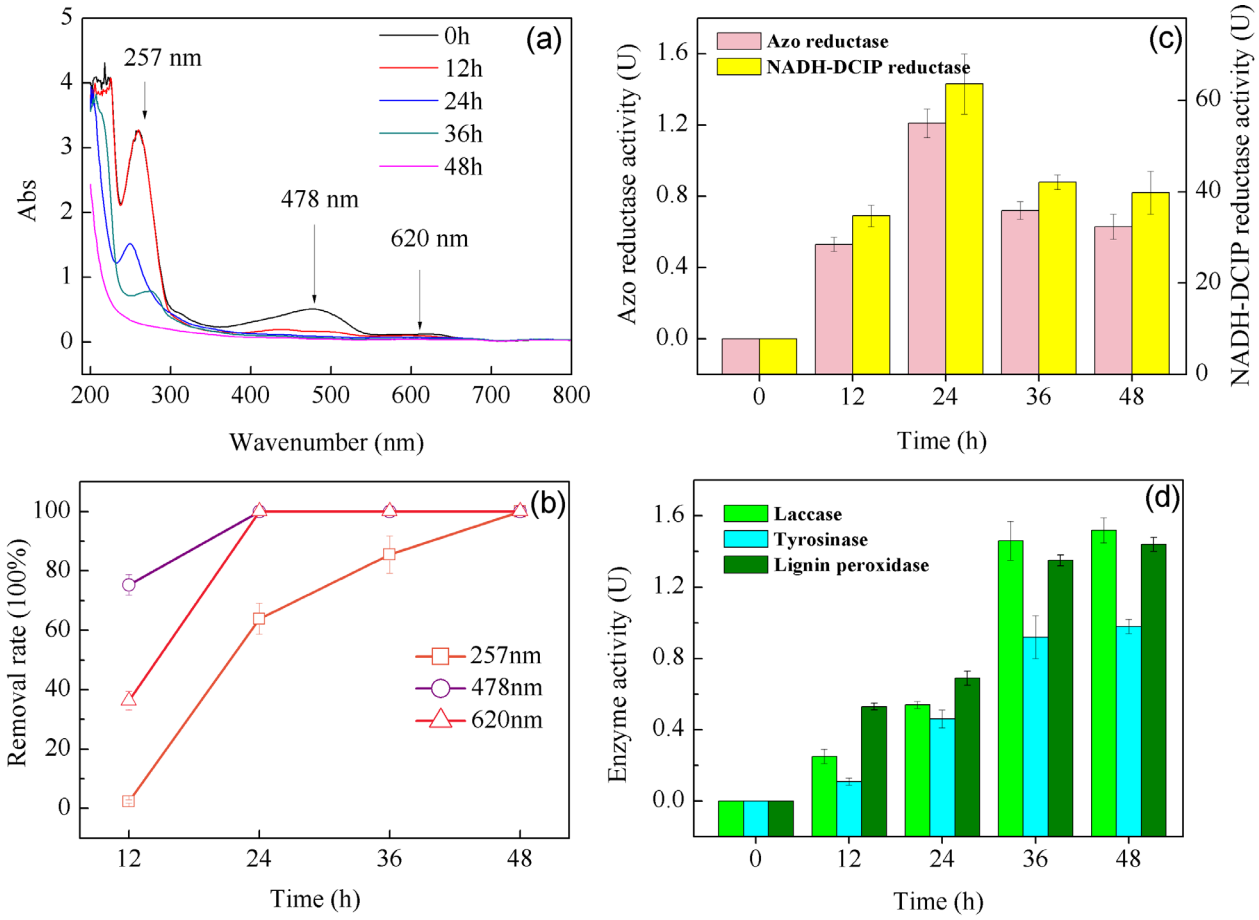

Fig. 4. UV-Vis spectra (a) and removal rates (b) of azo dyes degraded products at varied time intervals under optimum condition. The activities of reductases (azoreductase and NADH-DCIP reductase) (c) and oxidases (laccase, tyrosinase and lignin peroxidase) (d) during the degradation of azo dyes.

bance at 616 nm due to -N=N- azo bond in visible region. While, in the spectra of the four mixed azo dyes (Fig. 4a), two peaks at 478 nm and 620 nm in visible region assigned to -N=N- double bonds were detected and only one peak at 257 nm in UV region was identified. Thus, in our study, peaks at 478 nm and 620 nm were used to characterize -N=N- double bond, while peak at 257 nm was employed to characterize aromatic amines.

In anoxic phase, -N=N- double bonds in the four azo dyes were broken down and the two peaks in visible region gradually decreased and disappeared at 24 h. The absorbance value at 478 and 620 nm were used to calculate the removal rate. The removal rates of the two peaks reached 100% at 24 h. The concentration of aromatic amines did not change at 12 h, about 63.85% of them were removed at 24 h with little shift in λ_{max} and completely degraded in aerobic phase at 48 h (Fig. 4c). It can be seen that, broken of -N=N- in azo dyes happened under anoxic circumstances. Aromatic amines such as aminobenzene, aryl sulfonates and aminonaphthyl sulfonates which were difficult to degrade were obtained [28]. Cleavage of naphthalene and benzene rings was detected in anoxic and aerobic phases, leading to generation of low-molecular-weight organic acids such as 2-ethyl-hexanoic acid, octanoic and benzeneacetic acid. According to other reported researches, further degradation may happen and CO_2 was formed [29,30].

3.3. Enzymes involved in degradation of azo dyes with mixed cultures

Generally, enzymes play important roles in decolorating and mineralizing azo dyes process. It has been reported that two main kinds of enzymes were involved in the degradation process, which were reductase and oxidase [31,32]. In order to reveal the mechanism of azo dyes decoloration and mineralization by mixed cultures, enzyme activities of reductase (azoreductase, NADH-DCIP reductase) and oxidase (laccase, tyrodinase, lignin peroxidase) were determined every 12 h.

Fig. 4c shows that the two reductases activities increased in the 24 h under anoxic circumstance and then decreased significantly in the next 24 h in aerobic environment. It can be seen that anoxic situation was more suitable for the yield of azoreductase and NADH-DCIP reductase. Azoreductase activity of 1.2 U and NADH-DCIP reductase activity of 61.3 U were observed for 100% dye decoloration at 24 h. It has been suggested that azoreductases produced by a num-

ber of bacteria can cleave the azo dye to form hydrazine intermediates first, and the generated intermediates can be further reduced to quinoneimine, quinones or phenols through acid or base catalyzed reactions. The major role of azoreductase in the biological process is detoxification of quinones and should be grouped to NAD(P)H quinone oxidoreductases (NQOs) family [33].

Oxidase including laccase, tyrodinase and lignin peroxidase were involved in oxidation of aromatic amines, which was the crucial step of mineralization of azo compounds. The enzyme activities were observed to increase from 0–48 h with strategic anoxic/aerobic phasing (Fig. 4d). The highest levels of the three oxidases were found at 48 h with 100% of aromatic amines removed from the solution. And the enzyme activity of 1.52 U, 0.98 U and 1.44 U corresponding to laccase, tyrodinase and lignin peroxidase was observed. In the researches focused on degradation of azo dyes with single strains, only one or two of the above oxidases were involved in the biological process [34,35]. While, when mixed cultures were applied in the biodegradation, all of the three oxidases were included. It may account for the high mineralization efficiency of the mixed cultures.

3.4. Broad-spectrum azo dye degradation ability of mixed cultures

Azo dyes are generally classified to reactive dyes, direct dyes, acid dyes and disperse dyes based on their properties. The degradation ability of mixed cultures to a broad-spectrum of azo dyes was also evaluated. Eight azo dyes assigned to above four groups (i.e Direct Brown, Direct Blue 5G, Acid Light Yellow 2G, AO7, Reactive Red X-3B, Disperse Orange, Reactive Black KN and Disperse Scarlet) containing different molecular structures and functional groups were used for the test (Fig. S4). From the UV-Vis spectra (Fig. 5), it can be seen that except for Acid Light Yellow 2G, the maximal wavelength in visible region of different azo dyes disappeared at 24 h, indicating that cleavage of -N=N- happened under anoxic circumstance. As time went by, the peaks which characterize aromatic amines in ultra-vision decreased with little shit, and disappeared in the spectra of Direct Browns, Direct Blue 5G, AO7, Reactive Red X-3B, Disperse Orange and Reactive Black KN, which revealed complete mineralization of the above six azo dyes. It is speculated that maybe due to low water solubility of Acid Light Yellow 2G, mixed cultures could not degrade it effectively. In the rest of the seven azo dyes, only Disperse Scarlet GS 200 cannot be completely mineralized with the mixed cultures. The special functional group in Disperse Scarlet GS 200, i.e., presence of trichlorobenzo[d]thiazole may be attributed to the variation in the mineralization efficiency. It can be seen that the mixed cultures were competent for decoloration and mineralization of textile wastewater containing various types of azo dyes.

3.5. Changes of microbial community in anoxic and aerobic phases

Totally, 40199-59989 sequence reads were yielded by high-throughput sequencing of the 16S rDNA gene in four mixed cultures samples collected at 12, 24, 36 and 48 h during the decoloration and mineralization process (Table 1). The rarefaction curves of four samples reach plateau, which revealed that the sequencing depth was sufficient to cover the majority of bacterial diversity (Fig. S5). The number of OTUs, diversity (Shannon and Simpson indexes), richness (ACE and Chao 1) and coverage for each sample were calculated and demonstrated in Table 1. The coverage of four samples ranging from 99.62% to 99.76% implied that the constructed library covered the 16S rDNA sequences in each sample very well. Shannon and Simpson indexes were used to evaluate the internal complexity of individual microbial communities. It can be seen that the biodiversity increased from 12 h to 24 h in anoxic phase, then decreased slightly when DO was increased in the solution at 36 h. The results indicated that microbial community might be changed from anoxic phase to aerobic environment. Based on RDP Classifier, the effective sequences were assigned to

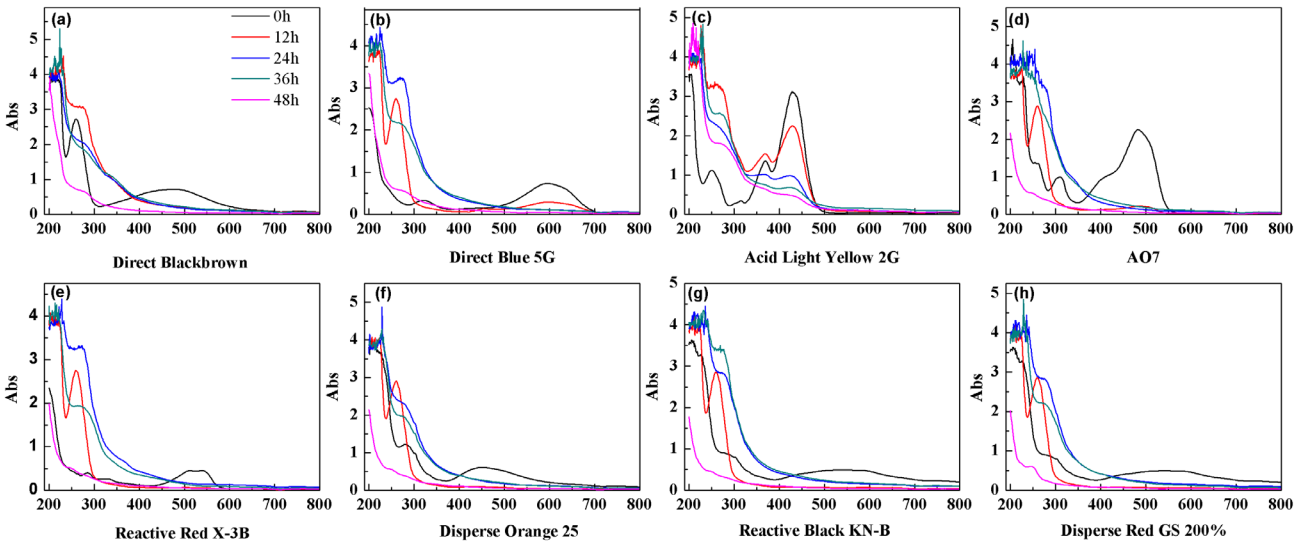

Fig. 5. UV-Vis spectra of Direct Black brown (a), Direct Blue 5G (b), Acid Light Yellow 2G (c), AO7 (d), Reactive red X-3B (e), Disperse Orange 25 (f), Reactive Black KN-B (g) and Disperse Red GS 200% (h) degraded products at varied time intervals.

different phylogenetic. The top 50 genera belong to 14 phyla were identified, in which *Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes* and *Bacteroidetesn* were the most abundant.

At the genus level (Fig. 6), the top seven genera in the four samples were *Aridibacter*, *Comamonas*, *Gemmatimonas*, *Brevundimonas*, *Petrimonas*, *Nannocystis* and *Chryseobacterium*. Although the main genera were the same, the proportions were significantly different in anoxic and aerobic conditions. At 12 and 24 h, the relative abundance of *Aridibacter* was 5.96% and 5.67%, respectively. While the value was increased to 8.53% and 8.04% at 36 and 48 h. Similar changes were observed in the genera of *Comamonas*, *Gemmatimonas* and *Nannocystis*. However, as to the genera of *Brevundimonas*, *Petrimonas* and *Chryseobacterium*, their relative abundance decreased sharply when DO was increased in the solution. For example, the relative abundance of *Brevundimonas* was 14.35% and 15.39% at 12 and 24 h and decreased significantly with the value of 8.26% and 7.89% detected at 36 and 48 h. It must be pointed out that the microbial community analysis was conducted using DNA samples, in which DNA was not easily gone during the shift of conditions. Thus, only abundance change of the main genera was detected.

It can be speculated that the genera of *Aridibacter*, *Comamonas*, *Gemmatimonas* and *Nannocystis* might prefer to inhabit under anoxic circumstance, while the genera of *Brevundimonas*, *Petrimonas* and *Chryseobacterium* were adapt in aerobic environment. Anoxic circumstance was maintained for hydrolysis of high molecular weight contaminants into low molecular weight compounds, and aerobic condition was beneficial for the aromatic amines degradation bacteria. Generally, it is reported that, bacteria belonging to *Comamonas* genus own the potential ability in degrading azo dyes. Pathak et al. found that *Comamonas sp.*VS-MH2 had high metabolic activity towards mixture of four azo reactive dyes and degraded them completely [36]. Strains in *Comamonas* genus could yield veratryl alcohol oxidase and catalase-peroxidase which are key enzymes for desulphonation and oxidative cleavage of azo dyes [37]. Previous studies showed that members of *Petrimonas* and *Chryseobacterium* were responsible for the degradation of aromatic-carbohydrates detected in the oxic bioreactors [38,39], which are in accordance with our findings. However, few species in the genera of *Aridibacter, Gemmatimonas Nannocystis* and *Brevundimonas* were reported to have the ability of degrading azo dye. We found that the four genera were abundant in mixed cultures. Most of the stains in the four genera were facultative bacteria and are need further study.

3.6. Variations of HRTs in the SBR under different operational phases

In order to investigate the decoloration and mineralization efficiency of the mixed cultures in a large scale, a SBR was operated under anoxic-aerobic process. HRT is a cru-

Table 1
Number of sequence and OTUs, richness and diversity and Coverage of four samples

Sample	Number of sequences	OTUs	Diversity		Coverage(%)	Richness	
			Shannon	Simpson		ACE	Chao 1
12h	59989	629	3.57	0.068	99.67	852.16	791.52
24h	55831	470	3.00	0.116	99.76	617.05	575.43
36h	40199	389	3.35	0.066	99.62	718.99	606.78
48h	68442	503	3.44	0.058	99.76	817.04	658.51

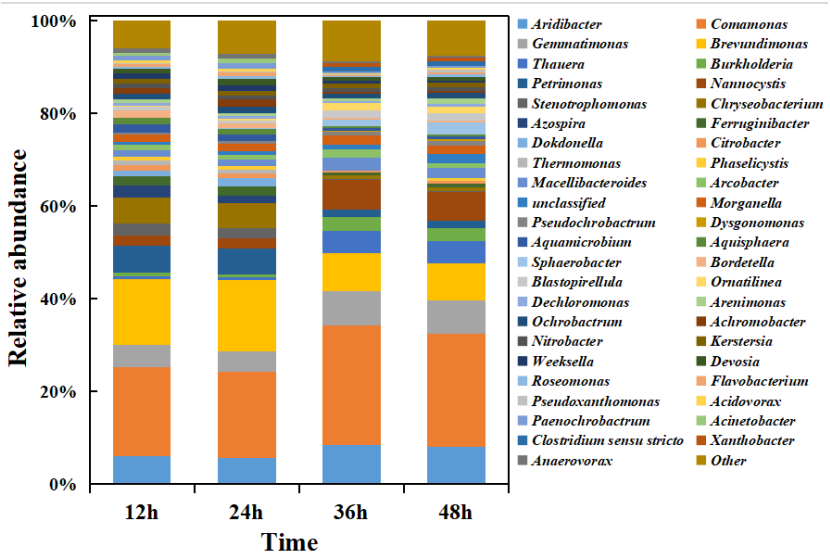

Fig. 6. Relative abundance of major genera in the mixed cultures at different biodegradation stages.

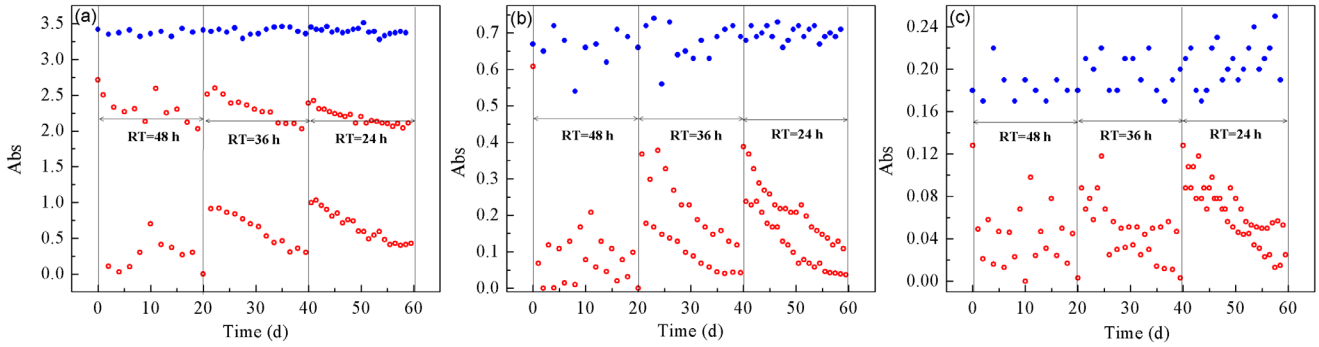

Fig. 7. Changes in -N=N- azo double bonds and aromatic amines in azo dye wastewater with variations of HRTs in the SBR under different operational phases.

cial operation parameter affecting both quantity of treated wastewater and removal rate of dye pollutants. An acclimation strategy was applied in this study to achieve high treatment efficiency with shortened HRT. The synthetic wastewater containing azo dyes (60 mg/L AO7, CR, MO and AB, respectively) and 5 g/L yeast extract provided nutrients for microbial growth. Different HRT strategies (48 h, 36 h and 24 h) were operated for periods of 20 d. 10% of the activated mixed cultures were inoculated in the SBR. Three parameters (620 nm, 478 nm, 257 nm) were selected to evaluate the decoloration and mineralization efficiency. Fig. 7 shows the variations of -N=N- and aromatic amines concentrations in the influent and effluent. During period I (RT = 48 h), SBR was operated under anoxic circumstance for 24 h and aerobic environment for another 24 h. The color (478 nm, 620 nm) removal efficiency of 85.86%–100% and 73.89%–100% was obtained after 48 h reaction. Aromatic amines removal efficiency demonstrated a delayed trend as the color removed, ranging from 78.91–100%. During period II (RT = 36 h), the color and aromatic amines removal rates decreased when the HRT was shortened to 36 h and were stable after ~15 d.

Afterward (Days 40–60, period III), the further shortening of HRT to 24 h was carried out by anoxic stirring for 12 h and aeration for 12 h. At the initial time of this phase, the removal efficiency deceased sharply with increased colour and aromatic amines detected in the effluent. Nevertheless, via hydraulic selection, the cultures of high biodegradation efficiency were remained and relieved in the bioreactor, improving the removal efficiency to ~90%. The results implied that accumulation strategy provided stable color and aromatic removal efficiency with shortened HRT.

4. Conclusions

With the anoxic-aerobic circle method, mixed cultures which could decolorate and mineralize azo dyes effectively in medium containing 5 g/L yeast extract at pH 6–7 in 48 h was developed via accumulation and acclimation process. Microbial community analysis showed that genera of *Aridibacter*, *Comamonas*, *Gemmatimonas* and *Nannocystis* may be responsible for decoloration in anoxic environment, while genera of *Brevundimonas*, *Petrimonas* and *Chryseobacterium* might play important roles in mineralization reaction under aerobic circumstance. Scaled up experiment with HRT shortened strategy was carried out. The mixed cultures could remove over 90% azo dyes from SBR in 24 h. The developed mixed cultures are promising candidates for the efficient treatment of azo dye wastewater.

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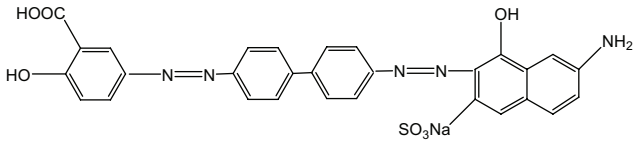
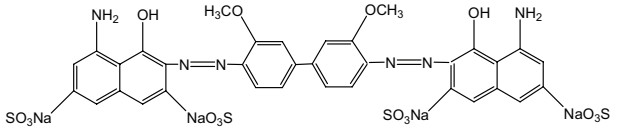
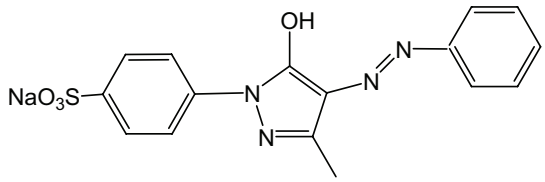
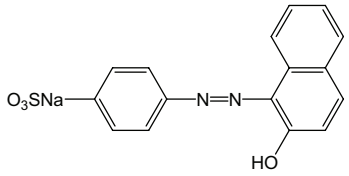
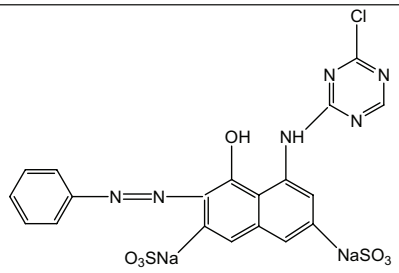
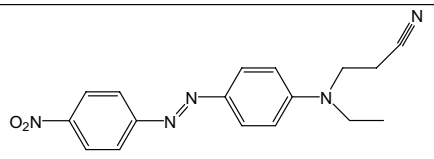
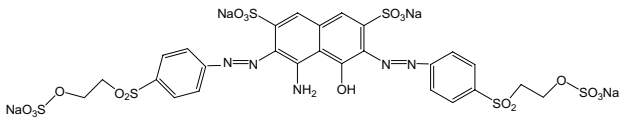
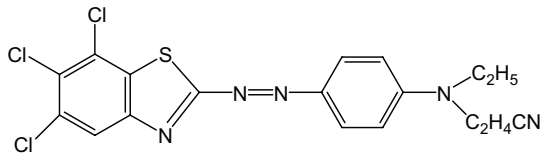
Supplementary material

Table S1
Chemical Structures of eight azo dyes

No.	Azo dye	Chemical Structure
1	Direct Darkbrown	
2	Direct Blue 15	
3	Acid Light Yellow 2G	
4	Acid Orange II	
5	Reactive Red X-3B	
6	Disperse orange 25	
7	Reactive Black KN-B	
8	Disperse Red GS 200%	

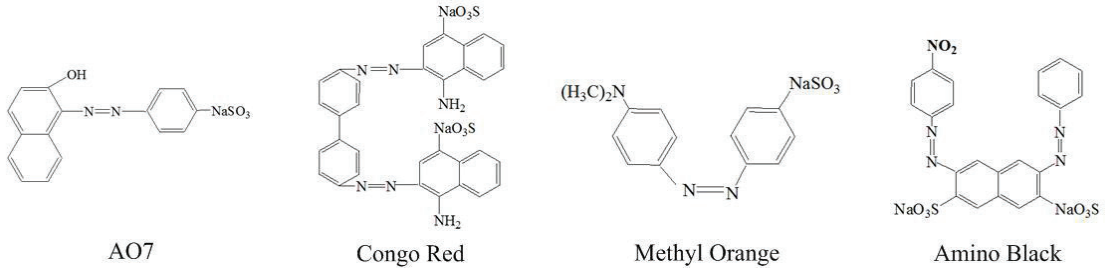


Fig. S1. Chemical structures of AO7, Congo Red, Methyl Orange and Amino Black.

Fig. S2. Degradation of four azo dye mixture with mixed culture. (a): Before degradation; (b): After degradation.

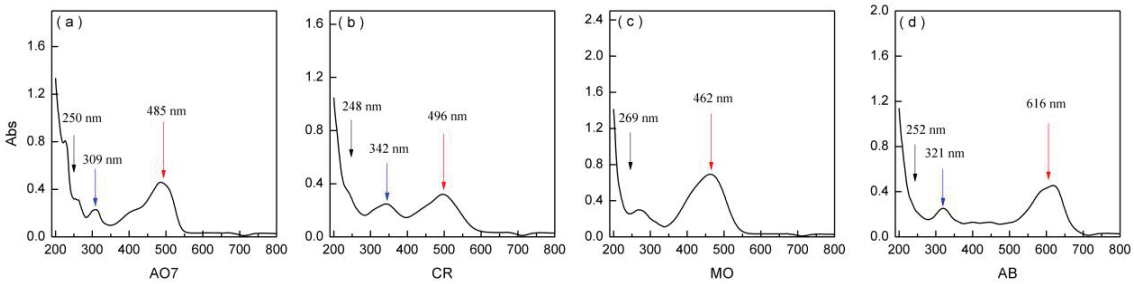


Fig. S3. UV-Vis spectra of AO7 (a), CR (b), MO (c) and AB (d).

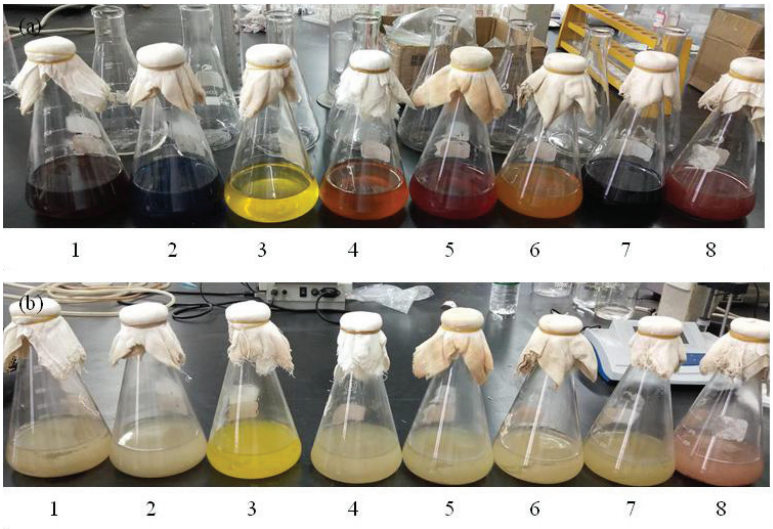


Fig. S4. Degradation of azo dyes 1-8 with mixed culture. (a): Before degradation; (b): After degradation. 1: Direct Darkbrown; 2: Direct Blue 15; 3: Acid Light Yellow G; 4: Acid Orange II; 5: Reactive Red X-3B; 6: Disperse orange 25; 7: Reactive Black KN-B; 7: Disperse Red GS 200%.

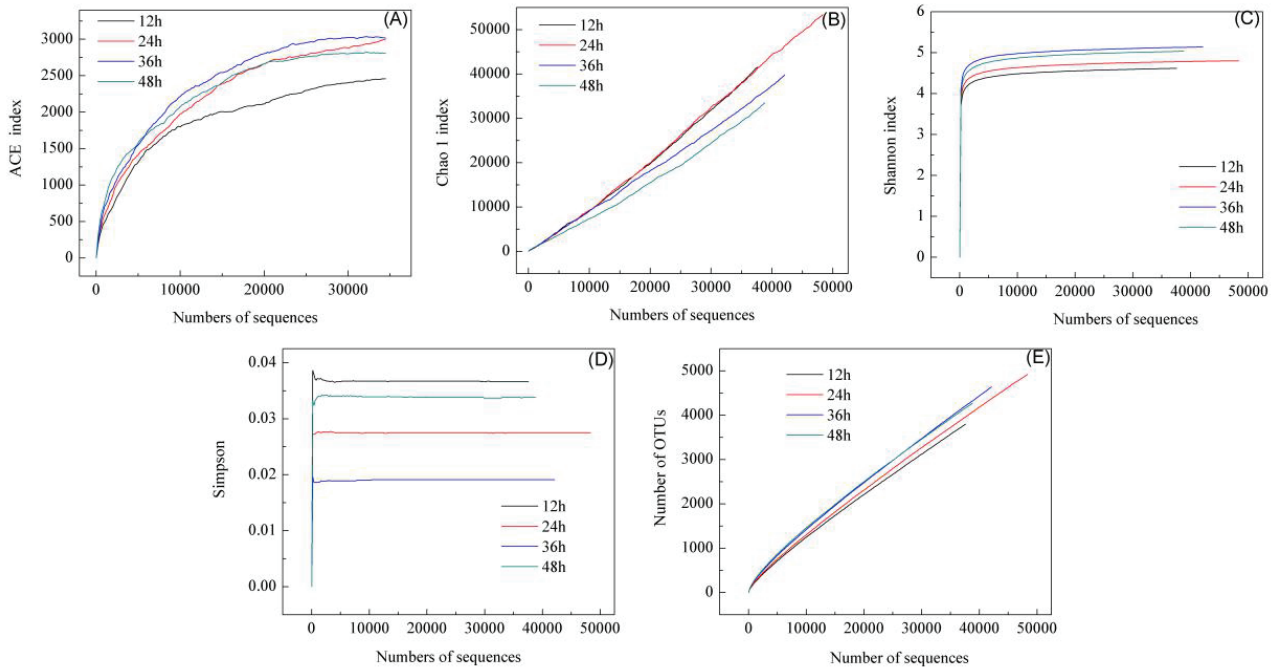


Fig. S5. ACE (A), Chao1 (B), Shannon (C), Simpson (D) indices and rarefaction curves (E) of four samples.