Azo Dye Decolorization by Consortium HR under High Temperature and Salinity

Keyuan Li, Yifan Lou, Lu Han, Chongyang Wang*
Miami College, Henan University, Kaifeng, Henan, 475000, China
*Corresponding author’s E-mail: wangchy18@henu.edu.cn

Abstract. Azo dye is a kind of most commonly used dyes in related industries. As the discharge from azo dye related industries is commonly found with high salinity and temperature, halo-thermophilic microbes able to decolorize azo dyes are required. Consortium HR enriched under 5% salinity and 50℃ in this study was mainly composed of *Ueribacillus* (70.4%), *Tepidimicrobium* (11.7%) and *Clostridium* (10.4%). The influence of salinity (1% to 10%) and pH (6 to 10) on the decolorization process were further measured. Meanwhile, consortium HR was identified with ability to decolorize several kinds of azo dyes and the phytotoxicity of the metabolic intermediates was decreased with the incubation time. All the results indicate that consortium HR has a good potential in the decolorization of azo dyes in hypersaline, high temperature and weak alkaline conditions.

1. Introduction
Since the first artificial dye synthesized, the dye related industries were developed rapidly in recent years. It was reported that about 0.7 million tons of dyes are synthesized each year. Among these synthetic dyes, azo dye was identified as the most commonly used dyes, containing one or more azo groups (-N=N-) in their molecular structures[1]. To data, approximately 10% to 15% dyes were wasted in their utilization process and discharged into the environment in the form of dissolved in the sewage[2]. These discharged dyes will have a direct toxicity to the ecosystem due to the benzene in their structure. Meanwhile, in the aqueous ecosystem, dyes may limit the efficiency of photosynthesis by preventing the sunlight into deep water[3]. Therefore, the decolorization and degradation of azo dyes in industrial discharge has been largely focused nowadays[4].

Several methods were investigated for elimination of azo dyes pollution, including Fenton oxidation, absorption and electrochemical oxidation[5]. However, these treatment methods were expensive and not suitable for large-scale utilization. Biological treatment was investigated as the most cost-effective and environmental friendly method. Enormous of bacteria able to decolorize azo dyes was isolated and investigated that aimed for azo dye decolorization in the discharges[6, 7].

In order to improve the efficiency and quality, dyeing was commonly processed under high salinity (3 to 10%) and high temperature. These two extreme conditions made the decolorization rate of neutrophilic microbes dropped dramatically in the practical application. Alternatively, extremophile, which was able to survive under extreme conditions, was identified with significant advantages in the treatment process[6]. To data, although several bacteria was isolated able to decolorization azo dye under hypersaline environment or high temperature, seldom research reported the isolation of bacteria resource under comprehensive conditions. Therefore, the research on halo-thermophilic bacteria that are able to decolorize azo dyes is of great significance[8, 9].

In this study, consortium HR able to decolorize Direct Yellow R was enriched under 5% salinity and
50°C. The community structure was further investigated using high-throughput sequencing. Combined with detection of decolorization rate under different salinity, pH and azo dye types, consortium HR was identified with a good tolerance to environmental variation and great application potential for industrial utilization.

2. Materials and methods

2.1. Azo dyes used in this study
The azo dye used in this study including Direct Yellow R (CAS: 1325-37-7), Acid Gold G (CAS: 587-98-4), Direct Blue 5B (CAS: 2429-74-5), Direct Scarlet 4BS (CAS: 3441-14-3), Direct Red 13 (CAS: 1937-35-5), Acid Black 10B (CAS: 1064-48-8) and Direct Yellow Brown D3G (CAS: 3811-71-0) were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All the azo dyes were reported with a wide utilization.

2.2. Enrichment process and DNA extraction
Consortium HR was enriched under 5% salinity using sea salt culture medium (SSDM) containing KCl 2.1g/L, (NH₄)₂SO₄ 2g/L, NaBr 0.25g/L, NaHCO₃ 0.1g/L, K₂HPO₄ 0.5g/L, CaCl₂ 0.75g/L, NaCl 40g/L, MgSO₄·7H₂O 5.25g/L, and MgCl·6H₂O 3.45g/L. The microelement solution SL7 and vitamin solution were also added as previously reported \[10\]. The fresh culture medium was also containing 1g/L yeast extract and 100mg/L Direct Yellow R. High purity N₂ was then used for blowing out the dissolved oxygen for constructing an anaerobic environment. 5g soil sample collected from a site chronically contaminated with organic matter was added into 200mL fresh medium and placed into an incubator at 50°C. 3 days after incubation, 10mL cell suspension was transferred into another 200mL fresh medium. The total enrichment process was lasted for 2 month for obtaining a stable halo-thermophlic consortium.

The genomic DNA of consortium HR was extracted using Fast DNA SPIN kit for Soil purchased from MP Biomedicals Co., Ltd (American). The extraction process was referred to the manufacture’s manual. After a detection of agarose electrophoresis, the DNA was further sent to Genewiz Co., Ltd (Hangzhou, China) for detection of V3-V4 regions of 16S rRNA. The obtained OTUs were separated with 97% identity and MAGE 6.0 was used for the construction of phylogenetic tree.

2.3. The measurement of decolorization rate under different conditions
Consortium HR was cultured under 5% salinity using sea salt culture medium (SSDM) containing KCl 2.1g/L, (NH₄)₂SO₄ 2g/L, NaBr 0.25g/L, NaHCO₃ 0.1g/L, K₂HPO₄ 0.5g/L, CaCl₂ 0.75g/L, NaCl 40g/L, MgSO₄·7H₂O 5.25g/L, and MgCl·6H₂O 3.45g/L. The microelement solution SL7 and vitamin solution were also added as previously reported \[10\]. The fresh culture medium was also containing 1g/L yeast extract and 100mg/L Direct Yellow R. High purity N₂ was then used for blowing out the dissolved oxygen for constructing an anaerobic environment. 5g soil sample collected from a site chronically contaminated with organic matter was added into 200mL fresh medium and placed into an incubator at 50°C. 3 days after incubation, 10mL cell suspension was transferred into another 200mL fresh medium. The total enrichment process was lasted for 2 month for obtaining a stable halo-thermophlic consortium.

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2.3. The measurement of decolorization rate under different conditions
Consortium HR was cultured under 5% salinity, 50°C. 5mL cell suspension were collected each 12 hours after incubation (namely 0h, 12h, 24h, 36h, 48h, 60h and 72h) and centrifuged at 12000 rpm for 5 min. The residual Direct Yellow R was measured using UV-Vis spectra at 411 nm. The decolorization rate was further calculated using following equation: Efficiency = (A₀-A)/A₀. A₀ stands for the absorbancy of sample collected at 0 hour. The cell growth was measured using OD600 methods as previously described \[8\].

The decolorization rate of consortium HR was also measured under different salinity. The salinity was adjusted by changing the concentration of NaCl, MgSO₄ and MgCl₂ in SSDM according to Wang et al \[11\]. The decolorization rate and cell growth were measured as mentioned above.

The effect of pH on decolorization process of consortium HR was also investigated. The pH in the fresh culture medium was adjusted using NaOH (0.5mol/L) and HCl (1:5 v/v) solution.

The decolorization ability of consortium HR was also measured using different azo dyes. Acid Gold G, Direct Blue 5B, Direct Scarlet 4BS, Direct Red 13, Acid Black 10B and Direct Yellow Brown D3G were selected and the decolorization rate was detected 72 hour after incubation at 425nm, 598nm, 508nm, 506nm, 619nm, 440nm, respectively.

2.4. Phytotoxicity analysis
In order to investigated the biotoxicity of the intermediates after decolorization, phytotoxicity analysis was performed on two seeds, namely Mung been and Zea mays. The generation rate, shoot length and radical length were selected as the indexes. 100mL cell suspension were collected 24, 48 and 72 hours
after incubation and then centrifuged at 12000 rpm for 5 min. The cell pellet was resuspended using 5mL distilled water and further disrupted by sonication. The cell debris was then extracted using ethyl acetate and then dried with NaSO$_4$ and N$_2$ as previously described[8]. The residual intermediates was dissolved in distilled water to make a final concentration of 100mg/L. 20 seeds of mung been and rice were steeped in 20mL final products and incubated for 5 days at 25℃. Then the generation rate (%), length of shoot and root were recorded.

3. Result and Discussion

3.1. Decolorization rate and community structure of halo-thermophilic consortium HR

Using 5% SSDM, the halo-thermophilic consortium HR was finally obtained by transferred at 3 days for nearly 2 months. The decolorization rate and cell growth curve were shown in Fig. 1. After 72h incubation, consortium HR was able to decolorize 90% of Direct Yellow R. The OD600 value was increased to about 0.35, indicating a good cell growth density.

Fig. 1 Decolorization rate and cell growth curve of consortium HR

The genomic DNA of consortium HR was extracted and sent to Genewiz Co., Ltd. The V3-V4 region of 16S rRNA was further sequenced and the OTUs was separated with 97% identity. All the important OTUs were further annotated using NCBI database. The community structure of consortium HR was shown in Fig. 2. Ueribacillus (70.4%), Tepidimicrobium (11.7%) and Clostridium (10.4%) were the three dominant genera in consortium HR. Ueribacillus was widely detected in high-temperature composting process which was identified able to survival at high temperature[12]. Tepidimicrobium was also detected in high-temperature and anaerobic environments. To data, it was the first time that Tepidimicrobium was found closely related with azo dye decolorization process[13, 14]. Clostridium was reported as a kind of anaerobic bacteria. As reported by Morrison et al. Clostridium was able to decolorize acid red using azo reductase (AzoC)[15]. Other genera such as Bacillus and Exiguobacterium were also found with the ability to decolorize azo dyes under anaerobic condition at high temperature or high salinity. The coexisting of these genera made consortium HR a rapid decolorization rate and cell growth rate.
Fig. 2 Community structure of consortium HR. The phylogenetic tree was illustrated using MAGE 6.0 software. All OTUs were separated at 97% identity and the proportion of each OTU were shown following the OTU name. 16S rRNA sequence of Methanococcus sp. SB was selected as the out group. The phylogenetic tree is constructed using Neighbor-Joining Method with bootstrap test (1000 replicates).

3.2. Decolorization of azo dyes under different environmental condition

The decolorization rate of consortium HR was detected under different salinity and pH. As shown in Fig. 3, consortium HR was able to decolorize Direct Yellow R under a wide range of salinity (1% to 8%). The decolorization process of consortium HR under 1%, 3% and 5% salinity demonstrated no significant difference. When salt content increased to 7%, the decolorization rate was limited and about 75% azo dye was decolorized 72 hours after incubation. When salinity increased to 10%, the consortium HR showed no decolorizing ability and no obvious cell grow was observed. Consortium isolated by Guo et al. which was mainly composed of *Bacillus* spp., was identified able to decolorize Metanil Yellow with a wide range of salinity (from 1% to 15%) at 50°C[9]. Consortium enriched by Fernando et al. was reported able to decolorize azo dye under 2.5% salinity at 50°C[16]. To the best of our knowledge, no other consortium was identified able to decolorize azo dye under saline condition at high temperature.
The influence of pH on the decolorization rate and cell grow were investigated. As shown in Fig. 4, consortium HR was able to decolorize azo dye at pH 7, 8 and 9, indicating consortium HR was suitable for slightly alkaline environment. When pH decreased to 6, only about 30% Direct Yellow R has been decolorized. When pH increased to 10, no significant decolorization process was observed.

The decolorization rate of Acid Gold G, Direct Blue 5B, Direct Scarlet 4BS, Direct Red 13, Acid Black 10B and Direct Yellow Brown D3G by consortium HR were measured at 5% salinity, 50℃ and pH7. The initial concentration of these azo dyes were all 100mg/L. As shown in table 1, consortium HR was able to decolorize Direct Blue 5B (81.79%), Direct Red 13 (65.07%) and Acid Gold G (66.36%). However, limited decolorization rate was occurred on Acid Black 10B (30.4%) and Direct Yellow Brown D3G (35.31%). Only 8.36% of added Direct Scarlet 4BS has been decolorized, indicating that consortium HR was not suitable for decolorization of this kind of azo dyes. Consortium HR was identified with ability to decolorize different kinds of azo dyes, indicating a potential use in the treatment process.

| Azo dyes        | Chemical structure | Photo at 0h | Photo at 72h | Decolorization rate |
|-----------------|--------------------|-------------|--------------|---------------------|
| Direct Blue 5B  | ![Chemical structure](image) | ![Photo at 0h](image) | ![Photo at 72h](image) | 81.79% |
3.4. Phytotoxicity assay

In order to evaluate the phytotoxicity of the metabolic intermediates after decolorization, seeds of *Mung been* and *Zea mays* were used. As shown in Table 2, the phytotoxicity of metabolic intermediates after 72 hour incubation was slightly lower than distilled water. The highest phytotoxicity was occurred in the treatment of metabolic intermediates 24 hours after incubation. With the incubation process, the phytotoxicity of the metabolic intermediates was decreased significantly as reflected by the data both from *Mung been* and *Zea mays*. Similar results were reported by several azo dye decolorization consortia[8, 17].

| Seeds          | Treatment                | Generation Rate (%) | Length (cm) |      |      |
|----------------|--------------------------|---------------------|-------------|------|------|
|                |                          |                     | Shoot       | Root |      |
| *Mung Been*    | Distilled water          | 100                 | 2.33±0.1    | 1.95±0.3 |      |
|                | 24h after incubation     | 50                  | 1.26±0.3    | 0.96±0.5 |      |
|                | 48h after incubation     | 75                  | 1.52±0.4    | 1.28±0.5 |      |
|                | 72h after incubation     | 100                 | 2.10±0.8    | 1.79±0.6 |      |
| *Zea Mays*     | Distilled water          | 100                 | 3.45±0.6    | 5.22±0.8 |      |
|                | 24h after incubation     | 60                  | 1.98±0.3    | 3.22±0.2 |      |
|                | 48h after incubation     | 80                  | 2.52±0.1    | 4.29±0.8 |      |
|                | 72h after incubation     | 100                 | 3.20±0.3    | 4.86±0.7 |      |

4. Conclusion

Consortium HR, which was mainly composed of *Ueribacillus* (70.4%), *Tepidimicrobium* (11.7%) and *Clostridium* (10.4%), was enriched under salinity and high temperature. This halo-thermophilic
consortium was confirmed to be able to decolorize azo dyes in a wide range of salinity (1% to 8%) and weak alkaline environment (pH 7 to 9). Consortium HR was also found to be able to decolorize several kinds of azo dyes including Direct Yellow R, Direct Blue 5B, Direct Red 13 and Acid Gold G. Meanwhile, the phytotoxicity of the metabolic intermediates was decreased with the incubation process. Therefore, consortium HR was investigated with a good potential to decolorize azo dyes under hypersaline, high temperature and weak alkaline conditions.

Author contribution
Keyuan Li and Yifan Lou were the joint first authors as they both contributed a lot in this research. Keyuan Li wrote the whole manuscript and determined the phytotoxicity. Yifan Lou enriched the consortium and measured the decolorization rate under different conditions. Lu Han assisted to modified the language. Chongyang Wang was the corresponding author. All authors revised the manuscript.

Conflict of Interest
Authors have no conflict of interest to declare

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