Isolation of microalgae with growth restriction and nutrient removal from alkaline wastewater

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Abstract: In order to isolate a well-tolerated microalgae strain and study its capability of wastewater treatment, a newly microalgae strain was isolated and identified from fresh water. The phylogenetic analysis indicates that this strain has a close relationship with *Desmodesmus* sp., named as EJ 9-2. The effects of temperature, pH value and NaCl concentration on growth of *Desmondesmus* sp. were investigated; the capability of nutrient removal from alkaline wastewater was also observed. *Desmodesmus* sp. EJ 9-2 had a wide pH adaptation range (3-12) and could remove nitrogen, phosphorus and COD which might substantially decrease the cost of biofuel production. The research can provide evidence for outdoor large-scale cultivation of microalgae.

Keywords: microalgae, isolation, growth restriction, alkaline wastewater, nutrient removal, algae-based fuel, wastewater treatment
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1 Introduction

Due to the rapid production rate, high lipid content, little land needed and other advantages, microalgae has become the candidate feedstock for the production of the third generation renewable energy. In next 10-15 years, microalgae-based biomass fuel might become an important form of energy supply[1]. Microalgae can utilize the sunlight, carbon dioxide in the nature and the nutrients in water to accumulate/generate biomass[2]. The growth of microalgae is affected by abiotic factors (light, temperature, nutrient, dissolved oxygen concentration, carbon dioxide concentration, pH value, salinity, etc.) as well as biotic factors (bacteria, fungi, viruses, etc.)[3-5].

In China, the current annual discharge of sewage has reached 36.5 billion tons (excluding townships), however the urban sewage treatment rate is only 70%; and 80% or even more of untreated sewage was discharged into water bodies, which could not be recycled and thus caused environmental pollution[6]. Especially for the paper mills, printing and dyeing mills, tanneries and other enterprises which have a large water consumption, their effluent contains high COD and alkalinity, and is difficult for treatment. If arbitrarily discharged into the environment, it may cause serious pollution[7-10]. Therefore, a more economical and effective approach is needed to deal with such high alkali wastewater.

Domestic and overseas researches indicate that the microalgae have a very strong ability of stress tolerance[11]. They can obtain the necessary nutrient source by using the nitrogen, phosphorus and other elements in the eutrophic wastewater and synthesize biomass[12,13].

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However, the metabolism and biomass cumulative effects of environmental factors on microalgae are synergistic. The environmental factors affect microalgae biomass production by affecting the photosynthesis of the microalgae. The temperature and water pH are the most direct factors that affect the microalgae biomass accumulation[14]. Either too acidic or alkaline environment will damage the cells of microalgae and can restrict the growth[15].

Microalgae have shown strong adaptability in wastewater and maintained a strong vitality, but different microalgae have different optimal growth conditions[16]. Ideal algal species not only need to have a higher lipid content, larger biomass production and higher resistance to the external environment, but also need to improve the potential of energy utilization of the microalgae. Although a large number of algae species have been screened, and several microalgae species libraries have been established at this stage[17], microalgae with well-tolerated and high energy production are still needed to be screened.

This research aimed to isolate well-tolerated microalgae species which could grow in wide ranges of temperature, pH value and NaCl concentration. Meanwhile, in order to save the costs of sewage treatment and microalgae cultivation, the ability to treat the high alkali wastewater was also evaluated.

2 Materials and methods

2.1 Isolation, identification and cultivation

The water sample was taken from the Olympic Forest Park, Beijing, China, which could isolate microalgae species after purification. Microalgae cultivation adopted the Blue-Green (BG-11) medium[18], which consisted of 1500 mg/L $NaNO_3$, 40 mg/L K_2HPO_4, 75 mg/L $MgSO_4·7H_2O$, 36 mg/L $CaCl_2·2H_2O$, 6 mg/L citric acid, 6 mg/L ferric ammonium citrate, 1 mg/L $EDTANa_2$, 20 mg/L Na_2CO_3, and 1 mL/L A5 trace metal solution. The recipe of A5 trace metal solution was: 2.86 g/L H_3BO_3, 1.86 g/L $MnCl_2·4H_2O$, 0.22 g/L $ZnSO_4·7H_2O$, 0.39 g/L $Na_2MoO_4·2H_2O$, 0.08 g/L $CuSO_4·5H_2O$, and 0.05 g/L $Co(NO_3)_2·6H_2O$. The medium was titrated to pH7.0 with 1 mol/L HCl.

The isolated microalgae were incubated in a growth chamber in the condition of (25±1)°C, light intensity (80±2) μmol/m^2·s and photoperiod 14:10 h (light : dark) for 14 d. Periodic agitations were performed for three times each day to prevent the microalgae precipitation or the attachment to the wall of the flasks. The microalgae were cultivated in 100 mL flasks with 50 mL effective volume. All experiment equipment and media need to be sterilized at 121°C by autoclave for at least 20 min prior to use.

2.2 Isolation and identification of algal species

The DNA extraction of microalgae species used the new plant genomic DNA extraction kit (Beijing ComWin Biotech Co., Ltd.), and the primer was designed by using DNAMAN and Primer 5.0 software and was synthesized by the Sangon Biotech (Shanghai) Co., Ltd, China.

18S rDNA genes were Polymeric Chain Reaction (PCR) amplified using the forward (5' AAGTATAAACTGCTTATACTGTGAA 3') and reverse (5' CCTACGGAAACCTTGTTACGACT 3') primers; ITS1genes were amplified by the forward (5' AGTCGTAACAAGGTTTCCGTAGG 3') and reverse (5' TATGCTTAAGTTCAGCGGGTAAT 3'). The DNA is amplified by PCR (Biometra Company, Germany). Comparisons for similar sequences were carried out using the BLAST Program (NCBI BLAST, USA).

2.3 Biomass production

The microalgae biomass adopted the nephelometry, which was determined by measuring the optical density of samples at 680 nm (OD_{680}) using a spectrophotometer (UV-7504PC, Xinmao Instrument, Shanghai, China)[19]. The culture medium with algae was filtered through a 0.45 μm glass fiber filter (Whatman Inc., USA), and the harvested cells were dried at 80°C for 24 h. The linear relationship with dry cell weight and OD_{680} was showed as Equation (1):

$$y=0.2993x-0.0365 \ (R^2=0.991, p<0.05) \quad (1)$$

where, y is the dry cell weight (DCW), g/L; x is the absorbance at 680 nm.

2.4 Microalgae growth restriction experiment

In order to figure out the culture conditions that restrict the growth and biomass production of microalgae, different temperatures, pH values and NaCl

concentrations were used in those experiments. The experiments change one of the conditions with other conditions were controlled in the same in the culture process. The temperatures used in current study were 5, 15, 20, 25 and 30°C, respectively; pH values were gradually adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0 with HCl or NaOH; and NaCl concentrations were 0, 5‰, 10‰, 20‰, 30‰ and 40‰, respectively.

2.5 Alkaline wastewater cultivation procedures

The artificial alkaline wastewater was prepared with 0.572 g/L glucose, 0.189 g/L NH_4Cl, KH_2PO_4 0.02 g/L, 75 mg/L $MgSO_4 \cdot 7H_2O$, 36 mg/L $CaCl_2 \cdot 2H_2O$, 5 mg/L $FeSO_4 \cdot 7H_2O$, 1 mg/L $EDTANa_2$, A5 solution 1 mL/L, and adjust pH value to 11.0 with 4 mol/L NaOH.

Selected microalgae were cultured in the artificial wastewater (pH11.0) for 5 d and the biomass, ammonia nitrogen (NH_4-N), phosphate (PO_4-P) and chemical oxygen demand (COD) contents in the solution were measured every day. The initial inoculum should be controlled at OD_{680} of 0.3. Each experiment is repeated for at least three times.

2.6 Nutrient analysis

After filtration with a 0.45 μm glass fiber filter, the filtrates were appropriately diluted and the nitrogen, phosphorus, and COD content were analyzed. Ammonia nitrogen (NH_4-N) and phosphate (PO_4-P) were measured following the UV/Vis spectrophotometric method (Chinese Environmental Protection Standard, HJ 536-2009 and Chinese National Standard, GB 11893-1989); COD was used dichromate method (Chinese National Standard, GB 11914-1989).

3 Results and discussion

3.1 Screening and identification of microalgae species

The microalgae EJ 9-2 was isolated and purified among 18 strains from the collected water samples, which has reasonable environment tolerance with relatively higher growth of biomass in extreme environments. There was a single green cell or cell group with no certain number with smooth surface in microscopic observation. The cells were planktonic and round with a diameter range of 4-6 μm.

The study showed that the 18s rRNA gene sequence of the algae strain was 1117 bp, while the ITS1 was 612 bp. By the phylogenetic analysis with the BLAST, the algae strain was found to have a close relationship with *Desmodesmus* sp. (Figure 1).

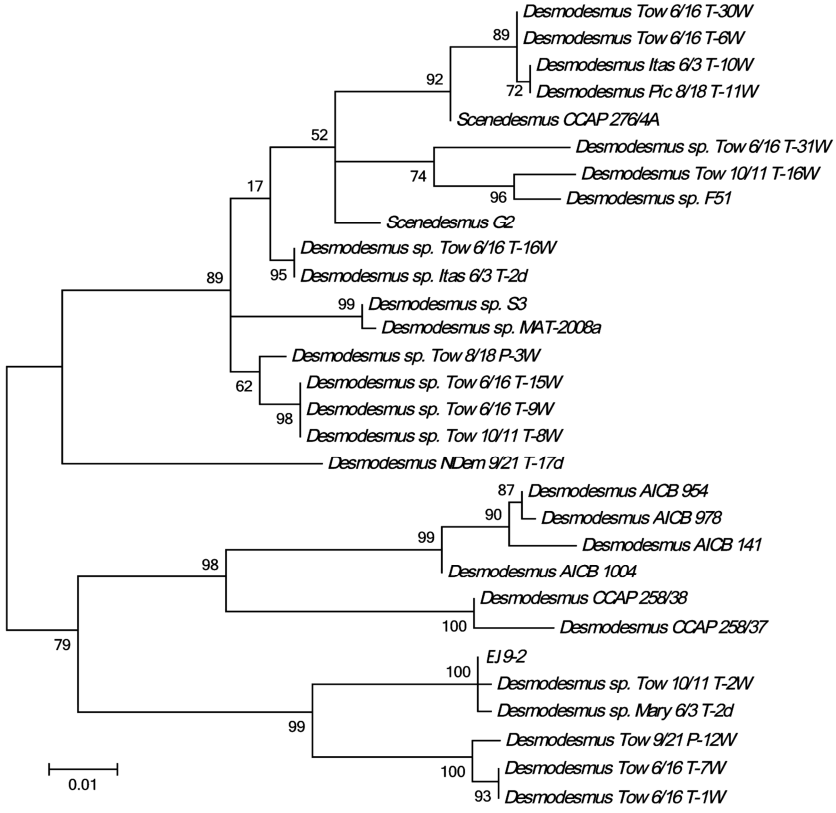

Figure 1 Dendrogram depiting the partial results of a neighbor-joining analysis of ITS1 sequences

3.2 Effects of different factors on algae biomass production of *Desmodesmus* sp. EJ 9-2

3.2.1 Effect of temperature on microalgae growth

Temperature is an important environmental factor for microalgae growth. Both photosynthesis and respiration of microalgae are affected by temperature[20]. The optimal growth temperature range is 20-25°C for most microalgae species. When the temperature exceeds a certain value, it will cause irreversible chemical destruction to the cellular components, leading to the rapid decline in cell function[21]. On the other hand, if the temperature dropped to a certain extent, the cytoplasmic membrane cannot normally transport nutrients and produce proton gradients, which can result in mechanical damage to cells and thus terminate the growth of microalgae[20-22]. Therefore, decreasing the ambient temperature can cause the reduction of microalgae biomass, while high temperature may cause the death of algae cells. As shown in Figure 2, the growth temperature range of *Desmodesmus* sp. EJ 9-2 was 5-35°C, and the maximum biomass reached 0.465 g/L at 25°C. However, all *Desmodesmus* sp. died 2 d after inoculation at 40°C. The biomass production of the microalgae at 5°C was also lower, with 0.005 g/L after cultured for 14 d.

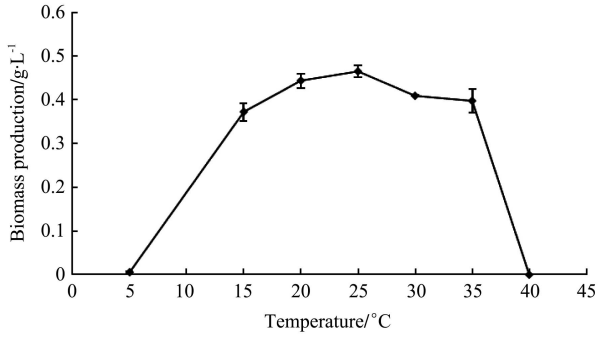

Figure 2 Effects of different temperatures on dry cell weight of *Desmodesmus* sp. EJ 9-2

3.2.2 Effect of pH value on micoalgae growth

As shown in Figure 3, *Desmodesmus* sp. EJ 9-2 could grow in a wider pH range (3-12); and different pH values cannot significantly affect its growth. The biomass production of microalgae maintained in the range of 0.413-0.511 g/L with pH 4-10 after being cultured for 14 d, while the maximum biomass production was 0.632 g/L at pH 11. Generally, microalgae can adapt acidic or alkaline growth environment[15,23], however there are rare reports about microalgae which can grow in such a wider pH range. This feature makes it capable of growing in various waters, which is extremely beneficial for large-scale microalgae cultivation.

The results of Xu et al.[23] showed that as long as the initial pH value of the water body did not cause much damage to the growth of microalgae, microalgae would change the pH value of water through its metabolic activity in order to reach the suitable scope for its growth. This research also showed that when cultivate the *Desmodesmus* sp. EJ 9-2 in the media with different initial pH values, the pH value of solutions would stabilize at around 11 after 14 d, which indicated that the *Desmodesmus* sp. EJ 9-2 could absorb or secrete certain materials that affect the pH value of the water solution during the growth process.

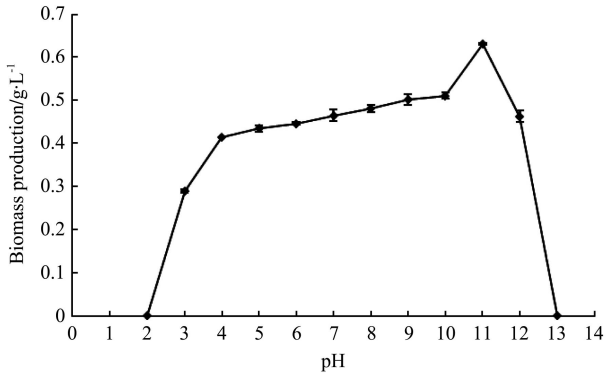

Figure 3 Effects of different pH value on dry cell weight of *Desmodesmus* sp. EJ 9-2

2.2.3 Effect of NaCl concentration on microalgae growth

The investigation of Erdmann[24] showed that under the stress of NaCl, microalgae can produce a type of small molecule carbohydrate, by which the microalgae can regulate the intracellular and extracellular osmotic pressure to adapt the high salinity environments. *Desmodesmus* sp. EJ 9-2 had a wider range of salinity and could grow within the range of 0-30‰ (Figure 4). However, relatively high NaCl concentrations could significantly inhibit the growth of microalgae cells. The biomass of *Desmodesmus* sp. EJ 9-2 reduced significantly with the increased of the increasing NaCl concentration. The biomass production could reach the largest value of 0.465 g/L when the medium without NaCl, however, the growth of microalgae was normal but the biomass was

accumulating slowly because of the long-term stress of high salinity when the NaCl concentration was 10‰, and the biomass dropped to 0.176 g/L. The microalgae cells died when the NaCl concentration increased to 40‰.

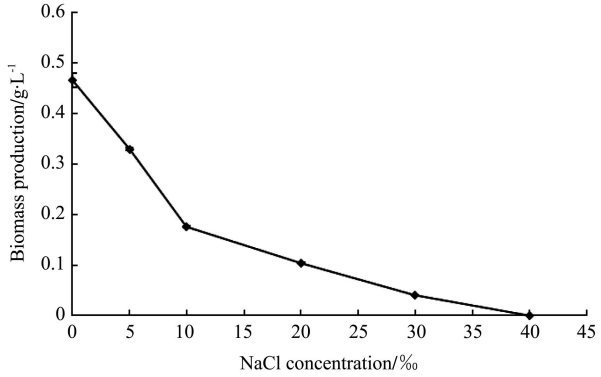

Figure 4 Effects of different NaCl concentrations on dry cell weight of *Desmodesmus* sp. EJ 9-2

3.3 Nutrient removal performance of microalgae in alkaline wastewater

2.3.1 Characteristics of the alkaline wastewater

The pH value of artificial alkaline wastewater was adjusted to 11 according to composition of high alkaline wastewater in paper mills and printing and dyeing mills, and so on[9]. Compared with the normal culture medium, high alkaline wastewater had higher NH_4-N and COD contents but lower PO_4-P content, and the N/P was higher (Table 1).

Table 1 Characteristics of high alkaline wastewater

Parameters	NH_4-N	PO_4-P	COD
Content/mg·L^{-1}	58.137±1.496	3.308±0.071	1298±38

2.3.2 Biomass production and nutrient removal

As shown in Figure 5, the biomass production reached 1.046 g/L after 5 d cultivation in wastewater, which indicated that this microalgae strain had a better adaptability in high alkaline wastewater. Nitrogen, phosphorus and COD were the main pollutants in wastewater and the necessary nutriments for the growth of microalgae. The nutrient removal condition of *Desmodesmus* sp. in alkaline wastewater after 5 d cultivation was also shown in Figure 5; and the nutrient removal performance was shown in Table 2. The nitrogen, phosphorus and COD concentrations in wastewater gradually decreased with the cultivation duration. The removal efficiencies of NH_4-N and PO_4-P were higher and completely removed in the third day and the fourth day, respectively, while the removal efficiency of COD was lower and reached 69.61% in the fifth day.

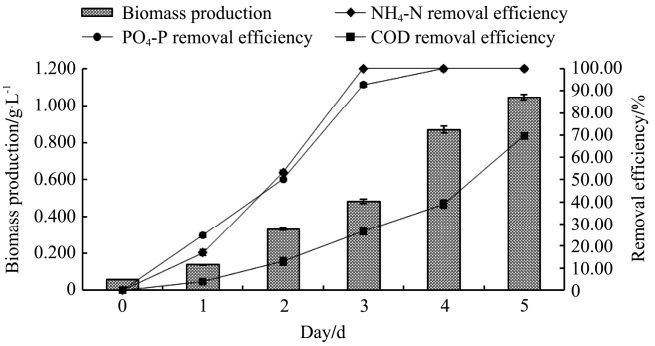

Figure 5 Nutrient removal and biomass production profile of high alkaline wastewater during *Desmodesmus* sp. EJ 9-2 cultivation.

Table 2 Nutrient removal performance in high alkaline wastewater

Removal amount/mg·L^{-1}			Maximum daily removal amount/mg·L^{-1}·d^{-1}		
COD	NH_4-N	PO_4-P	COD	NH_4-N	PO_4-P
903.5	58.137	3.308	181	19.379	1.022

Although NH_4-N is the favorite nitrogen source in the growth of microalgae, high NH_4-N concentration (>6 mM) could also inhibit the growth of microalgae[25]. Therefore, the wastewater needs to be diluted first, due to the toxic effect of high NH_4-N concentration on microalgae growth. Phosphorus, as another important element for the growth of microalgae, also had a high removal effieciency. On one hand, microalgae could absorb and utilize phosphorus in the growth process, high pH may cause part of phosphorus precipitation, which could also decrease PO_4-P concentration[26]. The COD cannot be completely removed because the dead cells are releasing organic matters while the new cells are absobing and utilizing the COD[27]. Meanwhile, under such extreme alkaline environment, *Desmodesmus* sp. EJ 9-2 still maintained a rapid growth rate and relatively high nutrient removal efficiency, which indicated that it could treat the high alkaline wastewater while producing the microalgae biomass.

4 Conclusions

A well-tolerated microalgae strain was isolated from fresh water and identified as *Desmodesmus* sp. by 18 s rRNA and ITS1, named as EJ 9-2. *Desmodesmus* sp. EJ 9-2 had a wide pH tolerance range (3-12) and can grow normally under the temperature of 5-35°C and NaCl

concentration of 0-30‰. Those features enable *Desmodesmus* sp. EJ 9-2 to adapt to most water environment and make it a dominant species for large-scale microalgae cultivation. The *Desmodesmus* sp. EJ 9-2 could remove most of the pollutants in alkaline wastewater and produce biomass which could greatly reduce the processing costs of organic wastewater treatment.

Acknowledgment

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