A new species of *Desmodesmus sp*. from high altitude area was discovered and its isolated, purification, amplification culture and species identification were studied

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Abstract

Plateau microalgae play a key role in maintaining water quality in these lakes and rivers on the Qinghai-Tibet plateau. Plateau microalgae have wide application prospects in environmental purification, biotechnology, medicine and cosmetics, food industry and renewable energy. In order to get the high biomass of microalgae that exist in nature, microalgae with the largest biomass was screened from natural water samples by filtration, preculture and plate scribing separation. Microalgae with the largest biomass was screened from natural water samples by filtering first, pre-culturing and agar plate scribing separation. Identified by 18S rRNA as *Desmodesmus sp.* and constructed an neighbor-joining phylogenetic tree based on 18S rRNA sequences; Further studies have been made on the light-absorbing properties of plateau *Desmodesmus sp.*. The characteristic absorption peak of *Desmodesmus sp.* on the plateau was found at 689nm in the visible region by full wavelength scanning with UV-Vis spectrophotometer. For *Desmodesmus sp.* which is easy to settle in the process of amplification culture. By monitoring the change trend of total nitrogen, total phosphorus, pH and electrical conductivity in algae solution system, it was determined that logarithmic phase of the growth and the best transfer time of *Desmodesmus sp.* were 15-20 days.

1. Introduction

The Qinghai-Tibet Plateau is the highest plateau in the world, with an average altitude of over 4000m. It is characterized by high cold, hypoxia, little precipitation, long sunshine and strong radiation, and has a unique physical and geographical environment (Yue et al. 2005). Large and small rivers and lakes are distributed on different elevation gradients, and plateau microalgae play a key role in maintaining the water quality of these lakes and rivers (Yang et al. 2003).

In recent years, wastewater treatment based on microalgae has attracted more and more attention due to its environmental friendliness and potential economic benefits (Martin et al. 2015).Studies have proved that microalgae can remove various pollutants from wastewater, such as oxygen consuming pollutants, nitrogen, phosphorus, heavy metals, organic matter, and absorb harmful gases such as NO_X, SO_X, and H_2S at a certain concentration.The biomass harvested from wastewater treatment can also be used as raw materials for biofuel, feed and chemicals(Ivanova 2021; Pancaldi 2020; Ec et al. 2020; Jakkapong et al.; Camacho et al. 2019; Fa et al. 2005). Microalgae can use CO_2 as the only carbon source for heterotrophic growth through photosynthesis, and can also use external carbon sources for heterotrophic growth to improve the biomass yield and oil content of microalgae(Yong et al. 2019; Nzayisenga et al. 2020), which is of great significance to reduce global emissions of greenhouse gas carbon dioxide(Jutidamrongphan et al. 2014).

Desmodesmus sp. was collected, purified, amplified and cultured from Yamdroktso lake, Langkazi County, Shannan City, Tibet Autonomous Region Named YH-1. This area belongs to the semi-arid climate area of the plateau sub cold zone, with low annual average temperature, low air density, low oxygen

content and strong solar radiation. The growth rate and cell components of vegetation and algae growing in this area are very different from those of crops in plain areas (CHU et al. 2007).

On the basis of previous studies, a plateau microalgae with the highest biomass was screened by filtration, pre-culture and plate scribing separation method. It was identified as *Desmodesmus sp.* by 18S rRNA. Genotyping was determined by PCR amplification and mutation locus was determined by GATK analysis. The characteristic absorption peak was determined by studying the light absorption characteristics of *Desmodesmus sp.* on plateau. The linear relationship between the concentration of *Desmodesmus sp.* solution and the absorbance and turbidity was established by the optical density method, and the regression equation was established. By analyzing the change trend of total nitrogen, total phosphorus, pH and electrical conductivity in the solution system during the amplification culture of *Desmodesmus sp.*, the logarithmic growth end and the optimum transfer time of *Desmodesmus sp.*, were determined.

The research results are helpful to comprehensively understand the isolation, purification, amplification and culture methods of microalgae in high altitude areas. For some microalgae species that are easy to settle and affect the accuracy of absorbance value (such as *Desmodesmus sp.*)(Joni et al. 2007), the best transfer species or harvest time can be accurately determined by monitoring the indicators of total nitrogen, total phosphorus, electrical conductivity and pH value during the culture process; Through the linear relationship between microalgae concentration and absorbance and turbidity, the microalgae concentration can be determined quickly and easily through the regression equation, which can provide the basis for the follow-up large-scale production and application research.

2. Results

From the water samples collected from Yamdroktso lake (4400 meters above sea level) in Langkazi County, Shannan City, Tibet Autonomous Region, the method of filtration, pre-culture and plate separation was adopted to obtain the algae strain with the maximum biomass after separation and purification. Then, after amplification and culture,the algae strain was microscopically observed, and one algae strain with relatively high biomass was selected.

2.1 Species identification by morphology

The method of first filtration, then pre-culture and then plate separation has the advantages of simple equipment, simple operation and small workload. The separated samples are likely to come from two or more cells. The isolated single species of microalgae may not be the target species, but other species or new species maybe isolated. It is especially suitable for the preliminary separation of water samples from natural water areas, and there is no limit on the species and size of microalgae.

The algal strain with the largest biomass was obtained after isolation and purification. After amplification and culture, the algal strain was microscopically observed. The algal body was yellow green, which was a

typical morphological pattern of four cells together, and had a spike. Through its morphological analysis, the species were identified as *Desmodesmus sp.*(Joni et al. 2007; John 2011*)* (Figure. 1a).

2.2. Species identification by 18S rRNA

Using the new generation sequencing method, DNA was extracted from the samples using the test kit produced by Shanghai Sangon Biotech. The amplified PCR products were detected and purified through PCR amplification, agarose electrophoresis detection, gel recovery and other steps. The PCR products were sequenced using the 3730xl sequencer produced by ABI company of the United States.

After PCR amplification, 18S rRNA was obtained. The length of PCR amplification product: site sequencing primer was about 150 ~ 300 BP, 80 ~ 150 BP away from the site, and exon detection primer was about 150 BP away from the upstream and downstream of exon; The PCR product band of target gene sequencing is generally no more than 1200 BP. The login number is PRJNA810921/SAMN26292020/SRR18173251, by analyzing the sequence homology with NCBI, it was found that it had the closest genetic relationship with *Desmodesmus sp.*, and the homology of 18S rRNA sequence reached 99.6%.(Figure. 1b).

2.3. Determination of Amplification Culture Cycle

Desmodesmus sp. belongs to algae with good sedimentation. At this time, there will be a certain error in determining its logarithmic growth end by absorbance through simple optical density method. By detecting the change trend of total nitrogen, total phosphorus, pH and electrical conductivity in the water during the amplification culture of *Desmodesmus sp.*, we can accurately judge the end of logarithmic growth, the best time of Transfering species and picking.

From the monitoring data of total phosphorus, it can be concluded that the best transfer time of *Desmodesmus sp.* is 15 ~ 20 days after amplification culture.Over 20 days, microalgae grow in apoptosis, which is manifested in the increase of total phosphorus index in the solution system. The main reason is the release of organic matter and metal elements caused by the decomposition of algal cells. Therefore, over 20 days, it is necessary to replanted or harvest again.(Figure. 2a).

Through the monitoring data of total nitrogen, it can be concluded that the best transfer time of *Desmodesmus sp.* is 15 ~ 20 days after amplification culture. Over 20 days, microalgae grow in an apoptotic state, which shows that the total nitrogen index of the solution system increases. The main reason is the release of organic matter and metal elements caused by the decomposition of algal cells. Therefore, over 20 days, it is necessary to replanted or harvest again.(Figure. 2b).

The pH value of microalgae fluctuates in the process of amplification culture, mainly because the growth process of microalgae needs to consume CO_2 in the atmosphere. The consumption of CO_2 in the process of dissolution and photosynthesis in water will cause the change of pH value of water. It can be seen from the graph that between 15–23 days, microalgae reached the balance of CO_2 adsorption and consumption during the growth process, and microalgae were in a stable growth state. Through the

monitoring data of pH value, it can be concluded that the best transfer time of *Desmodesmus sp.* is 15–20 days after amplification and culture. More than 20 days, it needs to be replanted or harvested again. (Figure. 2c).

The conductivity value of microalgae fluctuates during the amplification culture process, mainly because the growth process of microalgae needs to consume the metal nutrients in the culture medium, resulting in the decrease of conductivity. At the same time, the natural growth cells of microalgae will also produce certain conductivity, which will also lead to the increase of electrical conductivity, especially after the end of logarithmic growth cell wall breaking will lead to the release of metal elements and the increase of electrical conductivity. It can be seen from the change of conductivity value. The best transfer time of Streptococcus is 15–20 days after amplification culture. More than 20 days, it needs to be replanted or harvested again.(Figure. 2d).

After 1:5 amplification culture, the changes of total nitrogen, total phosphorus, pH value and conductivity were monitored in real time for 30 days. It was concluded that the best transfer time was 15–20 days after amplification culture. More than 20 days, it needs to be planted or picked again. It provides better data support for the expanded culture of microalgae.

2.4. Study on light absorption characteristics

After full wavelength scanning with UV-Vis spectrophotometer, the stock solution of *Desmodesmus sp.* culture solution has no obvious absorption peak in the UV region, and there is an obvious absorption peak when the wavelength in the visible region is 689 nm, that is, its characteristic absorption peak; The supernatant (background liquid) obtained by centrifugation of algal liquid culture medium was scanned in the same way, and the absorption curve was relatively stable without obvious absorption peak; Therefore, the characteristic absorption wavelength was selected at 689 nm to study the relationship between the concentration of *Desmodesmus sp.* and the absorbance of culture solution.(Figure. 3a; Figure. 3b)

2.5. Relationship between algae concentration and absorbance of culture solution

The results show that there is a good linear relationship between the concentration of plateau *Desmodesmus sp.* in the culture solution and the absorbance within a certain concentration range(10^6-10^8/L) and at the maximum absorption wavelength of 689 nm. The concentration of microalgae in the culture process can be accurately reflected by absorbance.(Figure. 4a)

2.6. Relationship between algae concentration and turbidity of culture solution

The results show that there is a good linear relationship between the concentration of *Desmodesmus sp.* in the culture solution and turbidity in a certain concentration range(10^6-10^8/L). The concentration of microalgae in the culture process can be accurately reflected by turbidity.(Figure. 4b)

3. Materials And Methods

3.1 Collection of microalgae

100 L of water was collected from Yamdroktso lake (25 times by water collector), the phytoplankton were filtered through a phytoplankton net of 500 mesh, and the water sample was poured into a teflon bucket of 50 L for preservation. 200g of sediment from the bottom of the lake was randomly collected at various points, and stored in a TEflon bottle with a breathable film tied at the mouth. Transport to the laboratory within 6 hours.

3.2 preparation method of culture medium

Preparation method of liquid culture medium: weigh the BG-11culture medium1.7g add it into a 1000 mL conical flask, add water to a constant volume,shake until they are completely dissolved, use 1M HCl or NaOH to adjust the pH to 7.2, cover the bottle mouth of the conical flask with a rubber stopper, and then use a 0.22 μm sterilization membrane for filtration in a clean workbench for later use.

Preparation method of solid culture medium: add 25 g of nutrient agar medium to the liquid culture medium prepared above, heat it to 60℃ in water bath, and shake it until it is completely dissolved. Then use 0.22 μm sterilization filter membrane to filter in a clean workbench, and use it for later use. After the culture medium is cooled to 35℃, it is poured into a sterile Petri dish and cooled continuously to obtain a solid culture medium.

3.3 Pre-culture after filtration

Under the condition that the purification workbench is opened, the collected water samples are poured into the Brinell funnel in turn, and filtered under the suction filtration of the water ring vacuum pump, and the filter paper is a 0.45 μm water-based filter membrane.

A water sample with a total volume of 100 L is filtered for three times, and a new filter membrane is replaced each time, and the replaced filter membrane is put into a liquid culture medium prepared in advance for culture. Three groups of parallel cultures can be obtained by three times of filtration. There is no need to filter the bottom mud collected from the water bucket, and about 30 g of bottom mud is taken from each group and put into the liquid culture medium separately, which requires three groups in parallel. The top of the conical flask is sealed with a breathable sealing film to prevent external bacteria from entering.

Shake it three times a day in a light incubator with a temperature of 25 ± 1℃ (light-dark ratio of 12 h:12 h, light intensity of 2000 Lx), and place each sample at random so that the sample can receive light evenly. After culturing for 7–15 days until the whole solution system is yellow-green or green, microscopic examination and dilution are carried out to separate algae strains.

3.4 Isolation and purification of microalgae

The preparation method of culture medium adopts the solid medium separation method. When the purification workbench is turned on, dip the above-mentioned fresh algal liquid at the end of logarithmic growth for about 10 days with the inoculation ring, and draw a dense line on the solid medium. Invert it into a 25 ± 1 ℃ light incubator for culture (light dark ratio 12h: 12h, light intensity 2000 Lx). The formation of algae strains with large biomass can be seen in 7–10 days. Pick out large algae colonies for microscopic examination. If a single species is detected by microscope, it indicates that the separation is successful. At this time, the purpose of separation and purification has been achieved, and expanded culture can be carried out.

3.5 The scale-up cultivation of *Desmodesmus sp.*

 in a 1,000 mL beaker, prepare 500 mL BG-11 liquid medium according to the formula of BG-11 medium, and adjust the pH to 7.1. 200 mL of BG-11 liquid medium is filled in each 500 mL conical flask, or 50 mL of BG-11 liquid medium is filled in 150 mL conical flask;

 in the operation of above, the BG-11 culture medium should be filter–ed by 0.22 micron sterilization filter membrane under the clean workbench, and then the bottle mouth should be bandaged for later use; gas permeable membrane, sterile filter membrane, filter device, conical flask and inoculation ring, etc. should be sterilized by steam pressure sterilization pot at 101.33 kPa and 121℃ for 30 minutes before use;

 under the clean workbench, select the microalgae with good growth by sterile inoculation ring and put them into 10 mL BG-11 liquid culture medium after sterilization and filtration, at 25 ± 1℃, the light intensity is 2,000 Lx (light ratio is 12 h:12 h), shake or stir for 2–3 times every 12 h, and the culture time is 7–15 days; when the biomass is obviously increased (> 10^6 /L), amplification culture can be carried out in time;

 under the clean workbench, 10 mL of cultured algae liquid is added into the liquid medium containing 20 mL BG-11 for culture (1:2 amplification),the temperature is 25 ± 1℃, the light intensity is 2,000 Lx (the light ratio is 12 h:12 h), and the culture time is 7–15 days by shaking or stirring for 2–3 times every 12 h, when the bottle mouth is sealed with a gas permeable membrane and the biomass is obviously increased;

 normal culture conditions: temperature 25 ± 1℃, light intensity 3,000 Lx (light ratio 12h: 12 h), shaking or stirring for 2–3 times every 12 h; when the culture volume is greater than 1 L, consider adding stirring to assist the culture, and the culture time is 7–15 days; when microalgae species with long culture period are used, the culture period can be appropriately prolonged;

 then, add the culture solution in into 150 mL BG-11 medium for amplif-ication culture (1:5 amplification), and the culture period is 15–20 days; when microalgae species with long culture period are used, the culture period can be appropriately extended to about 30 days;

 when the volume of amplification culture medium is greater than 1 L, the amplification culture conditions are 25 ± 1℃, the light intensity is 3,000 Lx (light ratio is 12 h: 12 h) and 100–200 r/min; when microalgae species with long culture period are used, the culture period can be appropriately extended to about 30 days;

 after the logarithmic phase of the growth of plateau green algae, when the final optical density OD689 value is ≥ 0.700 (the maximum absorption wavelength of each algae species can be obtained by full-wavelength scanning with UV-spectro-photometer), take the algae solution after amplification culture, centrifuge or filter, and wash it with BG-11 culture solution for 3 times for later use.

3.6 Species identification of 18S rRNA

Using the extracted microalgae DNA as the template, primers (positive 1143-510-2-F(AATTGACGGAAKGGCA) and reverse 1637-510-2-R (CGACGGGCGGTGTGTA) were designed by primer premier 5.0 software.The 18S rRNA gene was amplified by PCR. 25μL PCR reaction system: DNA template 1μL. Primer each 1μL, dNTP 1μL,Taq Buffer (with $MgCl_2$)2.5μL. Taq enzyme 0.25μL, add ddH_2O to 25μL. The PCR reaction conditions were: pre denaturation at 95℃ for 5 min, denaturation at 94℃ for 30s, annealing at 63℃ for 30s (0.5℃ per cycle), extension at 72℃ for 30s, 10 cycles; Denaturation at 95℃ for 30s, annealing at 58℃ for 30s, extension at 72℃ for 30s, 30 cycles; Recover and extend at 72℃ for 10min and keep warm at 4℃. After PCR products were detected, the sequences were blast compared in GenBank database. Construct a neighbor-joining phylogenetic tree based on 18S rRNA sequences.

3.7 Determination of Amplification Culture Cycle

Microalgae need to consume nutrients such as N, P and heavy metals in the growth process(Xenopoulos et al. 2002; Shekarabi et al, 2021). Considering the easy sedimentation characteristics of *Desmodesmus sp.*, the logarithmic logarithmic phase of the growth, transfer and harvest time of this species are determined by detecting the change trend of total nitrogen, total phosphorus, pH and conductivity in the culture solution of *Desmodesmus sp.* in the amplification culture stage, so as to avoid the error caused by the optical density method(Tan et al.2020).

The water quality multi parameter tester(KN-MUL20, kenuokeyi instrument, Beijing) and intelligent digestion instrument(KN-HEA12, kenuokeyi instrument,Beijing) are used to determine the total nitrogen and total phosphorus. The water samples for the determination of total nitrogen and total phosphorus need to be filtered with 0.22 μm filter in advance. The conductivity (cond) and pH were measured with a multi parameter test pen (PCSTestr-35).

3.8 Experiment on correlation between algae concentration and absorbance and turbidity

For the determination method of algal concentration, the blood cell counting plate is used to count under the optical microscope, the average value is taken for three counts, and the average deviation is controlled within 10%. Before counting, the algal solution needs to be vibrated with an oscillator to ensure the uniform dispersion of microalgae, and the initial algal concentration (PCS / L) is determined by taking the stock solution of *Desmodesmus sp.* culture solution as the test water sample.

Method for preparing water sample containing algae. Dilute 10mL of the same volume of algal solution with ultrapure water successively, and the volume is 20mL, 40mL, 80mL, 100mL and 200mL. Dilute it into a series of algal solutions with different concentrations at constant volume for cell counting and turbidity detection, there are three parallel samples. Dilute 10mL of the same volume of algal solution with ultrapure water to 20mL, 30mL, 40mL, 50mL and 100mL respectively, and dilute it into a series of algal solutions with different concentrations at constant volume for cell counting and absorbance (689nm), there are three parallel samples.

According to the above values, fit the absorbance algae concentration curve, establish the linear regression equation, fit the turbidity algae concentration curve, and establish the linear regression equation(Tsenkova 2020; O'Donoghue et al. 2022).

4. Discussion

By detecting the change trend of total nitrogen, total phosphorus, pH value and conductivity of *Desmodesmus sp.* in the process of scale-up cultivation, the end of logarithmic growth, and the best time of seed transfer and harvest can be accurately judged.

Desmodesmus sp. has characteristic absorption peak at 689 nm. In a certain dilution concentration range, the algae concentration in the culture solution has a good linear relationship with absorbance and turbidity; Therefore, absorbance or turbidity can be used to determine the concentration of algae in the process of algae culture.

During the experiment, the bubbles brought by shaking and shaking and the sedimentation of *Desmodesmus sp.* itself will also have a certain impact. In addition, when detecting absorbance and turbidity, it is necessary to shake the algal liquid evenly as much as possible to reduce the detection error caused by sedimentation of microalgae with strong sedimentation.

Declarations

Author Contributions: Conceived and designed the study: W.J., B.D. Performed the study: W.J., Z.Q., C.N., C.J., L.J., W.Y. Analyzed the data: W.J., Z.Q., Z.J., W.Y. Wrote the paper: W.J., B.D. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Sample Availability: Not applicable.

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Figures

Figure 1

(a) Morphological graph of *Desmodesmus sp.*(YH-1); (b)Neighbor-joining phylogenetic tree based on 18S rRNA sequences.

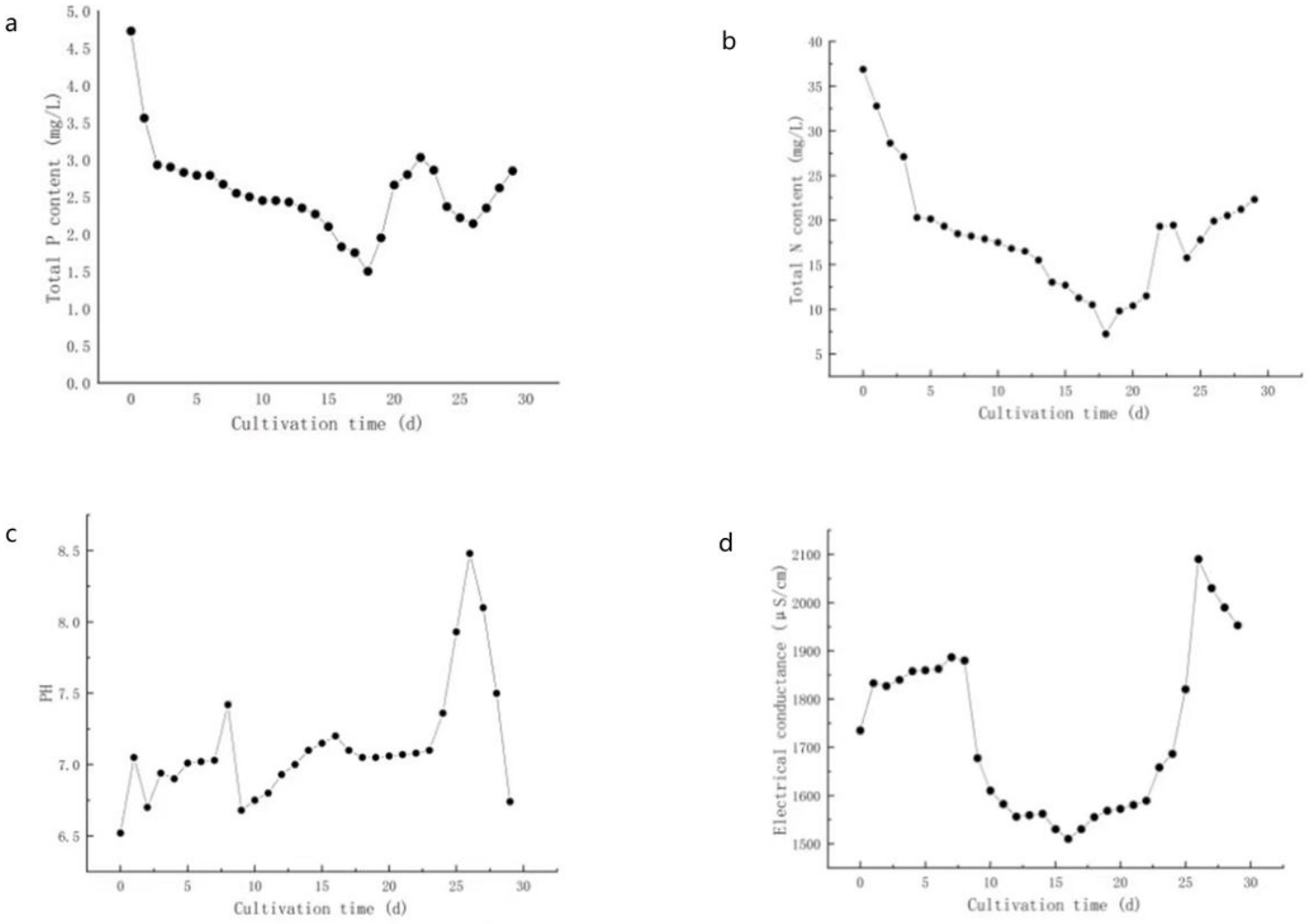

Figure 2

(a)Trend diagram of total phosphorus during microalgae amplification culture stage; (b)Trend diagram of total nitrogen during microalgae amplification culture stage; (c)Variation trend of pH value in microalgae amplification and culture stage; (d)Variation trend of electrical conductivity in microalgae amplification and culture stage.

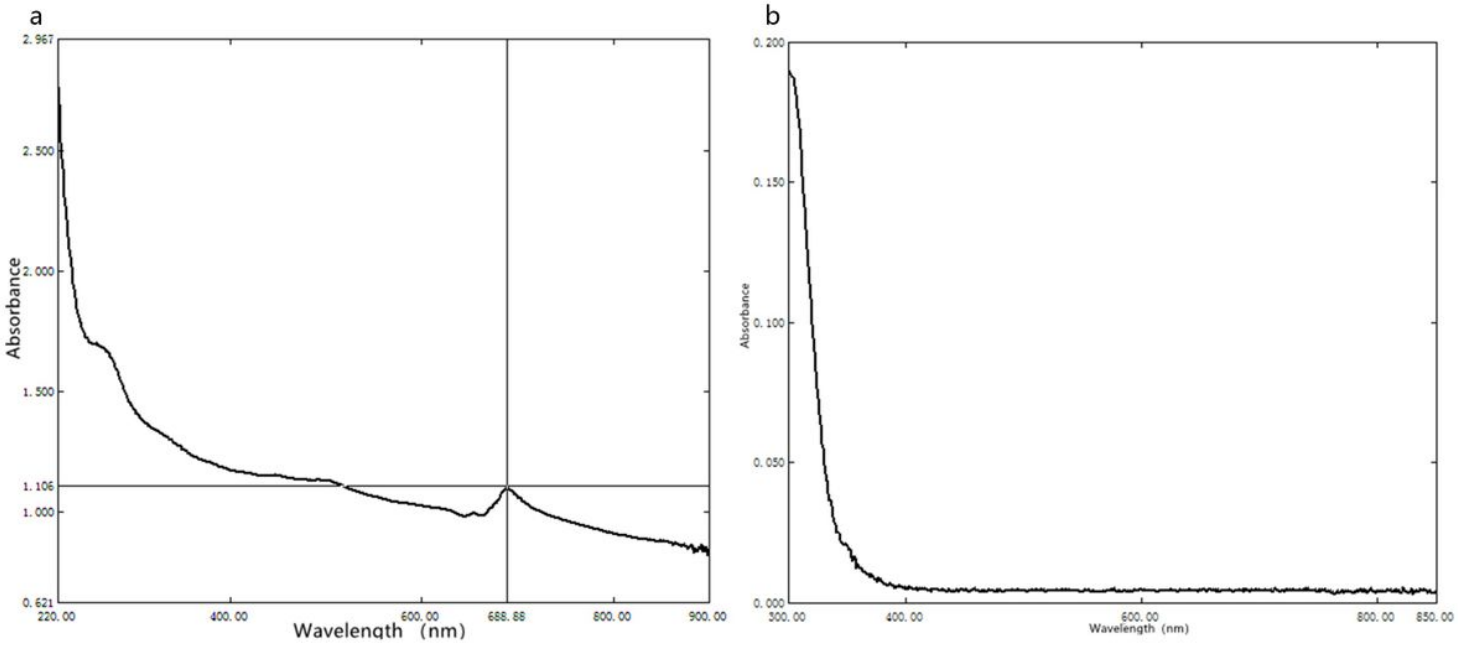

Figure 3

(a) Full wavelength scanning spectrogram of *Desmodesmus sp.* ;(b) Full wavelength scanning spectrum of culture medium background solution

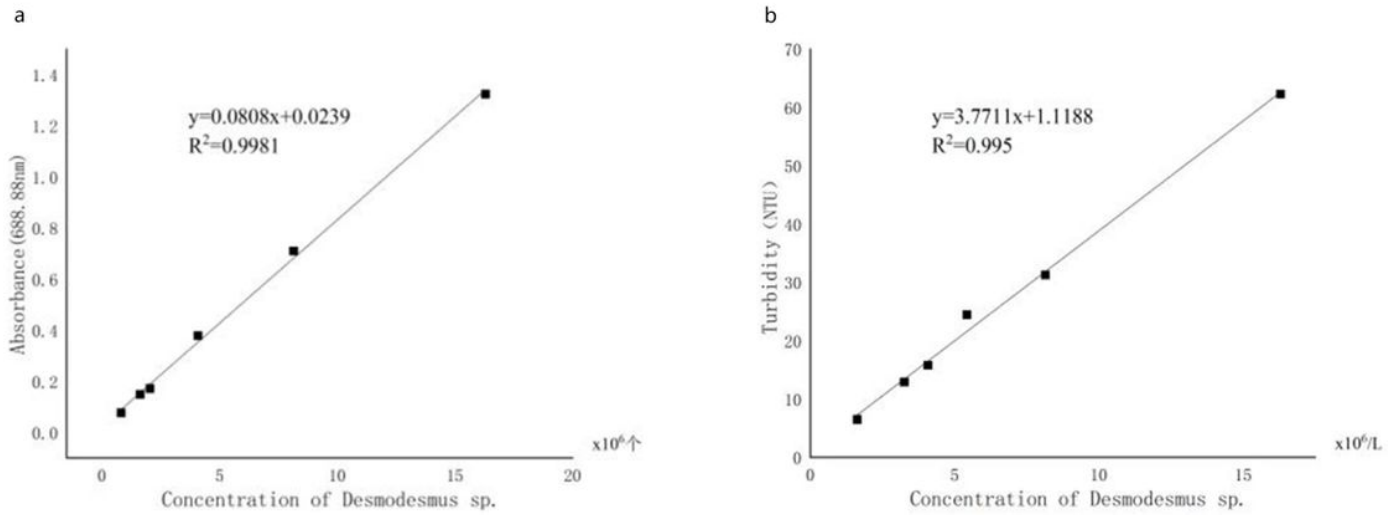

Figure 4

(a)Relationship between algae concentration and absorbance of culture solution; (b)Relationship between algae concentration and turbidity of culture solution