

STUDY ON IMMOBILIZED *CHLORELLA SP.* BY NANO MUSSEL-SHELL POWDER AND CHITOSAN

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Abstract: Shells were used to produce biomass materials through calcining due to their excellent mechanical properties. In this paper, the mussel shells were calcined by the vacuum tube furnace and muffle furnace at 600 °C, 700 °C and 800 °C respectively, and that prepared under optimum conditions was activated by K₂CO₃ in the hydrothermal method. The pore structure and morphology of the sample were characterized by specific surface area analyser (BET) and scanning electron microscopy (SEM). Then the *Chlorella sp.* was immobilized by mussel shell powder and chitosan, which was co-cultured for 60 days. The results showed that calcined shells at 700 °C in a vacuum tube furnace have an optimal specific surface area, up to 4.107 m²/g. K₂CO₃ hydrothermal modification method could effectively enlarge the pores of shell powder, from 4.107 nm up to 16.557 nm. The shell powder modified by chitosan had positive charges, which could attract the microalgae with negative charges, could live for up to 60 days. This article aims to use waste shells to immobilize microalgae, which is not only of great importance to the protection of the environment but also provides a novel way to immobilize microalgae.

Keywords: *Chlorella sp.*, immobilization, nanoporous mussel shell materials, microstructural characterization, recovery growth rate

Introduction

Biomass has been assigned numerous roles to human beings from ancient times because of its massive and recyclable nature, however, with the rapid expansion of man-made materials, like alloy, plastic fibre, and so on, a great number of natural materials have been neglected and abandoned. Most of the non-renewable and non-biodegradable synthetic materials were used and subsequently became wastes, resulting in seriously environmental “white pollution” at present (Wang et al., 2017). Therefore, high-value utilization of biomass resources has been paid more and more attention to researchers

because of their renewability and biodegradability (Wang et al., 2016). Shells are often used to prepare porous materials by calcination as carrier-loaded functional ions due to its main component is 95% CaCO_3 and a small amount of organic matter (Currey et al., 1977; Okumura and Gennes, 2001; Currie et al., 2007). The previous study was performed a shell-based catalyst by the impregnation process using potassium hydroxide to extract economical biodiesel fuel synthesis from castor oil (Nuridin et al., 2015). The current use of seashells is limited to feed additives (J. et al., 2013), adsorbents (Paradelo et al., 2016), cement reinforcing phase (Martinez-Garcia et al., 2016), antimicrobials (Li et al., 2016), and carriers for functional materials (Edralin et al., 2017) at present.

Chlorella sp. is a kind of unicellular green microalgae that around 40% of global photosynthesis is performed by microalgae (Myers, 1980). From the 1960s, Oswald et al. first found the *Chlorella* in the oxidized pond had an excellent removal effect on nitrogen and phosphorus in the water (Oswald et al., 1957). However, due to its small size of single cells, the presence of free-living microalgae in treated effluents remains a problem (Chevalier and Noüe, 1985), so researchers focused on the use of immobilized microalgae (Jiménez-Pérez et al., 2004). Immobilization techniques can be primarily divided into two groups: “passive” and “active” immobilization (Moreno-Garrido, 2008). Because the microalgae tends to adhere and naturally grow on the surface of natural organisms, the “passive” immobilization mostly uses it as a carrier (Robinson et al., 1986), but this process is easily reversed. Therefore, the “active” immobilization method, such as chemical attachment (Moreira et al., 2006), flocculant agents (Seki and Suzuki, 2002), and gel entrapment (Solé and Matamoros, 2016), are most widely used. Alginate gel, a gel entrapment type, has been most widely used because the immobilized cells do not undergo extreme physicochemical changes during the immobilization process (Smidsrød and Skjåk-Braek, 1990).

Most frequent current uses of *Chlorella sp.* are the metabolite production cultured, improvement of culture collections handling, obtaining of energy, nutrients, removal of metal or organic pollutant from aquatic media, measurement of toxicity and co-immobilization system production for different purposes (Abdel-Raouf et al., 2012). The use of immobilized algal cells in water purification processes has been reported previously (Bruun, 2013) as microalgae form part of the organisms fixed in percolating filters of wastewater treatment plants. At present, the immobilization technology of microalgae mostly uses natural polymer materials as carriers, such as agar (Kandilian et al., 2017), alginate (Smidsrød and Skjåk-Braek, 1990) and so on. The advantage of these materials is that they have good biocompatibility, but the immobilization power is not strong, as a result, the microalgae is easy to fall off. In addition, the polymeric polymer material as a carrier to immobilized microalgae has the disadvantages of low biocompatibility and toxicity to microalgae cells (Timofeeva and Kleshcheva, 2011). There are few reports on the use of porous biomass materials as carriers to immobilize microalgae.

In this paper, mussel shell was calcined, activated and modified by chitosan, used to be as a carrier, to immobilize microalgae. Because modified shell has positive charges, it could attract the microalgae with negative charges, and achieve microalgae immobilization with a strong immobilization power. At the same time, Chitosan has the dual functions of electric neutral flocculation and adsorption flocculation, which is not only can provide nutrients for the growth of microalgae, but also non-toxic and harmless. This material will has broad prospects in the field of water quality control.

Materials and methods

Materials

Mussel shell was provided by a food processing factory (Zhejiang province, China). *Chlorella sorokiniana* was obtained from the laboratory of Marine Biological Chemical Laboratory, Zhejiang Ocean University. BG-11 medium (NaNO₃, 1.5 g; K₂HPO₄, 0.04 g; MgSO₄·7H₂O, 0.075 g; CaCl₂·7H₂O, 0.036 g; Na₂CO₃, 0.02 g; H₃BO₄, 2.86 g; MnCl₂·4H₂O, 1.81 g; ZnSO₄, 0.222 g; Na₂MoO₄, 0.39 g; CuSO₄·5H₂O, 0.079 g; Co(NO₃)₂·6H₂O, 49.4 g; Citric acid, 0.006 g; ferric citrate, 0.006 g; ampicillin, 50 µg/mL; distilled water, 1000 mL; agar, 20 g; trace element solution A5, 1 mL). All chemical reagents were analytical grade. All solutions were prepared by using deionized water.

Microalgae cultivation

Chlorella sp. was cultured in the BG-11 medium. The culture broth and the container were sterilized in an autoclave (1.2 kg/cm²). Under sterile conditions, the algae seeds were evenly inoculated on the medium (300 mL), transferred to a conical flask (500 mL) and placed at 25 (± 1) °C with a sterile breathable film sealing with the bottle mouth; then the whole bottle was illuminated under a LED growth lamp with a red to blue ratio of 5:1 for 24 h, shaken three times a day at regular time.

Shell calcining

The cleaned shells were immersed in 1% HCl for 24 h and calcined at 600 °C, 700 °C and 800 °C for 2 h by using vacuum tube furnace (SKGL-1200-II, SIOMM, Shanghai) and muffle furnace (SX2-5-12, TAISITE, Tianjin), and sieved to < 100 µm (average particle size: 75 µm) mesh particle size by micro-nano-pulverizer (MKCA6-2J, Masuko, Japan; *Table 1*).

Table 1. Experimental design

Temperature	Muffle furnace	Vacuum tube furnace	Modified by K ₂ CO ₃
600 °C	MM600 °C	MT600 °C	MMM600 °C
700 °C	MM700 °C	MT700 °C	MTM700 °C
800 °C	MM800 °C	MT800 °C	MTM800 °C

Activation of shell powder

0.5 g shell powder and 30 mL, 1% K₂CO₃ were dissolved to 50 mL tube, sonicated for 30 min by using an ultrasonic cleaning machine (G-040S, Geneng Cleaning Equipment Co., Ltd., Shenzhen, China, 600 w) and the sample was dissolved. The mixture was placed into a Teflon reactor at 160 °C for 24 h. After filtering, precipitation was washed to neutrality and dried at 60 °C for 8 h.

Modification by chitosan

Chitosan, a linear polymer with high specific surface area, has the dual functions of electric neutral flocculation and adsorption flocculation, non-toxic and harmless,

which is not only can provide nutrients for the growth of microalgae, but also conducive to the immobilization of microalgae.

100 mg chitosan and 10 mL HCl (1%) were dissolved in 100 mL deionized water. The chitosan mixed with activated shell powder with a mass ratio of 1:2, stirring for 1 h and vacuum drying at 80 °C for 24 h. The sample was marked as CS-M (Fig. 1).

Preparation of immobilized microalgae

100 mL *Chlorella sp.*, growing in logarithmic phase, 20 mL, 2% tea extract solution, 0.5 g CS-M, stirring for 30 min, standing in layers, removing as much supernatant as possible, adding 0.2 g Sodium alginate, 2 mL, 3% glycerol, stirring for 30 min, standing for 2 h, centrifugal separation, storage of the precipitate in sterile, sealed, dark conditions, storage temperature were 25 °C, 4 °C, -24 °C, respectively. 0.1 g CS-M and 1 mL steamed water, diluted 20 times, injected on slides, covered with coverslips. Optical microscopy (OM) was used to observe the combined situation of microalgae and shell powder under the ratio of 10 ×10 (ECLIPSE Ti, Nikon, Japan).

Determination of chlorophyll-a (chl.a) content in immobilized microalgae

5 mL algae were centrifuged at 5500 r/min for 15 min, collecting the precipitate, adding 5 mL, 80% acetone solution, extracting the chlorophyll-a in darkness at 4 °C for 24 h until the chl.a and acetone were fully mixed, centrifuge at 6000 r/min for 15 min. Finally, measuring the absorbance (OD) of supernatant by using UV-vis (UV-2600, SHIMADZU, Japan) at 663 nm and 645 nm wavelength respectively, with the reference solution of 80% acetone. All tests are repeated three times.

Reference calculation formula of chl.a concentration (Gao and Sun, 2000) is as follows:

$$\text{Concentration of chl. a (mg/L)} = 12.71 \times OD_{663} - 2.59 \times OD_{645}$$

OD₆₄₅, OD₆₆₃: OD of the extract at 645 nm and 663 nm wavelengths, respectively.

Test methods of microalgae cell density

Take algae fluid, which cultivated in the BG-11 medium for 24 h, 48 h, 72 h, 96 h, 120 h, and 162 h. The absorbance (OD₆₈₀) of the algae was measured at a wavelength of 680 nm. After fixing with Rochelle's solution, the *microalgae* was diluted and counted its number using a hemocytometer (Shanghai refinement Biochemical Instrument Co., Ltd., Shanghai, China). Repeat three times and take the average. Establish a standard curve of OD₆₈₀ and the number of *microalgae* cells.

Characterization

The specific surface area and porosity tester (BET) was used to analyze the specific surface area, and pore size distribution of shell powder under 77 k with N₂ is the analysis gas (Quadrascorb SI, Quantachrome, USA). The surface morphology of the sample was observed by using JSM-7800F ultra-high resolution thermal field emission scanning electron microscope (JEOL, Japan).

Statistical analysis

Statistical analysis was performed using Origin 8.5 (Origin Corp., USA). All the experiments were performed independently at least three times.

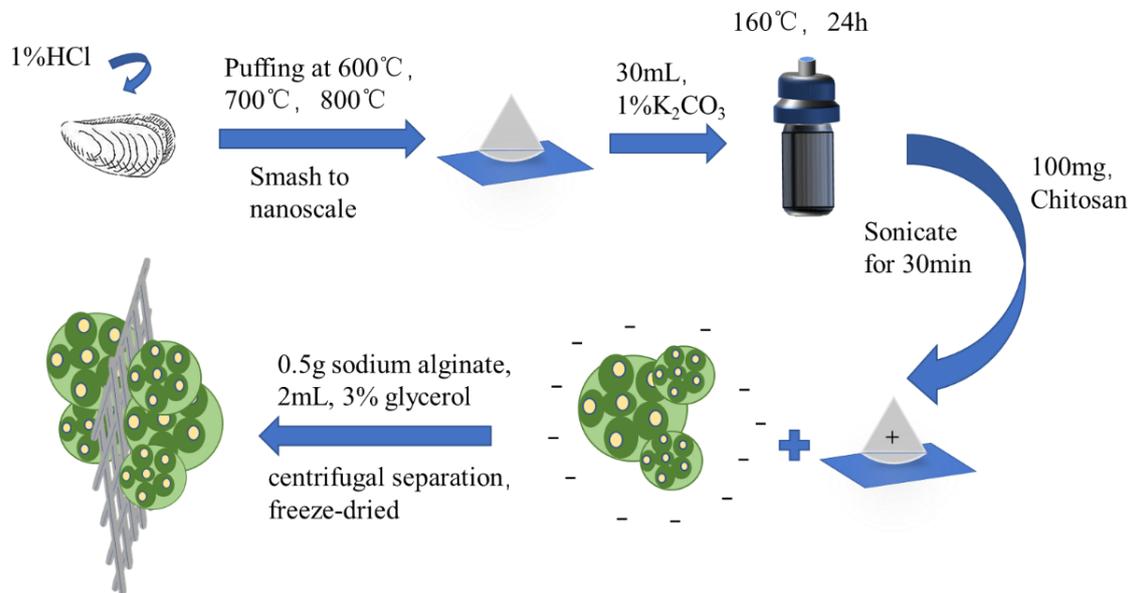


Figure 1. The schematic illustration of *Chlorella sp.* immobilized by nano mussel-shell powder and chitosan

Results and discussion

BET characterization

The curves of adsorption isotherms and pore diameter distribution of MM (mussel powder calcined by muffle furnace) and MT (mussel calcined by tube furnace) under 600 °C (a), 700 °C (b) and 800 °C (c) are as shown in *Figure 2*. The isothermal adsorption curves of MM and MT at 600 °C and 700 °C are similar, and all type III (Sing, 1985). The lateral interaction between the adsorbed molecules is greater than the interaction between the adsorbent surface and the adsorbate. Adsorption takes place in multiple layers, and the amount of adsorption increases with enhancement of pressure. While the specific surface area of MM is generally superior to MT under 600 °C, 1.308 m²/g vs 0.435 m²/g the average pore diameter of MT is about 5 nm, while that of MM is about 2 nm. The average pore diameters of MM and MT at 800 °C are not significantly different. The reason may be that the organic matter in shell powder has been completely decomposed since 700 °C. Adsorption isotherms of MM at 800 °C has a hysteresis loop, which belongs to type H₁, indicating that the pore distribution of the material is relatively narrow, the particle is spherical and its size is uniform. In general, the organic matter of shell powder will completely break down at 700 °C. According to the average pore size of each material, it can be proved that the shell powder will form a well-distributed, less porous mesoporous material after high-temperature calcination. The puffing effect of the tube furnace is slightly better than that of the muffle furnace (*Tables 2 and 3*).

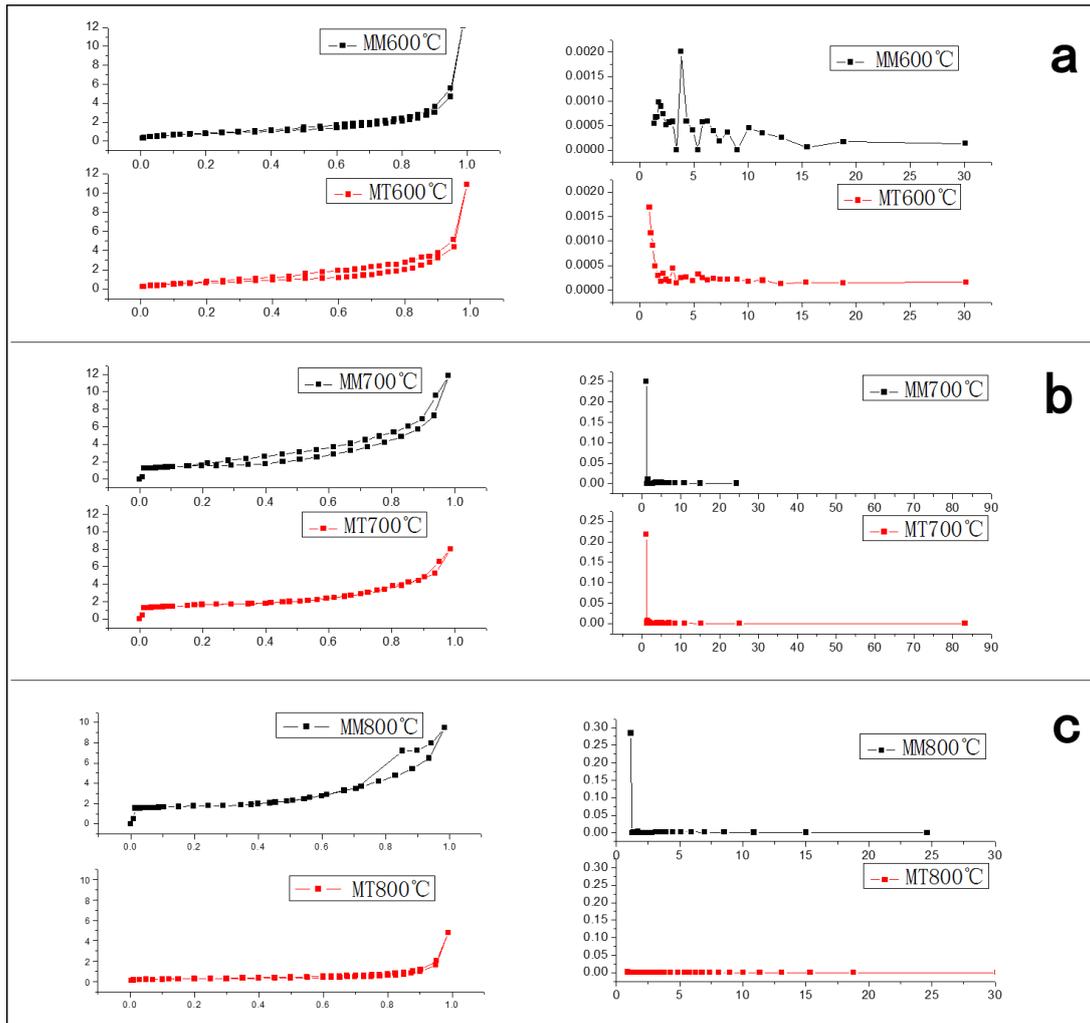


Figure 2. Adsorption isotherms and pore diameter distribution curves of MM and MT under different expansion temperatures (600 °C, a; 700 °C, b; 800 °C, c)

Table 2. The specific surface area and average pore size of MM

Temperature (°C)	600	700	800
Specific surface area (m ² /g)	1.308	5.538	12.45
Average pore size (nm)	7.627	11.1078	4.9343

Table 3. The specific surface area and average pore size of MT

Temperature (°C)	600	700	800
Specific surface area (m ² /g)	0.435	1.2	0.965
Average pore size (nm)	3.406	4.107	3.177

The curves of adsorption isotherms and pore diameter distribution of MMM (MM modified by K₂CO₃) and MTM (MT modified by K₂CO₃) are as shown in Figure 3,

whose calcined condition is 600 °C, 700 °C, 800 °C. All the adsorption isotherms of MTM are type IV (Sing, 1985), similar to type III at first, but at higher pressures, the adsorption increase rapidly with the hole is condensed in capillary. However, the hysteresis loops appeared on the adsorption isotherms of MMM, regardless of temperature, which belongs to type H₁. Comparing the surface area and average pore size of shell powder before and after modifying with K₂CO₃, we found that the average pore diameter of the shell powder, which calcined in the tube furnace, is greatly improved. The average pore size of shell powder increased from 3.406 nm to 15.176 nm at 600 °C, 4.107 nm to 16.557 nm at 700 °C, and 3.177 nm to 7.804 nm at 800 °C. But with the decrease of pore numbers, the specific surface area has reduced. The calcined shell powder in the muffle furnace does not have much difference before and after modification. We chose MTM (700 °C) as the best sample (Tables 4 and 5).

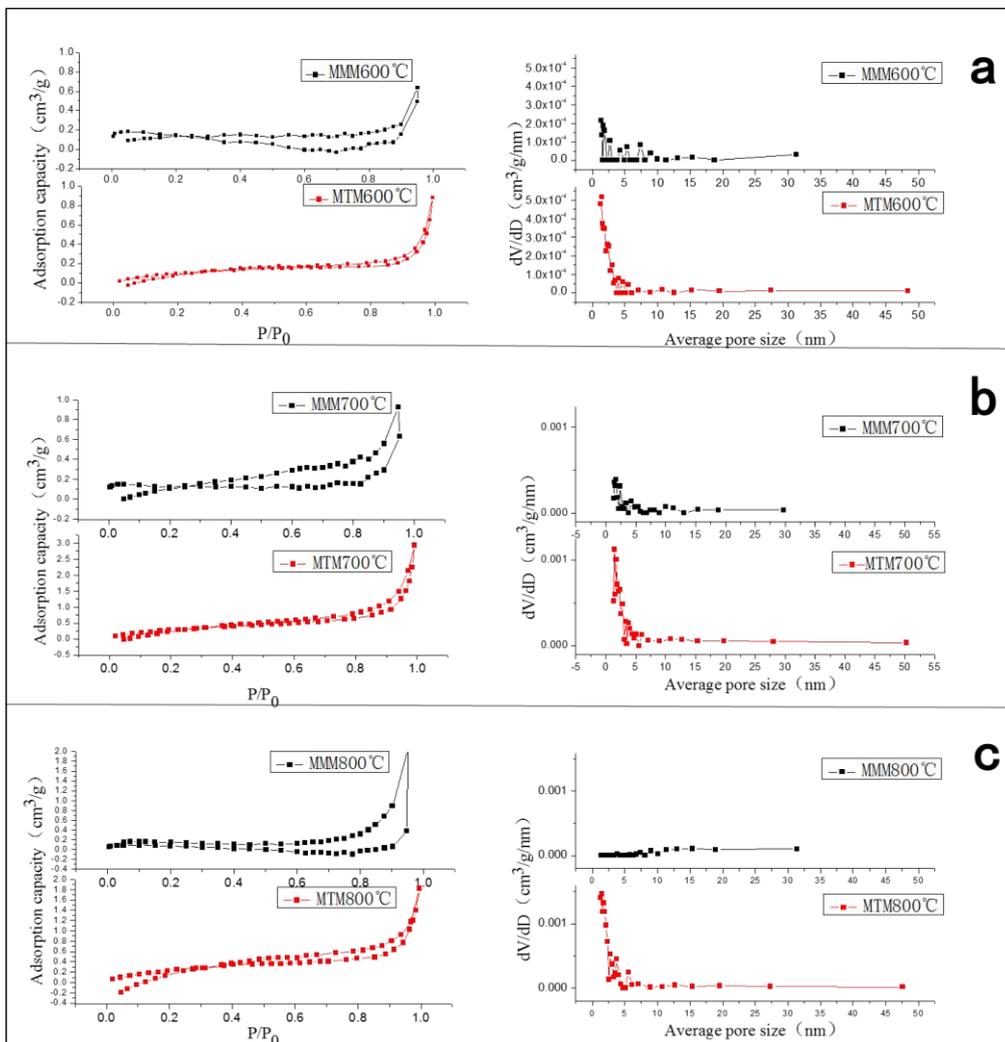


Figure 3. Adsorption isotherms and pore diameter distribution curves of MMM (MM modified by K₂CO₃) and MTM (MT modified by K₂CO₃) under different expansion temperatures (600 °C, a; 700 °C, b; 800 °C, c)

There are few reports on the use of biomass materials as carriers to immobilize microalgae. Shell powder, an excellent porous material, immobilizes microalgae by

means of charge attraction and physical adsorption, has strong immobilization power and can provide certain nutrients to microalgae and prolong its life.

Table 4. The specific surface area and average pore size of MMM

Temperature (°C)	600	700	800
Specific surface area (m ² /g)	2.894	5.4272	0.888
Average pore size (nm)	7.086	12.600	9.019

Table 5. The specific surface area and average pore size of MTM

Temperature (°C)	600	700	800
Specific surface area (m ² /g)	0.336	0.364	0.123
Average pore size (nm)	15.176	16.557	7.804

SEM characterization

Two SEM photographs of M (Mussel calcined at 700 °C in a vacuum environment), MTM as shown in *Figure 4*, the texture of M is loose, and there are several pores on the surface, with the pore diameter is about 0.5 μm, which determines the excellent adsorption performance of shell powder. The size of pores in MTM is increased, compared with M, which is 1 μm, because 1% potassium carbonate and water vapor have the good pore-forming ability, enhancing adsorption and sustained release capabilities of shell powder.

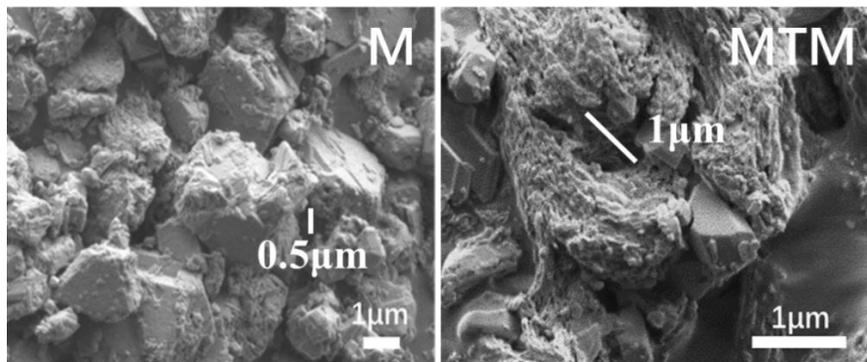


Figure 4. SEM images of M and MTM

OM characterization

Photomicrographs of immobilized microalgae under a bio-optical microscope are shown in *Figure 5*. It can be seen that the diameter of shell powder is about 200 μm, and the chlorella sp. cells are about 10 μm with a partial shell powder stacked. A large number of *Chlorella sp.* cells accumulate around the shell powder, indicating that modification of the shell by CS increased the “fixed” effect of the shell on the microalgae. It is beneficial to the dispersion of microalgae during the use of the immobilized microalgae. Sodium alginate also has a certain reinforcement and moisturizing effect.

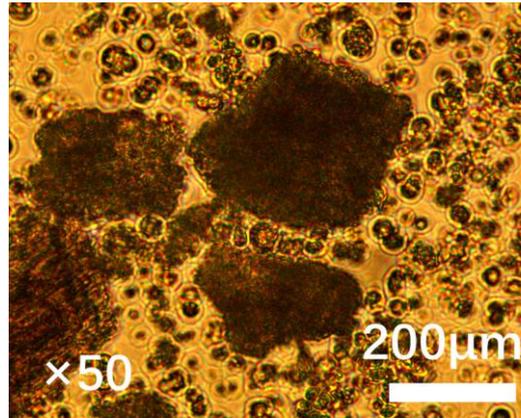


Figure 5. OM images of immobilized microalgae

The growth of immobilized microalgae

Growth curve of *microalgae* was determined in Figure 7a, during the preservation process, the chl.a content of all samples in the first 20 days changed slowly, however, after 20th day, decreased linearly. Loss rate of chl.a in immobilized microalgae after 60 days are respectively 59.04%, 50.22%, 42.77%, with the content of chl.a are respectively 3.41778 mg/L, 4.72185 mg/L, 5.31715 mg/L, which preserved in 25 °C, 4 °C and -24 °C. The changes of chl.a content in immobilized microalgae at 4 °C and -24 °C are similar. However, it can be observed that there was a greatly decrease from 30th to 40th days at 25 °C, from 6.88823 mg/L to 4.26672 mg/L, which is the best growth temperature of *microalgae*, with a highest activity, but it can only perform respiration and cannot synthesize chl.a due to the dark environment. The nutrients provided by shell powder are limited. Over time, *microalgae* lacks nutrient intake, resulting in the death of a large number of *microalgae* and the loss rate of chl.a increased rapidly. The loss rate of chl.a was the lowest after 60 days, which kept at 24 °C, 42.77%, probably because of the low activity of *Chlorella sp.* at low temperature. In general, *microalgae* can survive 60 days in immobilized *microalgae*, which is rarely founded in recent research (Fig. 6 and Table 6).

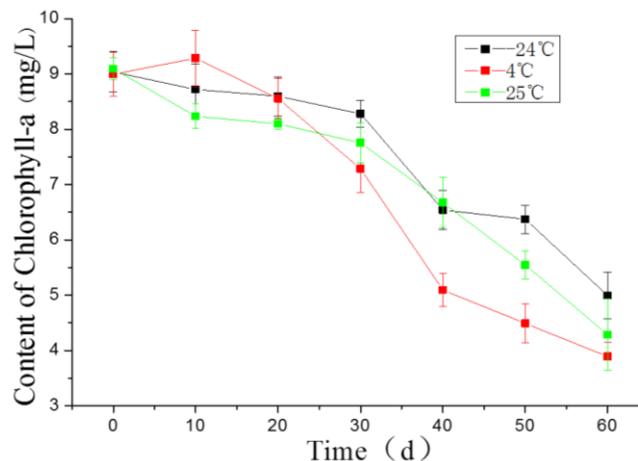


Figure 6. The curve of microalgae growth within 60 days under different storage conditions (-24 °C, 4 °C, 25 °C)

Table 6. The loss rate of chl.a (%) during 60 days in immobilized microalgae

Storage conditions (°C)	25	4	-24
Loss rate of chlorophyll-a (%)	59.04	50.22	42.77

Regrowth of immobilized microalgae

Regrowth curve of *microalgae* was determined in *Figure 7b*, the reactivation rate of *microalgae* in the immobilized microalgae at 4 °C is the fastest and the highest, with the cells number is up to 3.41769×10^6 cell/mL and the recovery rate is 26% (*Table 7*). The curves of *Chlorella sp.* cells number in immobilized microalgae are similar at 4 °C and -24 °C, which have a rapid recovery period of microalgae from the 15th day to 19th day, and reaching the stable at the 9th day. However, the overall growth rate is slow at 25 °C. The reason may be the ultimate mortality of *Chlorella sp.* is high in the preservation process of immobilized microalgae, at 25 °C. Generally, the resuscitation curve of microalgae in immobilized microalgae was consistent with the Logistic growth curve.

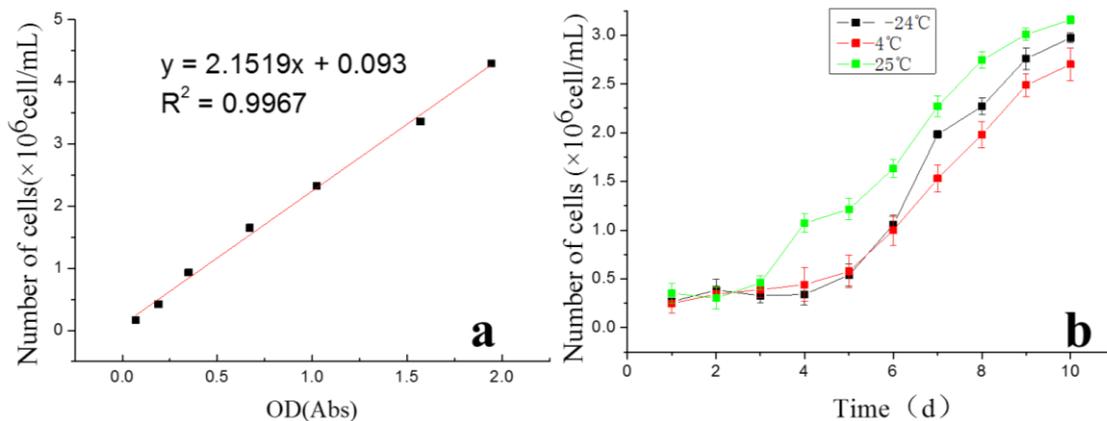


Figure 7. **a.** The stand curve of microalgae cells numbers. **b.** The regrowth curve of microalgae within 10 days under different storage conditions (-24 °C, 4 °C, 25 °C)

Table 7. Recovery growth rate of microalgae (%) during 60 days

Storage conditions (°C)	25	4	-24
Microalgae specific growth rate (%)	23	26	26

Conclusion

In summary, it was found that the shell powder calcined at 700 °C in a vacuum tube furnace and hydrothermal modification with K_2CO_3 has the optimum pore structure with an average pore size of 16.557 nm. The OM picture of CSM clearly shows that the *Chlorella sp.* is fixed with chitosan-modified shell powder, whose surface is positive. This structure can extend the shelf life of *Chlorella sp.* to 60 days and the best sample of the resurrection rate is 26%, the number of cells is 3.41769×10^6 cell/mL, reviving at 4 °C. With the rapid development of industrialization, this material has bright prospects. This is a new technology for immobilizing microalgae that can be used in water

purification, energy harvesting and other fields, not only has the great significance for environmental protection but also for abandoned resources reuse.

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