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The High Additional Value Products and Antimicrobial Activity of *Chlorella protothecoides*

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Microalgae are a potential biodiesel feedstock, as well as an important source of many high value-added of biological products. In this work, lutein, polysaccharide and lipid of *Chlorella protothecoides* were studied to improve the comprehensive utilization value. The lutein in *Chlorella protothecoides* was mostly in the form of free lutein, rather than lutein esters. It could maximally improve the comprehensive utilization value of *Chlorella protothecoides* in the extraction sequence of lutein> polysaccharide> lipid with lutein extraction ratio 66.21%, polysaccharide content 6.45% and lipid content 37.26%. Under optimal extraction conditions (ultrasonic power 280 W, ultrasonic broken time 10 min, solid to liquid ratio of 1:40 (w/v), and extraction 3 times), the lutein extraction ratio and content were 94.59% and 0.012%, while polysaccharide content was 6.43% and lipid content was 33.56%. The polysaccharide of *Chlorella protothecoides* had the highest antimicrobial activity against *Escherichia coli* with inhibition halo diameter 15.84 mm, and weak antimicrobial activities against *Bacillus subtilis* and *Pseudomonas aeruginosa*. The fatty acid composition of *Chlorella protothecoides* had no significant change after the extraction of lutein.

Key words: Lutein, Polysaccharide, Lipid, Orthogonal array design, Antimicrobial activity, Fatty acid composition.

Since the industrial revolution, the increased consumption of energy leads to a massive influx of carbon into the atmosphere mediated through the burning and consumption of petroleum-based products. As a potential substitute of petroleum, renewable biofuels such as ethanol, butanol, hydrogen, methane and biodiesel have drawn a lot of attention[1].

In recent years, technological developments for large-scale production of algae have gained commercial relevance. The microalgae have many advantages such as its high photosynthetic efficiency, fast growth rate, no restriction of season and climate, easy to realize the large-scale production and so on. Currently, the market for microalgae is mostly based on two applications. The production of biomass and the marketing of high added-value products obtained from them. Cultivation of extremophile microorganisms has gained interest due to their ability to accumulate and produce high value molecules, such as, enzymes, metabolites and surfactants[2]. Microalgae are among the most potentially significant source of sustainable biofuels in the future of renewable energy[3].

Since microalgae are still not competitive to be biofuels, the biomass after lipid extraction should be valorized in a biorefinery context. It is important that the remaining coproducts have a useful and safe purpose for the economic feasibility[4]. Compounds such as PUFAs[5], carotenoids[6], protein, antioxidants, astaxanthin[7], phycobiliproteins[8], dinoflagellate toxins, active polysaccharides[9] and bioactives in general, for food, feed, cosmetics, and medical industries, are well quoted and, consequently, could turn the process economically feasible.

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Carotenoids with a 40-carbonbackbone that contains a large conjugated double-bond system play a key role in photosynthesis and photoprotection[10]. Lutein is one of the carotenoids most widely distributed in frequently consumed fruits and vegetables. Humans are not capable of synthesising carotenoids de novo, and thus, their presence in human tissues is entirely of dietary origin. The current commercial source of pure lutein is marigold. However, this producing source presents some disadvantages, including the labor-intensive extraction process and the extremely low lutein content of the plants (as low as 0.03%)[11]. In recent years, microalgae have been viewed as a promising alternative to produce lutein, such as, *Muriellopsis* sp[12], *Scenedesmus* sp.[13], *Chlorella zofingensis*[14], and *Chlorella protothecoides*[15]. In the autotrophic cultivation of *Scenedesmus* for lutein production, the lutein content was generally less than 0.5% (5 mg lutein/g dry cell weight). The best results of heterotrophic *Cp* depicted by several authors revealed a high production of lutein (0.53 %)[16] and fatty acids (23 %)[17], suitable for biodiesel production.

Microalgae can be used to produce a variety of polysaccharides, which have significant biological activity. Since the earliest screening research of algae antibacterial activity by Pratt (1951), many scholars in the world have carried out extensive screening of the algae antibacterial activity, and they have found a lot of seaweed extracts that can inhibit bacteria and fungi. Based on these studies, the further researches find that the antibacterial compounds are mainly lipid and fatty acid, polysaccharide and terpenoid, etc. Padmakumar and Ayyakkannu[18] reported that the antibacterial activity of the algae extracts was greater than antifungal activity. The experimental results showed that the microalgae extract had inhibition to gram positive bacteria rather than gram negative bacteria[19].

At present, most of the researches focus either on the microalgae lipid, lutein or polysaccharide production, and only few studies combine these fields together. In order to improve the comprehensive utilization value of microalgae and reduce production cost, these two or more high value coproducts are necessary to study together. In this paper, the lutein, lipid and polysaccharide content of *Chlorella protothecoides* were studied; the extraction of lutein was optimized. In order to achieve the best comprehensive utilization value, the extraction sequence of lutein, lipid and polysaccharide was discussed. The antimicrobial activities of polysaccharide were made a preliminary exploration.

MATERIALS AND METHODS

Dry algae powder of *Chlorella protothecoides* was provided by Professor Qingyu Wu of Tsinghua University.

Bacillus subtilis (SCTCC 100034), *Staphylococcus aureus* (SCTCC 100048), *Staphylococcus albus* (SCTCC 100044), *Escherichia coli* (SCTCC 100005), *Salmonella typhimurium* (SCTCC 100403), and *Pseudomonas aeruginosa* (SCTCC 100200) were provided by Sichuan Type Culture Collection.

The extraction and saponification of lutein

0.1g dry algae powder was accurately weighed, adding extraction solvent, ultrasonic broken, centrifugation at 6,000 rpm for 10 min, collecting the supernatant, constant volume, then the content of lutein was determined. The entire extraction process was carried out in low light, no more than 30min, in order to minimize the loss of pigment.

The saponification agent was added to the supernatant collected after ultrasonic treatment for saponification reaction: 20% NaOH solution as saponification agent extract to saponification agent ratio of 4:1(v/v), saponification temperature 50°C, saponification time 5 h. After the reaction, constant volume, the content of lutein was determined.

The conditions of HPLC

The concentration of lutein was determined by HPLC. The conditions of HPLC were: column, Nova-PakC[18] (4.6 mm×l50 mm, 5 µm); temperature, 25 °C; mobile phase A, methanol/ acetonitrile/water (80:10:10, v/v/v); mobile phase B, methanol/acetonitrile (40:60, v/v); flow rate, 0.8 ml/min; sample size, 10 µl. The gradient elution program was presented in Table 1.

The extraction of polysaccharide

Ultrasonic-associated hot water extraction was used to extract polysaccharide: extraction medium of NaOH (4%), solid to liquid ratio of 1:25 (w/v), ultrasonic extraction 10 min,

ultrasonic power 200 W, ultrasonic broken for two times at the same conditions. Then, it was heated in a water bath at 70 °C for 180 min, immediately centrifuged for the supernatant. The supernatant was precipitated by ethanol of 3 times volume, kept overnight, centrifuged for precipitation, then adding 3% TCA, fully stirring, until no more precipitate dissolved, repeating the above operation, while the precipitation was the coarse polysaccharide.

In this experiment, anthrone-sulfuric acid colorimetric method was used to determine the polysaccharide concentration[20].

Lipid extraction

Acid-heating extraction was used to extract the lipid: dry algae powder weighed, adding 4 mol/L hydrochloric acid, oscillation and mixing, standing at room temperature for 30 min; a boiling water bath for 10 min, then, quickly transferred to a low-temperature refrigerator for 20 min, the process of freezing and thawing was repeated three times. About 2 times the volume of chloroform: methanol (1:1, v/v) extract was added, oscillation and mixing, centrifugation, drawing the lower chloroform layer into the new container, dried in vacuum.

The optimization of extraction conditions of lutein

The single-factor tests were carried out with the four most important variables which were set as follows: ultrasonic power (160 W, 200 W, 240 W, 280 W, 320 W); ultrasonic broken time (5 min, 10 min, 15 min, 20 min, 25 min); solid to liquid ratio (1:20, 1:30, 1:40, 1:50, 1:60, w/v); extraction times (1, 2, 3, 4).

The extraction of lutein was optimized by an L_9 (3^4) orthogonal array design (OAD) [21] with four factors at three levels on the basis of single-factor tests.

Analysis of fatty acid composition

Gas chromatography (MADZU GC-14B) equipped with 30 m DB-5 capillary column was used for qualitative and quantitative determination of fatty acid composition. The oven temperature program started at 150 °C, increase by 4°C min^{-1} until 250°C, maintained for 2 min. Carrier gas, N_2, was kept at a constant rate of 15 mL/min. Injector and detector (flame ionization) temperature were kept at 250 °C[22]. The fatty acid methyl ester (FAME) was prepared by KOH-methanol methyl esterification method at room temperature[23]. The contents of the fatty acids composition were calculated by area normalization method.

The antimicrobial activity of polysaccharide *in vitro*

The polysaccharide was dissolved with sterile distilled water, filtration sterilization, split charging, and cryopreservation.

Antimicrobial activity was determined against the above bacteria using the paper disk assay method[24]. Filter paper disk of 6-mm diameter was sterilized by autoclaving for 20 min at 121 °C. Agar plates were surface inoculated uniformly from the culture of the tested microorganisms. The sterile disks impregnated with the polysaccharide solution were placed aseptically on the seeded agar plates. Control was made with the sterile distilled water. The plates of bacteria were incubated at 37 °C for 24 h. Inhibition results were expressed as width of the clear halo surrounding each disc on cultivated agar plats. All the assays were carried out in triplicate.

Date calculation and statistical analysis

The lutein concentration *Cs* (mg/L) of the sample extract was calculated according to the regression equation of the standard curve.

Lutein content (%) = *Cs* (mg/L) ×extract volume (L) /W_D(mg) × 100%

Polysaccharide content (%) = W_P (g) /W_D(g) × 100%

Lipid content (%) = W_L (g) /W_D(g) × 100%

Where W_P and W_L are the polysaccharide content and lipid weight and W_D is the weight of the corresponding dry algae.

The extraction ratio of lutein: multiple extractions until the sample was colorless under the optimum extraction conditions, merging all the extracts, constant volume, and the lutein content was determined as complete extraction. The extraction ratio was determined by lutein content of each experiment comparing to the complete extraction.

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

Statistical analysis was done using the SPSS software package. One way analysis of variance (ANOVA) was used to establish whether the difference among the three groups was statistically significant. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of saponification on the extraction ratio of lutein

In nature, Lutein can exist in the form of free lutein and lutein esters. Body can directly absorb free lutein, but the lutein esters must be hydrolyzed into lutein in vivo in order to be absorbed. Research has shown that, lutein exists in the form of lutein esters in some *Chlorella*, and the strong alkaline substances are needed for saponification reaction which can transform lutein esters into lutein[25].

The extraction ratio of lutein basically had no change after the saponification reaction which indicated that the lutein in *Chlorella protothecoides* was mostly in the form of free lutein, rather than lutein esters (Table 2). Therefore, the

Table 1. The Program of Gradient Elution

Time(min)	Mobile phase A (%)	Mobile phase B (%)
0	75	25
5.00	60	40
8.00	0	100
12.00	0	100
13.50	75	25
20.00	75	25

Table 2. The extraction ratios of lutein before and after the saponification reaction

	Before saponification reaction	After saponification reaction
Lutein extraction ratios (%)	66.21±1.89	66.26±1.92

Table 3. The lutein extraction ratio, polysaccharide and lipid content under different extraction sequences

Extraction sequence	Lutein extraction ratio (%)	Polysaccharide content (%)	Lipid content (%)
Lipid> Lutein> Polysaccharide	negligible	negligible	43.75±1.28
Polysaccharide> Lutein> Lipid	negligible	6.82±0.28	37.97±1.25
Lutein> Polysaccharide> Lipid	66.21±2.14	6.45±0.23	37.26±1.16

Table 4. Orthogonal array design and results for the four variables studied

Run	Ultrasonic power (W)	Ultrasonic broken time (min)	Solid to liquid ratio (w/v)	Extraction times	Extraction ratio (%)
1	240	5	1:30	1	46.57±0.61
2	240	10	1:40	2	80.81±0.74
3	240	15	1:50	3	91.74±0.68
4	280	5	1:40	3	93.57±0.69
5	280	10	1:50	1	56.86±0.70
6	280	15	1:30	2	86.67±0.62
7	320	5	1:50	2	70.52±0.57
8	320	10	1:30	3	89.67±0.77
9	320	15	1:40	1	43.54±0.82
K_1	219.12	210.66	222.91	146.97	
K_2	237.10	227.34	217.92	238.00	
K_3	203.73	221.95	219.12	274.98	
1	73.04	70.22	74.30	48.99	
2	79.03	75.78	72.64	79.33	
3	67.91	73.98	73.04	91.66	
R	11.12	5.56	1.66	42.67	

saponification reaction was not required for the process of lutein extraction.

Effects of extraction sequence on lutein, polysaccharide and lipid extraction

The lipid content could reach 43.75%, but the lutein and polysaccharide were so low that could be negligible in the case of lipid extraction firstly. When polysaccharide was extracted firstly, the polysaccharide and lipid contents were 6.82% and 37.97%, but the lutein was negligible. The extraction ratio of lutein was 66.21% under the condition of lutein extraction firstly, while the polysaccharide and lipid contents had a little decline which were still 6.45% & 37.26 % (Table 3).

Table 5. An ANOVA table for normalized experimental responses in the L_9 (3^4) matrix

Source	Sum of squares	df	Mean square	*F*-value	Sig.
Ultrasonic power	371.931	2	185.965	48.135	0.000
Ultrasonic broken time	96.609	2	48.304	12.503	0.003
Solid to liquid ratio	9.045	2	4.523	1.171	0.353
Extraction times	5786.787	2	2893.393	748.924	0.000
Error	34.771	9	2.261		

Table 6. Antimicrobial activities of polysaccharide *in vitro*

Strains	Inhibition halo diameter (mm)
Escherichia coli	15.84±0.48
Salmonella typhimurium	
Pseudomonas aeruginosa	8.38±0.35
Staphylococcus aureus	
Staphylococcus albus	
Bacillus subtilis	7.36±0.29

Table 7. Fatty acid composition of *Chlorella protothecoides* before and after the extraction of lutein

Fatty acid (% of total fatty acid)	Sample	
	before lutein extraction	after lutein extraction
C14:0	0.17	0.18
C15:0	0.1	0.13
C16:0	6.15	4.77
C16:1	1.28	1.13
C17:0	0.69	1.06
C18:0	8.07	6.07
C18:1	65.18	70.8
C18:2	17.57	15.31
C19:0	0.06	0.28
C19:1	0.37	
C20:0	0.33	0.27
C22:0	0.03	
Saturated	15.6	12.76
Unsaturated	84.4	87.24

Lutein can dissolve in lipid and fatty solvents. Under the condition of lipid extraction firstly, lutein would dissolve in lipid and organic solvent in the long process of lipid extraction, resulting in the significant loss of lutein; meanwhile, lutein was unstable and easy to see light decomposition [26], which would also result in the loss of lutein in this process. In addition, the cells were ruptured in the process of repeated freezing and thawing which was conducive to the dissolving of lipid, but polysaccharide could also dissolve in hydrochloric acid at the same time which resulted in the loss of polysaccharide.

Lutein would be loss when polysaccharide was extracted firstly, because it could be decomposition in the long process of polysaccharide extraction. Lutein dissolved in lipid would not occur under the condition of lutein extraction firstly; polysaccharide couldn't be loss because it didn't dissolve in organic solvent; however, a small amount of lipid would dissolve in organic solvent in the short process of lutein extraction, resulting in a little loss of lipid.

Therefore, it could maximally improve the comprehensive utilization value of *Chlorella protothecoides* in the sequence of lutein> polysaccharide> lipid.

Effects of different solvents on the extraction of lutein

The methanol-dichloromethane mixed solvent had the best extraction effect, followed by methanol, and the effects of petroleum ether and

n-hexane were the worst (Fig.1). In addition, the volume ratio of methanol and dichloromethane also influenced the extraction of lutein. It could be seen that the extraction effect was the best when the volume ratio of methanol and dichloromethane was 2:1(Fig.1).

This is because that the polarity of lutein is stronger, so the extraction effect of stronger polarity solvent is better than the weaker polarity solvent, which is similar to the principle of like dissolves like.

The lipid contents of the remaining algae after the lutein extraction with different solvents were also shown in Fig.1. It could be seen that the lipid contents would have a certain degree of loss, but the level of the loss had no significant difference.

In conclusion, the extraction effect of lutein was the best when the extraction solvent was methanol-dichloromethane (2:1, v/v), and the lipid content of the remaining algae maintained at a very high level. Polysaccharide couldn't be loss because it didn't dissolve in organic solvent and the data were not shown in figure. Therefore, it was chosen as the extraction solvent of lutein.

The optimization of extraction conditions of lutein

With the increase of ultrasonic power, the extraction effect was the better (Fig.2A). The extraction ratio of lutein reached to a plateau when the ultrasonic power was 280 W. This was because that the higher the ultrasonic power, the better the crushing effect of cell walls, which was conducive to the dissolution of lutein. The polysaccharide content had no significant change with the increase of ultrasonic power, while the lipid content of the remaining algae had a little reduction with the increase of the extraction ratio of lutein. The loss of lipid would be reduced when the crushing effect was ineffective. Therefore, the best ultrasonic power was 280 W.

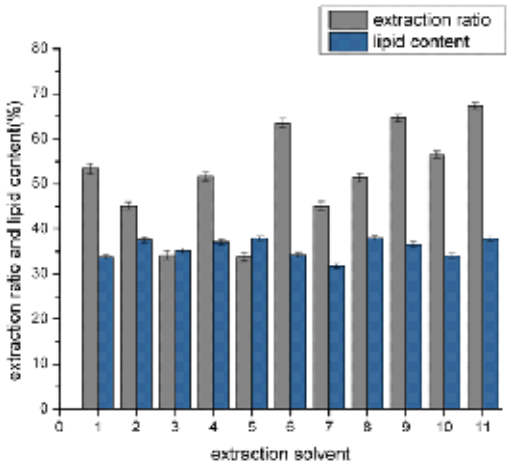

Fig. 1. The effects of different solvents on the extraction of lutein and the lipid content of the remaining algae

1: ethanol; 2: diethyl ether; 3: petroleum ether; 4: acetone; 5: n-hexane; 6: methanol; 7: dichloromethane; 8: n-hexane-ethanol (1:1, v/v); 9: methanol-dichloromethane (1:1, v/v); 10: methanol-dichloromethane (1:2, v/v); 11: methanol-dichloromethane (2:1, v/v).

The extraction effect was the best of 66.29% when the broken time was 10 min, but then it was decreased (Fig.2B). This was probably because that the crushing of cell walls was enough and lutein could also be sufficiently dissolved in extraction solvent when the broken time was 10 min, but lutein might be decomposed with the increase of time. Therefore, the broken time shall be controlled within 10 min. The polysaccharide content also reached the maximum when the broken time was 10 min. The lipid content of the remaining algae had a little reduction with the increase of ultrasonic broken time. The loss of lipid would be reduced when the crushing effect was ineffective, but the longer broken time could cause the loss of lipid. Therefore, the best broken time was 10 min.

The extraction ratio was 70.9% when the solid to liquid ratio was 1:40 (w/v), and then the extraction ratio had no significant increase (Fig.2C). The excessive extraction solvent would cause the increase of the cost, resulting in waste. Therefore, it was appropriate to choose solid to liquid ratio 1:40 (w/v). In addition, the polysaccharide and lipid content of the remaining algae had no significant change with the increase of extraction solvent. So, 1:40 (w/v) was chosen as the solid to liquid ratio.

The extraction ratio of lutein was 92.59% after three times extraction, and then it only increased 1.03% with the increase of extraction times (Fig.2D). The effect was not obvious in this case, but it would increase the solvent consumption and the cost. The polysaccharide content had no significant change with the increase of extraction times. With the increase of extraction times the loss of lipid was increased. It is inevitable, because there would be a small amount of lipid dissolved in the extraction

solvent with each extraction of lutein.

After implementing 9 experimental trials which were designed according to the L_9 (3^4) orthogonal array design (OAD), the corresponding results of output response for each experimental trial were calculated and listed in Table 4. To verify whether the effect of individual factors on the extraction of lutein was statistically significant, the analysis of variance (ANOVA) was used to interpret the experimental data obtained from the OAD optimization. The significance of each factor was evaluated by calculating the *F*-value and the results were summarized in Table 5.

The influence by the parameters on the extraction of lutein decreased with the order of : extraction times, ultrasonic power, ultrasonic broken time and solid to liquid ratio according to the *F*-values and three factors were all statistically significant ($p<0.05$) (Table 5). The factors of extraction times and ultrasonic power were statistically the most significant at $p<0.01$ while the factor of ultrasonic broken time was statistically significant at $p<0.05$. Compared to other experimental variables there was no significant difference in solid to liquid ratio.

Based on the above mentioned discussion, and considering the extraction ratio, the cost and the feasibility of experiment, the optimum conditions of extraction were therefore determined as follows: ultrasonic power 280 W, ultrasonic broken time 10 min, extraction times 3, and solid to liquid ratio of 1:40 (w/v). Under the optimum conditions, the extraction ratio and content of lutein were 94.59% and 0.012%, while the polysaccharide content was 6.43% and the lipid content of the remaining algae was 33.56%.

The antimicrobial activities of polysaccharide *in vitro*

The polysaccharide of *Chlorella protothecoides* had different inhibition against the tested bacteria *in vitro* (Table 6). Among them, polysaccharide had the highest antimicrobial activity against *Escherichia coli* and the inhibition halo diameter was 15.84 mm. In addition, it had weak antimicrobial activities against *Bacillus subtilis* and *Pseudomonas aeruginosa*. However, there was no inhibition against *Salmonella typhimurium*, *Staphylococcus aureus* and *Staphylococcus albus*. The results showed that polysaccharide from *Chlorella protothecoides* had

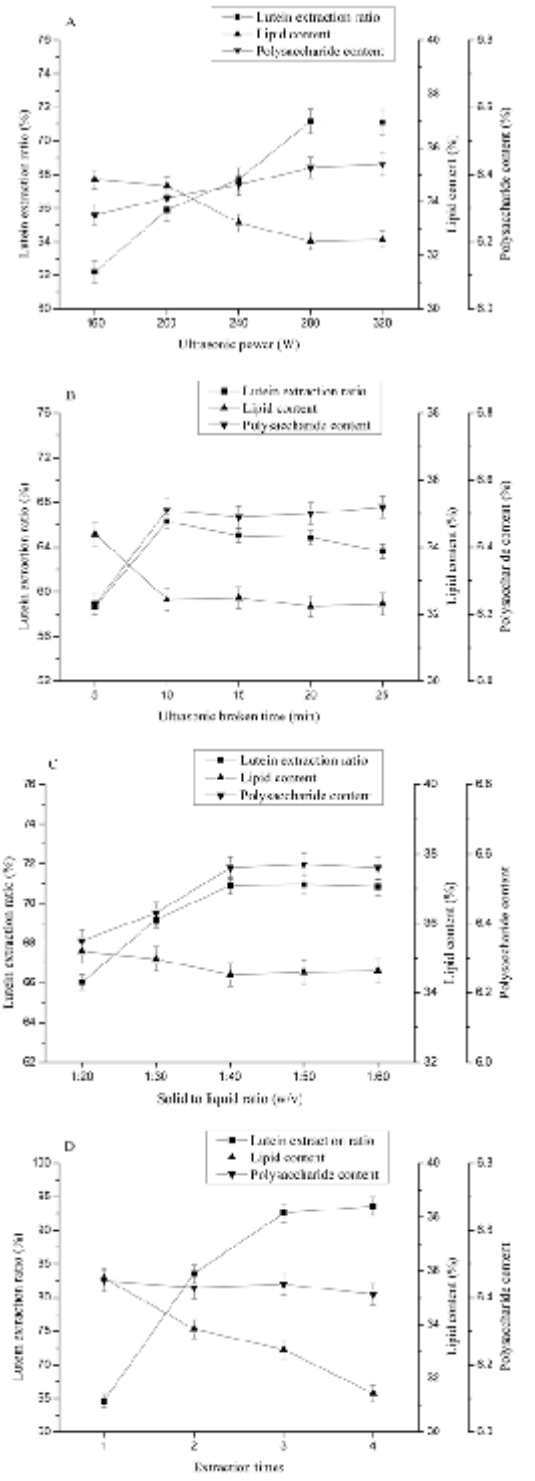

Fig. 2. Effects of ultrasonic power (A), ultrasonic broken time (B), solid to liquid ratio(C) and extraction times (D) on the extraction of lutein, and the polysaccharide and lipid content of the remaining algae

stronger inhibition against gram negative bacteria than gram positive bacteria.

Some studies have shown that the extracts in organic solvent of microalgae had antimicrobial activity. The antibacterial and antifungal activity can be different due to the type of extract and different microalgae. The antimicrobial activity of microalgae may be influenced by some factors such as the habitat and the season of algal collection, different growth stages of the algal, experimental methods.

Fatty acid composition of *Chlorella protothecoides*

The gas chromatography was used to analyze the fatty acid composition. The fatty acids of the lipid before the extraction of lutein were mainly C16 (7.43%) and C18 (90.82%); it was rich in unsaturated fatty acids, accounting for 84.4% of the total fatty acids, in which C18:1 and C18:2 were 65.18% and 17.57% respectively. The fatty acids of the lipid after the extraction of lutein were also mainly C16 (5.9%) and C18 (92.18%); the unsaturated fatty acids accounted for 87.24% of the total fatty acids, in which C18:1 and C18:2 were 70.8% and 15.31% respectively (Table 7). The results showed that the fatty acid composition of the lipid before the extraction of lutein had no obvious change, in other words the effect of lutein extraction on the fatty acid composition was no significant.

According to the quality standards of biodiesel from European Standards[27], the linolenic acid (C18:3) content has a limit of 12% for a quality biodiesel. As shown in Table 6, there was no linolenic acid in the lipid.

CONCLUSION

The lutein in *Chlorella protothecoides* was mostly in the form of free lutein, rather than lutein esters. It could maximally improve the comprehensive utilization value of *Chlorella protothecoides* in the extraction sequence of lutein> polysaccharide> lipid with lutein extraction ratio 66.21%, polysaccharide content 6.45% and lipid content 37.26%. The extraction effect of lutein was the best when the extraction solvent was methanol-dichloromethane (2:1, v/v), and the lipid content of the remaining algae maintained at a very high level. Under the optimal extraction condition (ultrasonic power 280 W, ultrasonic broken time 10 min, solid to liquid ratio of 1:40 (w/v), and extraction 3 times), the extraction ratio and content of lutein were 94.59% and 0.012%, while polysaccharide content was 6.43% and lipid content of the remaining algae was 33.56%. The polysaccharide of *Chlorella protothecoides* had the highest antimicrobial activity against *Escherichia coli* with inhibition halo diameter 15.84 mm, and weak antimicrobial activities against *Bacillus subtilis* and *Pseudomonas aeruginosa*. There was no significant change of the fatty acid composition after the extraction of lutein, which was rich in unsaturated fatty acids, accounting for more than 80% of the total fatty acids, in which C18:1 and C18:2 were more than 65% and 15%. The saturated fatty acids were mainly C16:0 and C18:0, which were about 10% of the total fatty acids.

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