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Purification of *Dunaliella salina* and Effects of Associated–Bacteria on its Physiological and Biochemical Characteristics

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Investigations of microalgae-bacteria relationship are important to studies of microbial ecology, red tide, aquaculture and pollutant degradation, etc. To date, microalgae-bacteria interactions are still not well understood, and purification of eukaryotic microalgae is also rarely mentioned. Here, bacteria-free *Dunaliella salina* was obtained with optimized antibiotic treatments. Axenic microalgal growth did not differ significantly with non-axenic algae in *f*/2 medium. However, when the contents of nitrogen and phosphorus were 10-fold higher of *f*/2, or there were no vitamins or trace elements in *f*/2 medium, the non-axenic algae grow much faster-increased 6.78%, 10.25% and 10.19% than axenic algal biomass, respectively. Associated bacteria haven't significant effect on microalgal intracellular gross fat and ash contents, while improved microalgal intracellular protein and intra- and extra-cellular total carbohydrate contents during most growth stages. Associated bacteria didn't impact algal nitrogen uptake. While under high nutrient concentration conditions, the phosphorus contents decreased more rapidly in non-axenic algal culture (decreased 40.48%) than in axenic algal culture (decreased 5.99%) within the first 4 days, suggesting that non-axenic algal phosphorus uptake was stronger. The study provided a simpler system and effective references to research of microalgae-bacteria relationship.

Key words*:* Antibiotics; Bacteria-microalga interaction; Biochemistry composition; *Dunaliella salina*; Inorganic nutrients uptake; Purification.

Many studies have indicated that microalgae and bacteria coexistence influences their mutual growth via both stimulative and inhibitory effects[1-7]. Accordingly, investigations of their relationship are important to studies of red tide[8-13], aquaculture[3,4,14] and pollutant degradation[7, 15-17], etc. Some compounds that affect the interaction of these organisms were isolated and identified[18-21], and bacteria community composition and species communities shift in non-axenic microalgal culture were investigated[7, 22-27]. At present, the topic of bacteria-algal association is also of great ecological interest[28]. In addition, some physiological effects of co-cultured systems, such as wastewater treatment[29, 30] and toxic production[8, 31-33] have been described. However, partly due to the complexity of bacteria-alga system, little is known about the microalgae and bacteria coexistence interaction mechanism, and reports about effects of associated–bacteria on microalgal physiological and biochemical characteristics were also few.

Microalgae axenic culture, providing a simple system for bacteria-microalga study, is very important to further investigation of microalgae physiology, genetic characteristics, toxin biosynthesis, heterotrophic cultivation and the relationship between bacteria and algae. Several physical and chemical methods are used to obtain axenic cultures, including differential

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centrifugation[34-37], serial dilution[38, 39], the spread plate method[35, 36, 40, 41], filtration[36, 37, 39, 42], sonication[36, 37, 39, 42], irradiation[43] and antibiotics or other germicidal chemicals treatments[12, 42, 44-48]. Since bacteria are often attached to the algal cells, antibiotic treatment is an efficient method that is normally combined with other physical dissociation methods, such as centrifugation, filtration, sonication and serial dilution plating[9, 34, 35, 37, 49, 50].

Dunaliella salina Teodoresco (*D. salina*), a type of haloduric green microalgae belonging to the Chlorophyceae, is among the most industrially important microalgae. To date, *D. salina* has also served as another potentially attractive bioreactor system [51], and its establishment generally requires bacteria-free culture. However, there have been few reports of *D. salina* axenic purification methods or comparisons of algal biochemical components and physiological characteristics in the presence and absence of bacteria. Therefore, in this study, the entire contaminant removal process was reported in detail to improve the axenic purification of other algae and also provide a simpler system for *D. salina's* transgenic engineering and further studies of the algae-bacterial relationship. Moreover, a preliminary study on effects of co-bacteria on microalogal morphology, growth, biochemistry components and uptake of inorganic nutrients, which will serve as an effective reference for study of the bacteria-microalga interactions, was also conducted.

MATERIALS AND METHODS

Sample and culture conditions

D. salina (strain 1009) was purchased from the Marine Microalgae Research Center, Ocean University of China and cultured in Erlenmeyer flasks with *f*/2 seawater medium[52] that had been added 30 g/ l NaCl and sterilized by filtration through a 0.22-µm filter, at 23±0.5°C under 60 mmol/m^2s^2 illumination with fluorescent lamps with a 14/10 dark/light cycle. Individual microalgal colonies were cultured in *f*/2 solid medium prepared from 12 g/ l agar.

Bacteria isolated from algal culture were cultured in ZoBell 2216E medium prepared from 15 g/ l agar and incubated at 28±0.5°C.

Microalgal biomass measurement

Colorimetric method and cell counts

The algal optical density was measured at a wavelength of 682 nm (OD_{682}) using an UV-VIS spectrophotometer (UV762, Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China), in that the algal absorption maximum lies at 682 nm based on our experiment. The algal cell counts were measured by hemacytometer. The algal optical density was converted to cell counts using the following formula based on our experiment: $y=0.0034x + 0.0047$, $R^2=0.9984$, where y is the algal OD_{682} and x is the algal cells per 1×10^{-4}mL. Growth rate K (d^{-1}) was calculated as $K = \ln (OD_2/OD_1) / (t_2-t_1)$[53], where OD_2 and OD_1 are the algal OD_{682} at time 2 (t_2) and time 1 (t_1) and t_1 *and* t_2 are the early exponential phase and the end of the exponential phase, respectively.

Dry weight method

Microalgal cells were harvested by centrifugation (2,680×g) for 10 min at room temperature, after which they were rinsed with 0.5 mol/ l ammonium formate and dried at 105°C for 6 h and then weighed.

Antibiotic sensitivity profiles of associated bacteria based on the K-B disc diffusion test

The bacterial density was adjusted to about 1.5×10^8 cells per ml by turbidimetric determination. The bacteria were then spread on Mueller-Hinton (M-H) solid agar media (Hangzhou Microbe Chemical Reagent Works, China), after which discs containing antibiotics (30 µg/ ml, Hangzhou Microbe Chemical Reagent Works, China) were placed onto the center of the media's surface. The samples were then incubated at 28±0.5°C for 24 h, after which the zones of inhibition were measured. The double-disc synergy method was used to detect the effects of multiple antibiotic antibacterial interactions under the same experimental conditions.

Reintroduction of bacteria to axenic cultures

Natural algal cultures were passed through 2-µm sterile filter membranes to remove the microalgal cells, after which the filtrate was passed through a 0.22-µm filter membrane. This resulted in almost all bacteria that were not attached to the algae being transferred to the membrane. The membrane was then placed in the axenic algal culture.

Purity tests

The purified algae were incubated in liquid and solid ZoBell 2216E medium, *f*/2 medium,

f/2 medium containing added nutrients (0.5 g/l tryptone, 0.5 g/l yeast extract) and fungal medium (1 g/ l glucose, 0.1 g/ l yeast extract, sea water preparing) at 28±0.5°C for 30 days. Further microscopic inspection was conducted using scanning electron microscopy (SEM, JEM-1200EX, Japan)[54]. The test of 6-diamidino-2-phenylindole (DAPI) staining was also performed[41].

Microalgal cell biochemical components test

10 ml of algal culture were centrifuged at 2,680×g, after which algal cells were subjected to ultrasonication on ice for 2 min, in phosphate buffer solution (pH 7.4) added protease inhibitor cocktail. Algal soluble intracellular protein content was measured by Coomassie Brilliant Blue staining [55, 56]. Bovine serum albumin was used to generate the protein standard curve. The optical density (OD_{595}) was then determined and compared to the curve using the following formula: $P = (A\text{-}0.0227) / 6.475$, R^2=0.9976, where P = the protein mass concentration (mg/ ml) and A = the sample optical density (OD_{595}).

Total carbohydrate content was measured by the anthrone colorimetry method[57]. Glucose was used to generate the carbohydrate standard curve. The optical density (OD_{620}) was determined and compared to the curve using the following formula: $S = (A\text{-}0.0267) / 6.425$, R^2=0.9991, where S = the carbohydrate mass concentration (mg/ ml) and A = the sample optical density (OD_{620}).

Algal intracellular gross fat content was measured by the Soxhlet extraction method. In brief, algae were harvested by filtration through the 0.45-µm membrane, after which the extraction with diethyl ether solvent was done in Soxhlet device at a temperature of 50°C for more than 10 h. Then the algal intracellular gross fat content was measured gravimetrically.

Ash was determined gravimetrically after heating at 550°C for 8 h.

Measurement of the extracellular $NaNO_3$-N and NaH_2PO_4-P

The contents of $NaNO_3$-N in the supernatant having removed the algal cells were determined by ultraviolet photometry and compared to the curve using the following formula: $C = (A\text{-}1.8106)/ 0.0856$, $R^2 = 0.9980$, where C = the $NaNO_3$ mass concentration (mg/ l) and A = the sample optical density (OD_{205}).

The NaH_2PO_4-P content was determined by the Molybdenum blue colorimetric method. The optical density (OD_{660}) was determined and compared to the curve using the following formula: $D = (A+0.0064)/ 0.0039$, $R^2 = 0.9992$, where D = the NaH_2PO_4 mass concentrations (mg/ l) and A = the sample optical density (OD_{660}).

Statistical Analyses

Experiments were repeated at least three times for each independent assay. Variations between means of axenic and non-axenic alage were analyzed by t test (95% confidence interval).

RESULTS

Antibiotic resistance profiles of co-bacteria and *D. salina*

Examination of a series of antibiotics was conducted to determine their potential to eliminate the commensal bacterial contaminants without affecting algal growth. Five strains of bacteria (A-E) categorized by their distinct morphotypes were isolated from *D. salina* culture. The sensitivity to seven antibiotics was then measured (Tab.1). The results showed that the bactericidal effect of neomycin was lowest among seven common antibiotics. Chloramphenicol and gentamicin very effectively inhibited four strains of bacteria, while ampicillin and cephalothin very effectively inhibited three. So the four antibiotics were selected. Considering the combined effects of antibiotics, streptomycin and kanamycin were also candidate antibiotics. The double-disc diffusion experiments revealed that any combination of the six selected antibiotics exerted a synergetic antibacterial effect (data not shown).

The effects of the six candidate antibiotics on the growth of *D. salina* are presented in Fig. 1. Chloramphenicol exhibited the highest toxicity toward *D. salina*, whereas the other five antibiotics could be tolerated at a wide range of concentrations by *D. salina*. Therefore, gentamicin, ampicillin, cephalothin, streptomycin and kanamycin were used in the subsequent experiments.

Determination of the appropriate purification method and Assessment of bacteria-free strains

Preliminary experiments revealed that treatments with single and double antibiotics did not have a good bactericidal effect (data not shown). To determine the appropriate antibiotic combinations, we designed four different

combinations: Combination 1, ampicillin, gentamicin and cephalothin; Combination 2, combination 1 plus kanamycin; Combination 3, Combination 1 plus streptomycin; Combination 4, Combination 1 plus kanamycin and streptomycin. Algae in the mid-exponential phase were subjected to two treatments with one of the four antibiotic cocktails (each antibiotic at 200 µg/ ml) at 2-day intervals. The bacterial concentrations after treatment with Combinations 1 to 4 were 7.40×10^4 cfu/ ml, 8.47×10^4 cfu/ ml, 7.28×10^3 cfu/ ml and 6.85×10^3 cfu/ ml, respectively. The concentration of a control group that was not subjected to any antibiotics treatment was 1.25×10^5 cfu/ ml. The results showed that Combination 3 and Combination 4 were most effective. Because there was no significant difference between these two groups, we selected Combination 3 as the antibiotics mix for subsequent experiments.

As shown in table 2, three applications of the antibiotics mix at an interval of 2 days using 800 µg/ ml or 1000 µg/ ml of each antibiotic appeared to be effective. However, when each antibiotic reached 1000 µg/ ml, the final algal optical density was only 61.8% of the control. Therefore, the purification procedure was determined to be three additions of the mixture of ampicillin, gentamicin, cephalothin and streptomycin at 800 µg m/ l with an interval of 2 days.

After optimized antibiotics treatments, the treated culture fluid was diluted appropriately and spread on *f*/2 solid media. The isolates were then transferred to *f*/2 liquid media, and the bacterial status was confirmed by cultivation in agar ZoBell 2216E media. Among 20 isolates, four strains remained bacteria-free through repeated testing for about 2 months.

Culturable bacteria were not detected from axenic isolates when cultured in a series of liquid and solid test media, and bacterial cells were not found upon careful observation of DAPI-stained purified algal cultures. Moreover, further observation by scanning electron microscopy revealed that purified *D. salina* was well dispersed, had a clear outline, and had no visible bacteria on the algal surface or near the algal cells (Fig. 2). However, non-axenic algae were adhered together and had many bacteria present on the microalgal surface and free-living in the culture fluid so the outline of the algal cells could not be distinguished (Fig. 2).

Effects of co-bacteria on microalgal growth

After subculturing the natural and axenic *D. salina* for about one month, their growth in *f*/2 media was compared. The non-axenic microalgae grew slightly faster than the axenic microalgae (Fig. 3), but this difference was not significant ($P>0.05$). It indicated that the inhibitory effects of the antibiotics on algal growth had been eliminated at that time. We also found that non-axenic algae tended to gather and sink, suggesting that they began aging earlier. Reintroduction of almost all bacteria to the axenic algae resulted in a similar aging phenomenon. The data in Fig. 3 also showed 5 bacteria strains (bacteria A-E) all appeared to promote the algal growth. To determine if the stimulatory effect of the bacteria was due to differences in the amount of microelements, vitamins, nitrogen and phosphorous absorbed by algae in the culture, algal growth in different amendments of *f*/2 medium was compared.

As shown in Fig. 4, the difference in growth became more obvious when there were no vitamins or microelements in the *f*/2 media. On day

Table 1. Sensitivity of bacteria in the culture of *Dunaliella salina* to antibiotics

	Chloramphenicol	Gentamicin	Ampicillin	Streptomycin	Cephalothin	Kanamycin	Neomycin
A	+++	+++	+++	++	-	+	+
B	+++	+++	+	++	++++	+	-
C	-	+++	++++	+++	+	++	+
D	++++	+++	+++	++	++++	++	+
E	++++	+	++	+	+++	-	-

Note: A-E, 5 bacterial isolates from non-axenic *Dunaliella salina*. Antibiotic sensitivity of associated bacteria was detected based on the K-B disc diffusion test. ++++, average zone of inhibition > 50 mm; +++, average zone of inhibition = 41-50 mm; ++, average zone of inhibition = 31-40 mm; +, average zone of inhibition = 20-30 mm; -, average zone of inhibition < 20 mm.

24, the non-axenic algal biomass increased 10.25% and 10.19% than axenic algal biomass without vitamins or microelements in the *f*/2 media, respectively. A similar situation, that the non-axenic algal biomass increased 6.78% than axenic algal biomass, was encountered when the concentrations of nitrogen and phosphorous in the *f/2* medium were increased by 10-fold (Fig. 5). Figure 5 also indicates that a certain amount of nitrogen and phosphorous are necessary; therefore, *D. salina* will grow very slowly when they are not present. In an attempt to explain the relationship between nutrients utilization by algae and co-existing bacteria, the uptakes of $NaNO_3$-N and NaH_2PO_4-P between axenic and natural algae were compared.

Effects of co-bacteria on microalgal uptakes of $NaNO_3$-N and NaH_2PO_4-P

Non-axenic and axenic algae exhibited similar changes in the uptake and release of N (Fig.6). They both began to release N from the 10th day following incubation, and their $NaNO_3$ mass concentrations showed no significant difference. However, the $NaNO_3$ content decreased more rapidly in *f*/2 containing 10-fold higher N/P nutrients during the first 2 days. Furthermore, the phosphorus uptake seemed to be stronger than N uptake, and the phosphorus contents decreased more rapidly in non-axenic algal culture (decreased

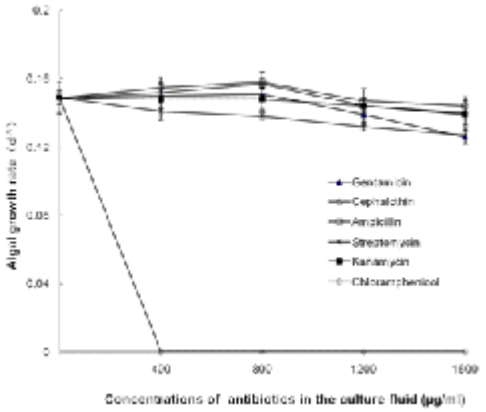

Fig. 1. Sensitivity of *Dunaliella salina* to six antibiotics. The algal growth rates at various concentrations of antibiotics were compared with those of the control without any antibiotics treatment (x=0). Growth rate K (d^{-1}) was calculated as $K = \ln(OD_2/OD_1) / (t_2\text{-}t_1)$, where OD_2 and OD_1 are the algal OD_{682} at time 2 (t_2) and time 1 (t_1).

Table 2. Bactericidal effect of different concentrations and treatment times of antibiotics cocktail in the culture of *Dunaliella salina*

	Concentration of each antibiotic (μg/ ml)					
	0	200	400	600	800	1000
1 Treatment[a]	$(6.8\pm1.19)\times10^5$	$(9.28\pm0.55)\times10^4$	$(7.56\pm0.89)\times10^4$	$(4.89\pm0.67)\times10^4$	$(3.65\pm0.024)\times10^3$	$(4.14\pm0.019)\times10^3$
2 Treatments[a]	$(1.2\pm0.36)\times10^6$	$(1.06\pm0.23)\times10^4$	$(8.92\pm1.13)\times10^3$	$(3.9\pm0.32)\times10^3$	178±15.6	144±14.1
3 Treatments[a]	$(8.3\pm1.00)\times10^5$	$(9.16\pm1.76)\times10^3$	$(3.9\pm0.45)\times10^3$	$(3.45\pm0.80)\times10^2$	0	0
Microalgal biomass[b] (OD_{682})	0.953±0.010	0.956±0.023	0.923±0.019	0.817±0.030	0.804±0.027	0.589±0.025

a: Bacterial concentration (cfu/ ml) under antibiotic treatments. Take antibiotic treatments at an interval of 2 days. Bacterial concentrations were measured in ZoBell 2216E agar on the 3rd day after antibiotics treatments. b: Microalgal biomass was detected on the 10th day after three antibiotics treatments.

40.48%) than in axenic algal culture (decreased 5.99%) within the first 4 days under high nutrient concentration conditions.

Effcts of co-bacteria on microalgal biochemical components

As shown in Fig. 7**,** the algal intracellular soluble protein mass fraction was highest in the exponential phase because that was when the most vigorous growth was occurring. On day 9, 14 and 25, the non-axenic algae protein mass fraction was higher by 10.59%, 31.34%, and 45.85%, respectively than that of the axenic algae ($P<0.01$), except during the early growth period, when the axenic algae protein mass fraction was higher ($P<0.05$).

Comparison of the gross fat illustrated that *D. salina* has rich gross fat with a mass fraction between 20% and 50%, especially during the exponential stage (Fig. 7). The gross fat content

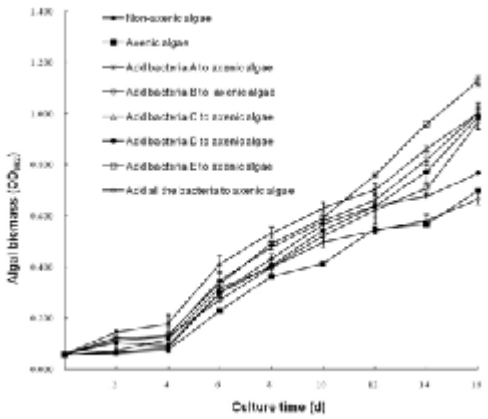

Fig. 3 Effects of bacteria on the growth of *Dunaliella salina*. Individual bacteria (bacteria A–E) were added to axenic algae cultures at about 2×10^5 cells per ml during the early algal growth stage. The algal biomass was then sampled and analyzed by the colorimetric method at intervals of 2 days.

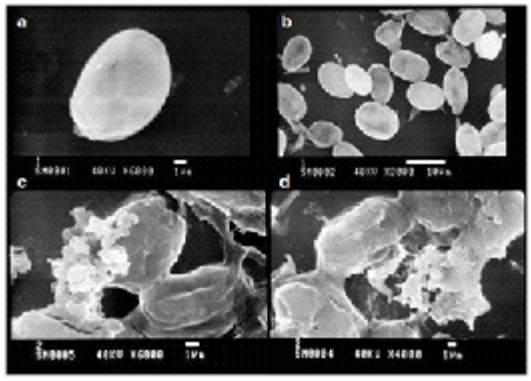

Fig. 2. SEM photographs of axenic and non-axenic *Dunaliella salina*. a: axenic *Dunaliella salina* (×6000 magnification). b: axenic *Dunaliella salina* (×2000 magnification). c: non-axenic *Dunaliella salina* (×6000 magnification). d: non-axenic *Dunaliella salina* (×4000 magnification). Scale bars represent: a, c, d: 1 μm; b: 10 μm.

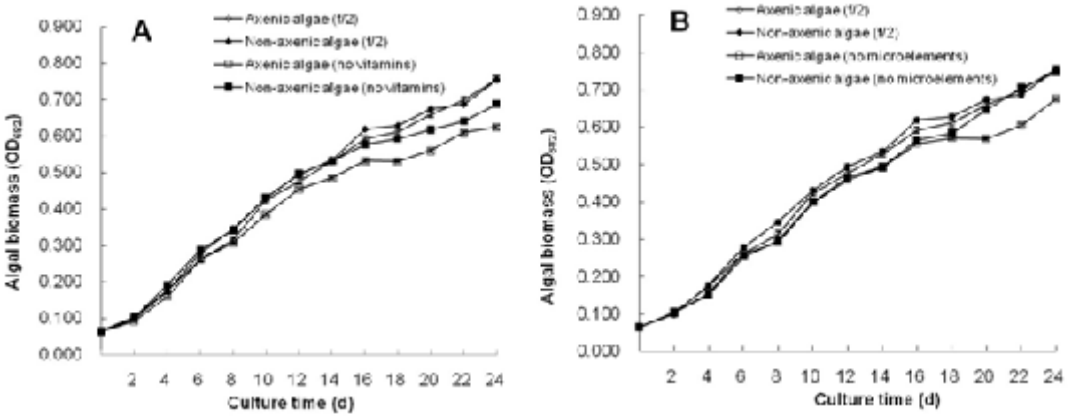

Fig. 4. Effects of vitamins and microelements on the growth of axenic and non-axenic *Dunaliella salina*. The algal biomass was sampled and analyzed at intervals of 2 days. a: Axenic and natural algae were cultured in *f/2* media and *f/2* media without vitamins, separately. b: Axenic and natural algae were cultured in *f/2* media and *f/2* media without microelements, separately.

did not differ significantly between non-axenic and axenic algae (P>0.05).

The intracellular total carbohydrate mass fraction of non-axenic algae was much higher than that of axenic algae in the early stage on day 4 (P<0.01). However, following further culture, the gap decreased until the algal senescence phase, at which point the intracellular total carbohydrates mass fraction of the axenic algae was slightly higher (P>0.05). The non-axenic algal extracellular carbohydrate mass fraction was higher except during the logarithmic phase on day 9 (day 25 P>0.05, others P<0.05).

The algal ash content was about 13.72–32.79%, with no significant difference observed between non-axenic and axenic algae (P>0.05).

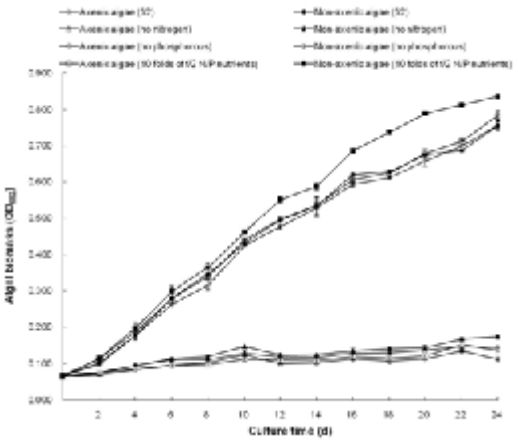

Fig. 5. Effects of N/P nutrients on the growth of axenic and non-axenic *Dunaliella salina*. Axenic and natural algae were cultured separately in *f*/2 media without N/P nutrients, *f*/2 media and *f*/2 media containing 10-fold higher N/P nutrients. The algal biomass was sampled and analyzed at intervals of 2 days.

DISCUSSION

Purification of *D. salina*

In the preliminary experiments for algae purification, fungus appeared in the solid media when some co-bacteria were diminished by antibiotics. The final bacteria-free assessment demonstrated that the fungal contaminants were removed successfully by spreading the diluted algae fluid on the plate and culturing monoclonal *D. salina* without fungal contaminants. These findings show that the dilution and spread plate technique without the use of fungicide is a simple and effective method for elimination of fungal contaminants. These findings also indicate that there are some interactions between different microbial contaminants. Therefore, we can expect isolation of more bacteria strains that grew relatively slower following the removal of predominant bacteria strains. However, the purification will be more complicated and time-consuming if all of these culturable bacteria are considered; therefore, the effects of antibiotics were investigated based on isolated predominant bacteria.

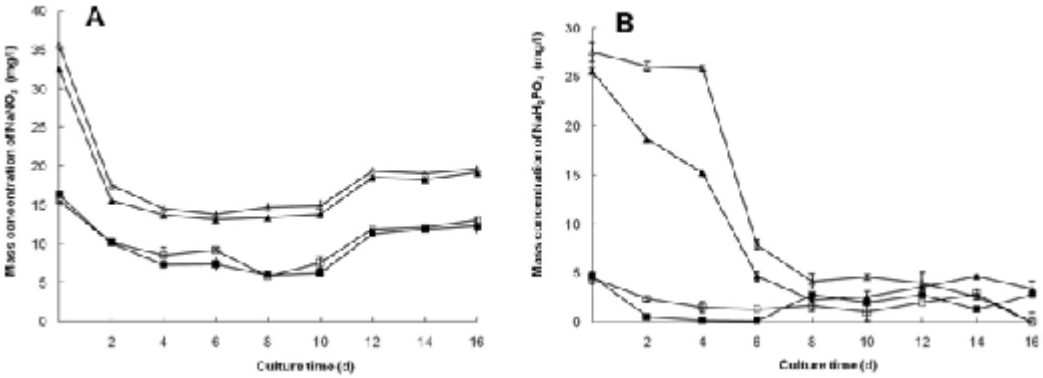

Fig. 6 Changes in extracellular $NaNO_3$ and NaH_2PO_4 mass concentrations in the culture of axenic and non-axenic *Dunaliella salina*. The initial algal cell density was about 2×10^5 cells per ml. The supernatant culture fluid was sampled and analyzed at intervals of 2 days. a: Change curve in extracellular $NaNO_3$ mass concentrations. b: Change curve in extracellular NaH_2PO_4 mass concentrations.

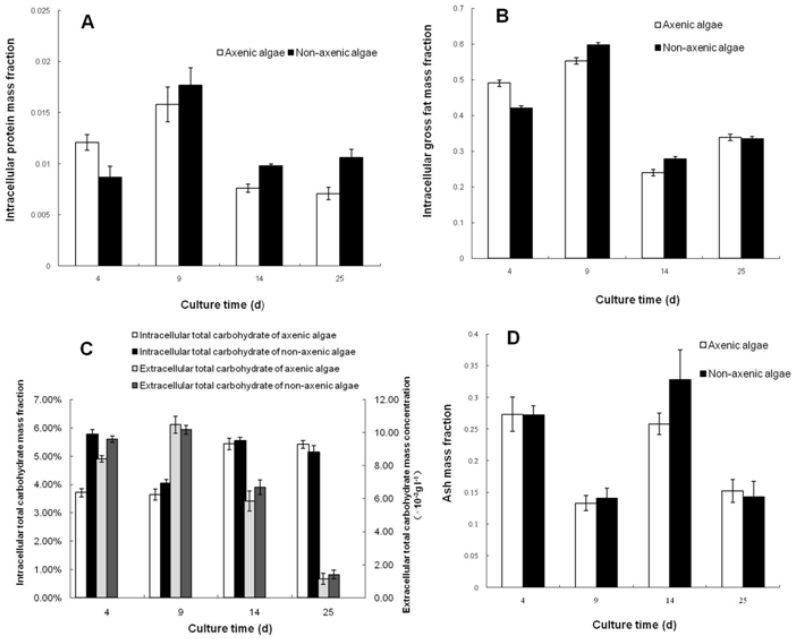

Fig. 7. Biochemistry contents comparison of axenic and non-axenic *Dunaliella salina* during different phases. The initial algal cell density was about 2×10^5 cells per ml. The algal biomass was sampled and analyzed on day 4, 9, 14 and 25. a: Comparison of intracellular protein mass fractions. b: Comparison of intracellular and extracellular total carbohydrate contents. c: Comparison of intracellular gross fat mass fractions. d: Comparison of intracellular ash mass fractions.

D. salina cells are highly sensitive to chloramphenicol; therefore, chloramphenicol can be selected as a screening marker for *D. salina* genetic transformation. In some recent studies, the chloramphenicol resistance gene (the CAT gene) was used in the transformation of *D. salina* cells [58]. These findings indicate that these strains of *D. salina* had similar sensitivities to chloramphenicol.

A high concentration and repeated antibiotic treatment will help prevent the development of resistant strains. Through drug sensitivity testing, we can improve the efficiency of removing bacteria while decreasing the likelihood of resistant strains developing. Cottrell and Suttle [46] obtained an axenic culture of *Micromonas pusilla* using a sequence of antibiotics and reported that the method of adding antibiotics in a stepwise fashion could prevent combined antibiotics antagonistic effects. However, our experiments showed that this method was not effective for *D. salina* (data not shown)**.** This may have been a result of synergistic effects of different antibiotics, which would also explain why various methods are suitable for different types of microalgae. The plate method is normally used as the final conventional process for axenic culture induction. Spreading plates in a timely manner can prevent co-bacteria, which was temporarily inactivated by antibiotics, from being reactivated in the liquid media. In addition, this can also diminish the effects of antibiotics on algal growth more rapidly.

Effects of co-bacteria on microalgal growth

Microalgae and coexisting bacteria communities likely engage in both positive and negative interactions; however, some bacterial effects of microalgae are species-specific [8, 15, 28], while others can affect many different algal species[2, 28, 59]. Some accompanying bacterial species have been identified based on the similarity of the partial sequence of 16S rRNA[22-24]. In our experiments, bacteria A-E all seemed to have a growth-stimulatory effect on the algae; these bacteria should be identified in future research. The antibiotics treatment likely removed the growth-stimulatory bacteria, but also the growth-inhibitory bacteria; therefore, axenic and non-axenic algae have similar growth curves.

To date, the mechanism of the microalgae-bacteria interaction has mainly focused on the interaction among extracellular compounds [60-62], some of which have been isolated and identified [18-21]. These products include vitamin analogs, enzymes, glycopeptides and antibiotics analogs, etc. As shown in Fig. 4, the axenic algal growth speed decreased more obviously when there were no microelements or vitamins in the *f*/2 media. These findings indicate that the co-existing bacteria supplied microelements or vitamins (or their analogs) needed by the algae. Haines et al.[63] found that some bacteria strains can utilize microalgae excretory products and provide vitamin B_{12} to coexisting microalgae. Croft[64] found that more than half of the algal species require exogenous vitamin B_{12} for growth, and that the source of cobalamin appears to be bacteria. However, few studies associated with bacteria providing microelements to algae have been conducted. More studies have focused on what effects co-bacteria have on the sensitivity of microalgae to metal microelements or algal-bacteria interactions during metal removal and degradation [65, 66].

Effects of co-bacteria on microalgal uptakes of $NaNO_3$-N and NaH_2PO_4-P

Algae and co-bacteria interactions occur not only through extracellular products but also via competition for inorganic nutrients, and both are closely interrelated [67]. As shown in Fig. 5, when N/P nutrients were both enhanced 10-fold in f/2 media, a significant increase in non-axenic algal growth occurred. This may have been due to nutrients competition between algae and accompanying bacteria. Elevated nutrient concentrations will ease nutrition competition. Nevertheless, there was no direct evidence of algae being negatively affected by competition with bacteria for nitrogen and phosphorus [68]. Our results also appeared to indicate that bacteria would not limit algal growth through nutrients competition when nitrogen and phosphorus appeared to be slightly limited (Fig. 5). Some studies have shown that the utilization of inorganic and organic nitrogen by microalgae or heterotrophic bacteria are different and interacting [41, 69]. Furthermore, utilization of nitrate and ammonium by algae also differed significantly and their mutual influence had also been proved[70]. These studies assumed that the uptake of nitrogen in non-axenic algal culture would depend on different sources of nitrogen. Further study is necessary to determine how inorganic nutrients affect the composition of the algae-bacteria community and associated biochemical processes. Nevertheless, it is accepted that the interactions between bacterial and microalgal phytoplankton uptake of inorganic nutrients are more complicated than simple competition[69, 71].

Effcts of co-bacteria on microalgal biochemical components

As shown in Fig. 7**,** the non-axenic algal intracellular soluble protein mass fraction was higher than that of the axenic algae at most times. This may have been due to bacterial production of rich proteins in the natural algae culture. Moreover, some proteins such as enzymes can also be produced actively during the interaction between algae and bacteria.

Comparison of the carbohydrates indicated that those produced by bacteria were likely the main portion of intracellular total carbohydrate during the early algal growth phase, when the bacteria were in their first growth peak. As algae grew, they became responsible for the majority of carbohydrate production through photosynthesis. Moreover, the bacteria could utilize the carbohydrate produced by algae. This may have caused the reduction in the difference in the algal intracellular carbohydrate content in the presence and absence of bacteria. Previous studies have also shown that carbohydrate accumulation in microalgal culture could be induced by nitrogen deficiency[72]. The accumulation may also reduce the influence of bacteria on the total carbohydrate to some degree. The non-axenic algal extracellular carbohydrate content was higher except during the logarithmic phase (the 9th day). This may have been caused by extracellular carbohydrates secreted by bacteria. The secretion can be affected by algal growth because bacterial metabolism and growth can use the carbohydrates produced by algae or be hindered by them.

The gross fat and ash mass fractions did not differ between non-axenic and axenic algae. This may indicate that the co-existing bacteria had little effect on the algal lipid metabolism. Furthermore, the high gross fat and ash content of algae would partially reduce the gap of their difference between natural and axenic algae.

CONCLUSION

In the early studies, Bell [60, 61] had pointed out that algae-bacterial biomass and their extracellular products did not have a simple linear relationship, although their complicated interactions have selectivity, associated bacteria appear to be able to adjust to the co-existing microalgae. The difference in the morphology, growth and biochemical composition between natural and axenic algae is undoubtedly closely related to their interaction. To date, investigations of the relationships between algae and bacteria have primarily been related to biological responses processes of some bacterial strains and microalgae communities and analyses of the structure and function of the microalgae-bacteria consortium. Indeed, few studies have compared biochemical components and physiological characteristics of such consortia. This lack of research is partially due to the relatively time-consuming and complicated work associated with removing bacteria from isolated microalgal culture. Therefore, the present study provides methods for the axenic culture of *D. salina* and other similar algal strains, as well as a simpler system for further investigation of microalgae physiology, genetic characteristics, toxin biosynthesis and the microalgae-bacteria relationship.These results can serve as a reference for further utilizing *D. salina* as a bioreactor of genetic engineering, as well as the future studies of the bacteria-microalgae relationship.

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