Starter Kit for the Production of Pure and High Quality Microalgae

Field of Invention

The present invention relates to a method used for preparation of culture medium for microalgae from marine aquaculture pond sludge. Said culture medium in turn can be used as a starter kit to initialize the growth of microalgae culture with pure microalgae isolates to produce high quality microalgae.

Background of the Invention

Pure algae are actually in great demand, especially in the field of aquaculture industry, research institutions and schools. This is because pure microalgae have high contents of important polyunsaturated fatty acids such as arachidonic acid (20:4n-6), EPA (20:5n-5), DPA (22:5n-3) and DHA (22:6n-3). Hence, microalgae are excellence sources of important nutrients in human and animal health. Furthermore, it also enhances the fish colour within the water habitats as well as being the live food for fish and shrimp larvae production. Besides, pure and high quality microalgae are commonly used for feed additives, pharmaceutical, neutraceutical and cosmetic products.

Due to the fact that obtaining and maintaining pure microalgae isolates are difficult because it requires expertise and special equipment. For example, the use of organic fertilizer for the production of microalgae results in contamination such as heavy

metals, antibiotics and other harmful organisms. In addition, the quality of algae produced is low and variable, depending on the source of organic fertilizer. The use of chemical fertilizers such as urea or triple super phosphate would not provide all the essential ingredients such as trace minerals found in interstitial water. In fact, a complete culture medium for microalgae widely used in laboratories has to be made up of 15 different chemicals. Thus it is essential to develop a cheap and easy way to start a pure algae culture.

Bottom sediment from aquaculture ponds generally receives large amount of high quality organic matter in the form of feed, fertilizers and excretory products resulting in enriched sludge. Although those bottom sediment sludge is rich in phosphates, nitrogen, carbons, silicon oxides and other micronutrients, untreated aquaculture sludge is actually a source of pollution to the aquatic environment. Discharge of this enriched sludge can cause serious eutrophication problems in public waters. This can undermine the sustainability of all aquatic-based industries including aquaculture.

On the other hand, organic matter from aquaculture practices enriched the bottom sediment. The availability of growth nutrients in the interstitial water extracted from the sludge, such as nitrogen, phosphorus and other micronutrients can be used to produce high quality microalgae. In fact, treatment or recycling of said sludge can be done by extracting the interstitial water containing these nutrients. Discharge of said treated sludge would minimize pollution in rivers and coastal areas.

Summary of Invention

The present invention provides a method for production of microalgae culture medium comprising the steps of obtaining pond sludge from aquaculture pond sediment followed by extracting the interstitial water through centrifugation or compression, filtering and sterilizing the extract and finally freeze-drying the concentrate extract to become powder. The produced culture medium can be used to start pure cultures with minimal contamination.

The main objective of said invention is to provide a cheap and easy method to produce pure and high quality microalgae. This is because obtaining and maintaining pure microalgae isolates are difficult, it requires expertise and special equipment. This culture medium preparation steps are relatively easy and its production cost is much cheaper than standard medium. The produced culture medium is made to powder form for easy handling and storage.

Apart from that, the culture medium prepared according to the disclosed method can initialize pure microalgae culture by adding the provided pure microalgae isolates into the diluted said culture medium in desired concentration. Mass culture of pure microalgae is made easy with minimal contamination. Microalgae grown in the said culture medium contains higher levels of nutritional value comparing to those grown in standard medium.

Brief Description of Figures

Figure 1 a schematic flow-chart of the disclosed method.

Detailed Description of Invention

Present invention provides a method for preparing culture medium from aquaculture pond bottom sludge for culturing pure microalgae species. The nutrient-rich interstitial water (IW) extracted from aquaculture pond bottom sediments is collected and undergone extraction followed by filtration and sterilization to obtain a nutrient concentrated extract. The nutrient concentrated extract is made to powder form by freeze-drying. Customized pure microalgae isolates is supplemented to said concentrated extract or powder to provide a microalgae growth initializing kit for microalgae culturing purposes. The steps of disclosed invention are illustrated as in Figure 1.

First, the enriched aquaculture pond bottom sediment, or referred as pond sludge hereafter, is collected either manually or with the aid of a suction pump. A kilogram of pond sludge can produce 500-1000 ml of concentrated liquid extract depending on the texture of the sediment. The amount of pond sludge to be collected is depending on the amount of extract required.

The collected pond sludge is subjected to extraction to obtain the pore water or interstitial water (IW), referred as liquid concentrate hereafter. The sludge is undergone centrifugation (using a centrifuge) at the speed of 6000 to 8000 rpm for 20 to 40 minutes. Centrifugation is undergone only once to extract the pore water or IW from each loading of pond sludge in the centrifuge container. Alternatively, the sludge can be undergone compression (using a compressor) to sediment down the sludge leaving the said liquid concentrate which is rich in micronutrients. During the process, no other additional chemical is added.

After the extraction process, said liquid concentrate is transferred to a clean container. Filtration is carried out through glass fiber filter followed by 0.45 and 0.22 μm membrane filters. Said filtering step is to filter out the bacteria and other living microorganisms. This step is best done in a sterile condition. Said filtered liquid concentrate is then subjected to chemical analysis to determine its nutrient content.

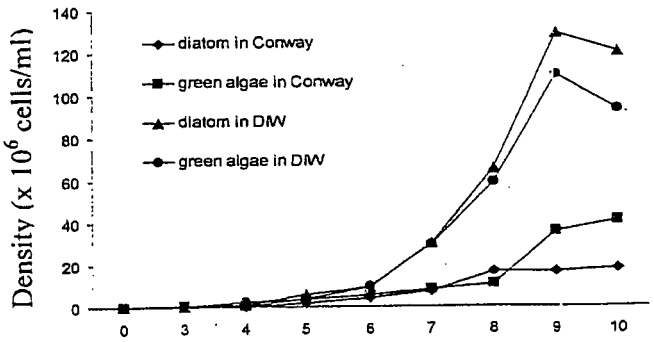
Next, said analyzed liquid concentrate is subjected to sterilization through ultraviolet (UV) light (far-UV range of 254 nm) exposure for 1-2 hours. UV light eradicates all those smaller microorganisms which are not being filtered out previously. Sterilization by this method but not autoclaving is to ensure all the enrich media are maintained and not destroyed by heat and pressure due to autoclave. The sterilized liquid extract is ready to use upon dilution to desired concentration depending on the nutrient content determined previously.

Said sterile liquid concentrate is transferred aseptically to ice cube maker to form ice cubes. Said liquid concentrated in ice cube form is then transferred in a sterile plastic sheet and placed into the freeze drier container. Said ice cube is freeze dried within 3 days in freeze drier at the temperature of -40 to -55 °C and vacuum pressure of 130-150×10^3 mbar. The nutrient rich powder obtained is ready to use after freeze drying. Said nutrient rich powder is stored in air tight containers at 20 to 30 °C. Depending on the determined nutrient content, 1 g of said nutrient rich powder can produce 5 to 10 liter of liquid culture media. Said liquid culture medium is stored in air tight bottles and store at 2-6 °C.

Moreover, the nutrient rich liquid concentrate or powder can be further processed in the form of a kit, a microalgae growth initializing kit. The kit comprises the nutrient rich liquid concentrate or powder and several types of customized microalgae isolates. Said customized microalgae isolates can be mixed with the nutrient rich liquid concentrate or powder and users can simply culture the customized microalgae by diluting said mixture according to the instruction given. The abovementioned customized microalgae are *Chaetoceros* spp., *Skeletonema* spp., *Isochrysis* spp., *Amphora* spp., *Cymbella* spp, *Navicula* spp., *Chlorella* spp., *Nannochloropsis* spp., *Tetraselmis* spp., *Oscillatoria* spp. *Spirulina* spp., and *Pseudanabaena* spp. In another preferred embodiment, said customized microalgae isolates can be provided as additional supplement without the nutrient rich liquid concentrate or powder or vice versa. The disclosed kit is ready to be used by anyone with or without expertise in culturing microalgae as the culturing step can be easily followed according to the instruction enclosed with the kit.

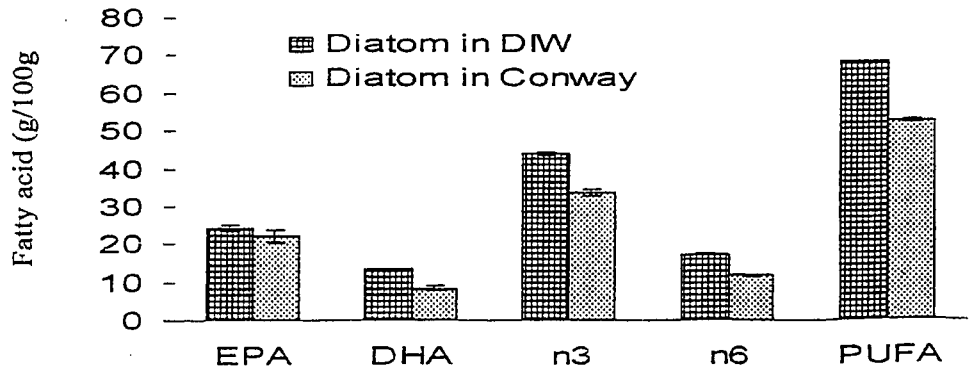
Example 1

The culture medium produced from the nutrient rich liquid concentrate or powder is rich in phosphorus, nitrogen, silica, essential minerals and micronutrients that meet the requirement for healthy production of high quality algal species. Said culture medium above can be used for mass culture of a pure species without contamination. On the exposure of the plastic bags to sunlight or other light sources, pure algal cultures will develop profusely reaching a plateau within 7 to 10 days. Experiments showed that marine algae (*Chaetoceros calcitrans* (a diatom), *Oscillatoria* sp. (a blue-green alga) and *Nannochloropsis oculata* (a green alga), *Tetraselmis tetrathele*, and *Spirulina* sp.) cultured in said culture medium produced significantly higher density ($p<0.05$) and culture rate than the standard culture medium (Conway). The graph below shows the culture of a diatom, *Chaetoceros calcitrans* and green algae, *Nannochloropsis oculata* in standard culture medium (Conway) and diluted interstitial water (DIW).



Example 2

Further bioassays demonstrated that diluted algal culture medium was the best medium for culturing pure stocks of microalgae. Algae cultured in the said culture medium showed significantly higher nutritional quality than those grown in standard (Conway) medium. For example, the cultured algae contain higher concentration of essential amino acids and polyunsaturated fatty acids (especially omega 3 and 6), compared to those grown in standard Conway medium which contains about 15 different chemicals. The graph below shows the fatty acid (g/100g lipids) profile of a diatom, *Chaetoceros calcitrans* in standard culture medium (Conway) and diluted interstitial water (DIW). (EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; n3: omega 3; n6: omega 6; PUFA: polyunsaturated fatty acids). The microalgae isolates are stored in dark bottles at 2-6 °C and can last for minimum 6 months. Algal culture can be easily initiated by diluting the liquid extract or powder in transparent containers such as plastic bags or bottles followed by inoculating algal cells provided. Said microalgae culture medium can be used as solid medium for growing microalgal stocks by adding agar into it.



Example 3

The filtered liquid extract is subjected to chemical analysis to determine its nutrient content. Table below shows the chemical and physical parameters of said filtered extract from shrimp ponds and Conway medium. Values are mean ± standard deviation.

Parameters	**Interstitial water from shrimp ponds**	**Conway**	**Conway + Silica**
Conductivity (mS/cm)	26.5 - 53.9	44.9	43.0
Salinity (ppt)	16.2 – 38.0	28.5	27.5
PH	7.9 - 9.8	8.0	8.0
Total ammonia-N (TAN) (mg/L)	18.5 – 55.1	10.3 ± 0.1	10.2 ± 0.1
Nitrate (NO_3-N) (mg/L)	0.1 - 12.1	< 0.1	< 0.01
Nitrite (NO_2-N) (mg/L)	<0.1 - 3.0	0.2 ± 0.1	0.2 ± < 0.1
Phosphate-P (PO_4-P) (mg/L)	4.2 – 12.7	6.1 ± 0.9	6.5 ± 1.5
Reactive Silica (mg/L)	32.1 - 71.8	21.0 ± 0.5	36.3 ± 0.3
Total Nitrogen (TN) (mg/L)	30.4 – 125.5	17.8 ± < 0.1	20.6 ± < 0.1
Total Phosphorus (TP) (mg/L)	8.1 – 46.5	10.9 ±< 0.1	10.6 ±< 0.1

Claims

1. A method for production of microalgae culture medium comprising the steps of:
   a) collecting of sludge;
   b) extracting the interstitial water from said collected sludge to obtain the liquid concentrate;
   c) filtering said extracted liquid concentrate;
   d) assessing nutritional content of said filtered liquid concentrate;
   e) sterilizing said filtered liquid concentrate; and
   f) concentrating said sterile liquid concentrate to obtain the nutrient rich powder.

2. A method as claimed in claim 1, wherein said extracting the interstitial water is done by centrifugation or compression.

3. A method as claimed in claims 1 and 2, wherein said extraction is performed at the speed of 6000 to 8000 rpm for 20 to 40 minutes.

4. A method as claimed in claim 1, wherein said filtering of said extracted liquid concentrate is glass fiber filtration and membrane filtration.

5. A method as claimed in claims 1 and 4, wherein said membrane filtration is done under the pore size of 0.22 and 0.45 μm.

6. A method as claimed in claim 1, wherein said sterilizing of said filtered liquid concentrate is exposing said interstitial water to UV light for 1-2 hours.

7. A method as claimed in claim 1, wherein said concentrating of said sterile liquid concentrate further comprising the steps of:

a) forming of ice from said sterile liquid concentrate;

b) freeze-drying of said ice in freeze drier to obtain the nutrient rich powder.

8. A method as claimed in claim 7, wherein the freeze drying is done under the temperature of -40 to -55 °C and vacuum pressure of $130\text{-}150\times10^{3}$ mbar.

9. A method as claimed in claims 7 and 8, wherein all steps of said methods are conducted under sterile condition.

10. A liquid concentrate or nutrient rich powder produced from any of the preceding claims.

11. Use of the liquid concentrate or nutrient rich powder as claimed in claim 10 in the method for producing microalgae culture medium.

12. A method for producing microalgae culture medium by mixing 1g of said nutrient rich powder, as claimed in claim 10, to 5 to 10 liters of water.

13. A kit to initialize microalgae growth comprising:

a) nutrient rich liquid concentrate and/or powder as claimed in claim 11, and

b) at least one type of microalgae isolates is supplemented with said nutrient rich liquid concentrate or powder.

14. A kit as claimed in claim 14, wherein said microalgae isolates are *Chaetoceros* spp. , *Skeletonema* spp., *Isochrysis* spp., *Amphora* spp., *Cymbella* spp, *Navicula* spp., *Chlorella* spp., *Nannochloropsis* spp., *Tetraselmis* spp., *Oscillatoria* spp. *Spirulina* spp., and *Pseudanabaena* spp.