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(54) **Title:** AN ECONOMICAL AND EFFICIENT METHOD FOR MASS PRODUCTION OF SPIRULINA

(57) **Abstract:** The present invention relates to an economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3 % w/v, phosphorus of concentration ranging between 0.1 to 0.3 % w/v, potassium of concentration ranging between 0.1 to 0.3 % w/v seawater and composition thereof.

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AN ECONOMICAL AND EFFICIENT METHOD FOR MASS PRODUCTION OF SPIRULINA

Field of the present Invention

The invention relates to a seawater based medium composition for Spirulina production.

Background and prior art references of the present Invention

Spirulina are blue-green algae belonging to the phylum: Cyanophyta, class Cyanophyceae, order: Nostocales, family: Oscillatoriaceae, genus: Spirulina or Arthrospira.

Spirulina, is one of the most comprehensive sources of nutrition has known to man. Algae are rich in gamma linolenic acid (GLA), linoleic and arachidonic acids. They are also rich in iron, protein, essential amino acids, vitamins, minerals and chlorophyll.

Different strains of Spirulina exist, notable ones are S. platensis and other species S. maxima; S. fusiformis (Bourrelly P. 1970. Les algues bleues ou cyanophyces, in "Les Algues d'eau douce, Tome III, Editions N. Boube).

Spirulina is a nutrient-rich (Table 1, Becker E. W. and Venkataraman L. V., 1982, Biotechnology and exploitation of algae – The Indian Approach, Publication "German Agency for technical co-operation,, D-6236, Eschborn 1, Federal Republic of Germany, Druckerei Gneiting GmBH Filmsatz+Druck, Tübingen) microalga and processed as dry powder. It can be consumed as powder or as tablets for availing health benefits. The powder may also be added to many dishes to enhance the protein and vitamin content. A variety of recipes are available to prepare Spirulina based dishes. It can also be added to fruit or vegetable juice. Worldwide, Spirulina powder is sold with recipes. which, include pasta, whole wheat Spirulina bread, Spirulina drink, scones, whole meat biscuits, soups, pastalina, fermented products like Dihe, Tofu, Bread spreads, sources, salad dressings, curries, herb filling and deserts. (United States Patent Application-20030017558 pham, quoc kiet; et al. - Method for mixotrophic culture of Spirulina for producing a biomass rich in omega 6 polyunsaturated fatty acids and/or in sulpholipids)

It has been proved experimentally that Spirulina can reduce serum cholesterol, triglyceride and LDL (undesirable fat) levels, and hence has a wide application in health foods. This may be due to its unusual and very high levels of gamma linolineic acid (GLA) and other organic substances. GLA, an essential fatty acid, is a precursor for the body's prostaglandins, master hormones that control many essential body

functions. Many degenerative diseases and health problems are associated with GLA deficiency. Clinical studies show dietary intake of GLA can help arthritis, heart disease, and obesity and zinc deficiency. It may also help premenstrual stress, depression and alcoholism. Spirulina contains about 5% essential fatty acids or lipids and of this about 20% is GLA (United States Patent Application-20030017558 pham, quoc kiet; et al. -Method for mixotrophic culture of Spirulina for producing a biomass rich in omega w6 polyunsaturated fatty acids and/or in sulpholipids).

Spirulina various health benefits.

Glycolipids extracted from Spirulina have been found to combat the AIDS virus (Boyd, et al, 1989). Phycocyanin, the blue protein in Spirulina, is demonstrating positive results with treating cancer and in stimulating the immune system (Iijima, et al, 1982). It stimulates the immature or damaged immune system to grow or to repair itself when injured or weakened by infection or toxic chemicals. GLA has been found to have a positive effect in the treatment of arthritis (Belch, et al, 1988), and premenstrual syndrome (Horrobin, 1983), and in protecting the body against degenerative diseases (Kendler, 1987).

Spirulina, which contains nutritional and highly valuable bioactive compound, can help to many health problems by one or the other mechanism. Researches done on Spirulina have revealed that it can be beneficial in following cases,

Anemia, β-carotene deficiency Cancer, Diabetics, Entrogastritis, Fertility, Gastric problems, Heart problems, Immune disorders, Jaundice, Kidney disorders, Liver problems, Malnutrition, Nutritional suppliment, Obesity, Protein suppliment Radiation sickness, Skin problems, T-Cell stimulation, Ulcers, Vision problems, Wound healing etc.,

Beta-carotene is one of the most well known anti-cancer substances. It is the precursor of vitamin A. Since beta-carotene is present in very high amount in Spirulina, a diet comprising of Spirulina will reduce cancer risks.

Table. 1: Composition of Spray dried Spirulina (Percentage on dried weight)

(Percentage DW)	g/l	Minerals	(mg/100g)
Moisture	6.8	Calcium	755
Total protein	64.5	Phosphorous	1455
Lipids	3.1	Iron	160
Carbohydrates	10.1	Sodium	330

Crude Fiber	4.2	Magnesium	890
Ash	7.4	Zinc	10
Nucleic acids	3.9	Potassium	1425

Spirulina has the highest protein content (60-70%) of any natural food. It has no hard cellulose in its cell walls, being composed of soft mucopolysaccharides. This ensures its protein is easily digested and assimilated in the human body. It is 85 to 95% digestible. Spirulina protein contains all essential amino acids in adequate quantities (Table-2, Becker E. W. and Venkataraman L. V., 1982, Biotechnology and exploitation of algae – The Indian Approach, Publication "German Agency for technical co-operation,, D-6236, Eschborn 1, Federal Republic of Germany, Druckerei Gneiting GmBH Filmsatz+Druck, Tübingen), which is comparable with any other vegetables and meat.

Table – 2. Amino acids composition of Spirulina (all in dry weights)

Essential amino acids	per 10 gm	Percent of total
Isoleucine	350 mg	5.6
Leucine	540 mg	8.7
Lysine	290 mg	4.7
Methinione	140 mg	2.3
Phynylalanine	280 mg	4.5
Threonine	320 mg	5.2
Tryptophane	90 mg	1.5
Valine	400 mg	6.5
Non Essential Amino acids		
Alanine	470 mg	7.6
Arginine	430 mg	6.9
Aspartic acids	610 mg	9.8
Cystine	60 mg	1.0
Glycine	320 mg	5.2
Glutamic acid	910 mg	14.6
Histidine	270 mg	4.3
Proline	270 mg	4.3
Serine	320 mg	5.2
Tyrosine	300 mg	4.8
Total Amino Acids	6200 mg	100.0

The vitamin content of Spirulina reflects another important benefit as a human food, representing a rich natural source of vitamins. A ten-gram serving of Spirulina supplies a rich profile of vitamins we need. It is the richest source of beta-carotene (precursor of

Recently it has been experimentally proven that aqueous extract of Spirulina is helpful in suppressing AIDS where it has been linked to the inhibition of HIV-1 replication (Ayehunie et al, 1998, J. of AIDS illumination Retrovirol, Vol. 18: 7-12).

Spirulina has also been implicated in immuno-modulating activities (Hirayashi et al, 2002, International Immunopharmacology, Vol. 2: 423-434) and against arthritis (Remerez et al, 2002, Mediators Inflamm. Vol. 11: 75-79).

Thus many Institutes / Corporate bodies have been involved in developing methods to produce Spirulina biomass to meet the ever-increasing market demand.

Drawbacks associated with hitherto known technology/process are, Spirulina biomass has been under production by several companies using Zarrouk's medium that results in the quality of Spirulina with nutritional composition as listed in Table. 4 where the total iron content in processed material is about 160 mg per 100 g dry weight.

To reduce the input cost, Becker and Venkataraman, (Becker E. W. and Venkataraman L. V., 1982, Biotechnology and exploitation of algae – The Indian Approach, Publication "German Agency for technical co-operation,, D-6236, Eschborn 1, Federal Republic of Germany, Druckerei Gneiting GmBH Filmsatz+Druck, Tübingen) simplified the earlier Zarrouk's medium and used the following medium composition, i, e., Sodium bicarbonate 4.5g, Sodium nitrate 0.5g, Sodium chloride 1.0 g, Potassium sulphate 1.0 g, Potassium hydrogen phosphate 0.5g, Magnesium sulphate 0.20g, Calcium chloride, 0.04g, Ferrous sulphate 0.01g and water to make up 1L was used which resulted in 47.7 mg of iron accumulation per 100g of dry weight of Spirulina.

Reference may also be made to Venkataraman L. V. (Mass production of the blue-green algae Spirulina: an overview, Biomass, Vol. 15: 233 – 247) wherein nutrient medium was further modified to contain a very simple nutrient mixture composed of the following commercial grade constituents: Sodium bicarbonate 4.0g, Urea ($CO(NH_2)_2$) 0.5g, Sodium chloride 1.0 g, Potassium sulphate 1.0 g, Potassium hydrogen phosphate 0.5g, Magnesium sulphate 0.20g, and water to make up 1L where though no additional ferrous sulphate in the medium was used, the resultant biomass contained 50 mg iron accumulation per 100g of dry weight of Spirulina which probably resulted from traces of ferrous sulphate contamination in the commercial grade chemicals.

Reference may also be made to (Mahadevaswamy, 1994, Production of blue-green algae, Spirulina platensis for biomass protein in clean water and integrated system, Ph.

vitamin A), with a ten times higher concentration than carrots. Vitamin-A is important in maintaining mucous membranes and pigments necessary for vision. Also Spirulina is one of the richest sources of vitamin B-12, higher than beef liver or sea vegetables. Spirulina vitamin content is given in Table-3 (Becker E. W. and Venkataraman L. V., 1982, Biotechnology and exploitation of algae – The Indian Approach, Publication "German Agency for technical co-operation,, D-6236, Eschborn 1, Federal Republic of Germany, Druckerei Gneiting GmBH Filmsatz Druck, Tübingen).

Table-3. Vitamin content of Spirulina platensis

Vitamins	Per 10 grams	US RDA
Vitamin A (beta carotene)	23000 IU	5000IU
Vitamin B 1 (thiamin)	0.31mg	1.5 mg
Vitamin B2 (riboflavin)	0.35 mg	1.7 mg
Vitamin B 3 (niacin)	1.46 mg	20 mg
Vitamin B6 (pyridoxine)	80 mcg	2 mg
Vitamin B12	32 mcg	6 mcg
Vitamin E	1 IU	30 IU
Folic acid	1 mcg	400 mcg
Panthothenic acid	10mcg	10mcg
Biotin	0.5 mcg	0.5 mcg
Inositol	6.4 mg	6.4 mg

Pigments help synthesis of many enzymes necessary for regulating the body's metabolism. Phycocyanin and phycocyanobilin from Spirulina has been linked to scavenging of peroxinitrite free radicals leading to protection against oxidative damage to Deoxyribonucleic acid (DNA) (Bhat and Madyastha, 2001, Biochem. Biophys. Res. Commun. Vol. 285: 262-266). Chlorophyll contains a magnesium ion at its core, giving it a green colour, and hemoglobin contains iron, giving it a red colour. The beneficial effect of Spirulina for anemic patients could be due to the conversion of chlorophyll into hemoglobin, as well as the high bioavailbe iron content of Spirulina (Puyfoulhoux et al, 2001, J. Agric. Food Chem. Vol. 49: 1625-1629).

Apart from these, Spirulina contains several useful enzymes in adequate quantities. Superoxide dismutase (SOD) enzyme activity ranging from 10,000 to 37,000 EU per ten grams in Spirulina powder is linked to its very high free radical scavenging effects in human body, imparting anti-cancerous property to Spirulina (Babu, 1995, Nutrition and Cancer Vol. 24: 197-202; Dasgupta et al, 2001, Mol. Cell Biochem. Vol. 226: 27-38).

D. Thesis, University of Mysore, India) where a commercial grade fertilizer by name "Suphala" (Madras fertilizers Ltd., India) was used having the following medium combination, all in grams per litre Sodium bicarbonate 10.0, Suphala (N: P: K = 15:15:15) 1.0, Magnesium sulphate 0.20, and water to make up to 1L.

Objects of the present Invention

The main object of the present invention is to provide a seawater based medium composition for Spirulina production.

Another object of the present invention is to obtain growth of Spirulina in synthetic or natural seawater.

Another object of the present invention is to produce Spirulina with most essential constituents comparable to the culture growth using Zarrouk's or CFTRI medium.

Summary of the Present Invention

The present invention relates to an economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v seawater and composition thereof.

Detailed description of the present invention

Accordingly, the present invention relates to an economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v seawater and composition thereof.

An economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v seawater, said method comprising steps of:

- growing the spirulina in agar slants using standard Zarrouk's medium at temperature ranging between 25 to 35^{0}C, illumination ranging between

1000 to 2000 lux, with photoperiod of 12 to 16 hours per day for total cultivation period ranging between 25 to 40 days to obtain a culture,

- Transferring the culture to seawater-based medium composition with initial optical density of about 0.1 at about 560 nm,
- growing the culture of step (b) at temperature ranging between 25 to 35^0C under 20 to 30 Klux illumination for a photoperiod ranging between 12 to 16 hours per day for a time duration that is required to reach the optical density of about 1.0 at about 560 nm,
- transferring the culture of optical density 1.0 to an open cement raceway filled with the seawater-based composition medium to make its optical density to about 0.1 at about 560 nm,
- agitating the culture of step (d) at the culture velocity ranging between 20-25 cms/sec, at temperature ranging between 25 to 35^0C, under illumination ranging between 30-45 Klux for time duration till the optical density of the culture reaches the value of about 2.0,
- harvesting the culture at to obtain the mass produce of the Spirulina.

In another embodiment of the present invention, wherein the time duration of step (c) is about 6 to 12 days.

In another embodiment of the present invention, wherein the culture is agitated with paddle wheel.

In another embodiment of the present invention, wherein the culturing is in carboy.

In another embodiment of the present invention, wherein the method using seawater for culturing makes the method economical.

In another embodiment of the present invention, wherein the seawater can be both natural and synthetic.

In another embodiment of the present invention, wherein the present invention also relates to a seawater based medium composition useful for mass production of spirulina, said composition comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v in seawater.

In another embodiment of the present invention, wherein the composition is of pH ranging between 6.5 to 8.0.

Seawater is one of the widely available natural resource, which can be utilized for various purposes such as , cultivation of marine organisms like, seaweeds, fish and marine algae. Seawater is rich in various nutrients, which supports algal life (eg. Dunaliella salina, Spirulina). Since seawater contains less sodium bicarbonate, sodium nitrate and phosphorous, therefore seawater supplementation with these nutrient can serve as complete medium and economic source of Spirulina cultivation. This facilitates cultivation of Spirulina in coastal region using seawater.

Accordingly, the present invention provides a seawater based medium composition for Spirulina production., which comprises of a growing the stock cell culture of Spirulina platensis in agar slants using standard Zarrouk's medium, at 25-35° C under 1000 – 2000 lux illumination for a photoperiod of 12-14h per day for a total cultivation period of one month, transferring the cells from stock culture to liquid medium in carboy where the chemical composition of the synthetic sea water / sea water supplemented medium containing Sodium bicarbonate 15.0 to 32.0, Sodium nitrate 2.0 to 9.0 , Sodium chloride 25 to 62, Magnesium sulphate 5.0 to 18.0 , Magnesium chloride 4.0 to 15.0, Potassium chloride 0.5to 3.4, Boric acid 0.01 to 0.10, Suphala 0.4 to 3.0 values are gm per liter basis of the total salt and the volume made up with domestic water, ensuring the initial optical density of 0.1 at 560nm and growing Spirulina cells at 25-35°C under 20-30 Kilolux illumination for a photoperiod of 12-16h per day for a total cultivation period of about one week or till the culture reaches 2.0 optical density at 560 nm, transferring the Spirulina platensis culture from carboy to open cement raceway tank of conventional type filled with liquid medium containing similar salt composition and filling the tank upto 12-20 cm depth , ensuring the initial optical density of 0.1 at 560nm and growing Spirulina cells at 25-35°C, and agitating the culture with a conventional paddle wheel at a speed of 8-12 rotation per minute under 30-45 Klux illumination for a photoperiod of 8-10h per day for a total cultivation period of about one week or till the culture reaches 2.0 optical density at 560 nm, harvesting the biomass followed by drying using standard process conditions, where the biomass is equally rich in nutrients as that of Zarrouk's medium.

In an embodiment of the present invention, the stock culture of Spirulina platensis selected from a group of stains grown in agar slants using standard Zarrouk's medium, at 25-35° C under 1000 – 2000 lux illumination for a photoperiod of 12-16h per day for a total cultivation period of one month.

In another embodiment of the present invention of the cultures are transferred to carboy where the chemical composition of the medium includes Sodium bicarbonate 15.0 to 32.0, Sodium nitrate 2.0 to 9.0, and Potassium phosphate 0.1 to 2 g/l in sea water. Initial optical density of culture 0.1 at 560nm and growing Spirulina cells at 25-35° C under 20-30 Klux illumination for a photoperiod of 12-16h per day for a total cultivation period of about 10 days or till the culture reaches 1.0 optical density at 560 nm.

In another embodiment of the present invention the cultures are transferred from stock culture to liquid medium in carboy containing sea water supplemented with Sodium bicarbonate 10.0g, Suphala (Agricultural fertilizer N:P: K::15:15:15)0.5g ,Urea 0.2g in 1L of sea water, ensuring the initial optical density of 0.1 at 560nm and growing Spirulina cells at 25-35° C under 20-30 Klux illumination for a photoperiod of 12-14h per day for a total cultivation period of about one week or till the culture reaches 1.0 optical density at 560 nm.

In yet another embodiment of the present invention the cultures of Spirulina platensis from carboy are transferred to open cement raceway tank of conventional type filled with liquid medium of chemical composition involving Sodium bicarbonate 15.0 to 32.0, Suphala (NPK 15:15:15), 1.0g/l , Sodium chloride 2.5 to 30, Magnesium sulphate 4.0 to 18.0, Magnesium chloride 4.0 to 15.0, Potassium chloride 0.5to 3.4, Boric acid 0.01 to 0.10, Suphala 0.4 to 3.0 and the volume made up with domestic water and filling the tank up to 12-20 cm depth, ensuring the initial optical density of 0.1 at 560nm and growing Spirulina cells at 25-35° C, and agitating the culture with a conventional paddle wheel at a speed of 8-12 rotation per minute under 30-45 K.Lux illumination for a photoperiod of 8-10h per day for a total cultivation period of about one week or till the culture reaches 2.0 optical density at 560 nm.

In yet another embodiment of the present invention, the harvesting and drying of the biomass was done as per the standard industrial procedure followed and where the biomass is analyzed for the nutritional composition (table 2).

The following examples are given by way of illustration of the present invention therefore, should not be considered to limit the scope of the present invention.

Example 1

Among the different strains of Spirulina platensis, the strain SP-6 was selected as a best performing strain under various light, temperature and nutrient conditions and preserved for future use.

The nutrient medium used for maintaining the stock culture, namely the Zarrouk's culture medium, was prepared using the following chemicals:

Composition of medium for maintenance of Spirulina strain on agar slants

Ingredients	Quantity (grams per Litre)	% total nutrients Wt/wt
Sodium bicarbonate	16.80	76.19%
Sodium nitrate	2.50	11.34%
Sodium chloride	1.00	4.54%
Potassium sulphate	1.00	4.54%
Potassium phosphate	0.50	2.27%
Magnesium sulphate	0.20	0.907%
Calcium chloride	0.04	0.182%
Ferrous sulphate	0.01	0.045%
A5 Solution	1 ml	1mL:1000mL

A5 solution - (all in g/L) H_3BO_3-2.86; $MnCl_2$ $4H_2O$ - 1.80, $ZnSO_4$ $7H_2O$- 0.22; MoO_3- 0.01; $CuSO_4$- $5H_2O$- 0.08

The chemicals are dissolved in distilled water and the final volume is made up to one litre, the pH is adjusted to about 8.5, after which 10 grams of agar is added, and agar slants preparation and Spirulina culture inoculations are done as per standard microbiological methods. Cultures are maintained at a temperature ranging from 20 to 22°C and an illumination cycle of about 16 h of white light of 1000 – 2000 lux and 8 h in dark is maintained. The cells are allowed to grow for a period of 25-40 days.

Example 2

For indoor liquid inoculum development, the Spirulina cells cultured in agar slants grown as described in example 1 are taken by aseptically scraping the surface of the medium with a spatula and the cells are suspend in sea water (obtained from coastal region of Mangalore, Karnataka State). However, studies were further carried with synthetic seawater prepared as per the composition below medium present in glass carboys of 5L capacity, where the medium has the following chemical composition:

Composition of sea water and synthetic sea water

Chemical	Composition of sea water* Gams per liter	Modified sea water medium gams per liter
Sodium bicarbonate	0.16	10.00
Sodium nitrate	0.42	2.5
Sodium chloride	23.38	23.38
Potassium hydrogen phosphate	0.06	----
Magnesium sulphate	4.9	4.9
Magnesium chloride	4.0	4.0
Boric acid	0.01	0.012
Potassium chloride	0.74	0.74
Calcium chloride	1.47	1.47
Suphala N: P: K (15:15:15)	---	1.0
Urea	---	0.1
Micronutrients	----	10 ml
Cheated iron	----	3ml

(* Applied Environmental Microbiology, 43:735-739).

The aforementioned table shows the standard composition of seawater (left column) and also, the final composition of the seawater after incorporating the supplements of the seawater-based medium composition.

Where the compounds (grams) are dissolved in domestic water to make up a final volume of 1 litre, and an appropriate volume of medium is thus prepared. The suspended cells are incubated at ambient condition with daily 3 to 4 times by manual agitation. The initial optical density of 0.1 at 560nm is ensured and further cell growth is allowed at 25-35°C under 20-30 Klux illumination for a photoperiod of 12-14h per day for a total cultivation period of about one week or till the culture reaches 1.0 optical density at 560 nm. (OD 1.0, which corresponds to 900-1000 mg dry algae/liter). For further increase in the inoculum volume, the culture broth of optical density 1.0 is diluted 10 times using synthetic sea medium, and the required level of inoculum is thus developed.

Further, the presence of urea and calcium chloride is optional in the final composition of the modified seawater.

Example 3

The cell culture broth developed as described in example 2 is subsequently used to produce large quantity of cell biomass in open conventional raceway ponds. The basic

design we suggest for mass cultivation of Spirulina consists of oblong, shallow raceway type pond / tank stirred with paddle wheel. Commercial pond area varies between 5 to 5000 m^2. The Spirulina cultures from several carboys is transferred to 5 m^2 tank with the addition of 0.90 m^3 water fortified with the nutrients of medium the composition of which as above:

The following factors are important for large-scale cultivation of Spirulina.

Optimum condition

a. Light (Kilolux)	35-45
b. Temperature	25-35°C
c. Inoculum size of OD	0.1
d. Nutrient	new seawater medium
e. Culture depth (cms)	18-20
f. Mixing/agitation using Conventional paddle wheel (culture velocity cms/sec)	20-25

Growth of Spirulina at sea water medium.

Media composition:

Seawater based media composition for the production of Spirulina comprising Sodium bicarbonate1.2-3.0 %; Nitrogen 0.1-0.3 %; Phosphorus 0.1-0.3 %; Potassium 0.1-0.3 % in seawater and pH of 6.5-8.0.

	Optical Density of culture on Day					
	Initial Day	2	4	6	8	10
CFTRI medium	0.1	0.2	0.25	0.30	0.42	0.55
Sea water	0.1	0.15	0.20	0.25	0.40	0.50
Synthetic sea water	0.1	0.15	0.20	0.30	0.40	0.50

Proximate composition of Spirulina cultivated in different media (in % value).

Constituents	Zorrouk's medium	Sea water medium	Synthetic Sea water medium
Moisture	8.0	6.2	6.9
Total protein	64.5	66.3	67.9
Lipids	3.1	4.8	4.9
Crude Fiber	4.2	3.8	3.6

Carbohydrate	10.5	9.6	8.9
Ash	7.4	8.1	7.9

Harvesting the Spirulina cells was done by gravity filter or any other available conventional method. Harvested biomass was washed with 0.01% hydrochloric acid in a solution in order to remove surface coated $NaHCO_3$ and bound minerals. Moreover, the salt concentration of the rinsing solution, which is similar to one of the culture medium, permits to avoid a breaking of the cellular membranes due to osmotic pressure. The biomass is finally rinsed with tap water.

The harvested Spirulina biomass can be lyophilized or sprayed, preferably immediately, in order to avoid a secondary contamination. Spirulina may also be dried by any other standard dying method such as spray-drying, vacuum-drying, cross-flow drying, sun-drying etc.

The main Advantages of present investigations are,

1. By incorporating seawater in place of normal water we can reduce the cost of production.
2. The yield and quality of the Spirulina produced by this media is as good as that of the conventional ones and this will make the production economical.

Claims:

1. An economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v seawater, said method comprising steps of:
   a. growing the spirulina in agar slants using standard Zarrouk's medium at temperature ranging between 25 to 35^{0}C, illumination ranging between 1000 to 2000 lux, with photoperiod of 12 to 16 hours per day for total cultivation period ranging between 25 to 40 days to obtain a culture,
   b. Transferring the culture to seawater-based medium composition with initial optical density of about 0.1 at about 560 nm,
   c. growing the culture of step (b) at temperature ranging between 25 to 35^{0}C under 20 to 30 Klux illumination for a photoperiod ranging between 12 to 16 hours per day for a time duration that is required to reach the optical density of about 1.0 at about 560 nm,
   d. transferring the culture of optical density 1.0 to an open cement raceway filled with the seawater-based composition medium to make its optical density to about 0.1 at about 560 nm,
   e. agitating the culture of step (d) at the culture velocity ranging between 20-25 cms/sec, at temperature ranging between 25 to 35^{0}C, under illumination ranging between 30-45 Klux for time duration till the optical density of the culture reaches the value of about 2.0,
   f. harvesting the culture at to obtain the mass produce of the Spirulina.
2. A method as claimed in claim 1, wherein the time duration of step (c) is about 6 to 12 days.
3. A method as claimed in claim 1, wherein the culture is agitated with paddle wheel.
4. A method as claimed in claim 1, wherein the culturing is in carboy.
5. A method as claimed in claim 1, wherein the method using seawater for culturing makes the method economical.

6. A method as claimed in claim 1, wherein the seawater can be both natural and synthetic.

7. A seawater based medium composition useful for mass production of spirulina, said composition comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v in seawater.

8. A composition as claimed in claim 7, wherein the composition is of pH ranging between 6.5 to 8.0.

AMENDED CLAIMS

[received by the International Bureau on 08 August 2005 (08.08.05);
original claims 1 and 7 amended; original claim 8 deleted; remaining claims unchanged (2 pages)]

Claims:

1. An economical and efficient method for mass production of spirulina using seawater-based medium composition of pH ranging between 6.5 and 8.0 comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v seawater, said method comprising steps of:
    a. growing the spirulina in agar slants using standard Zarrouk's medium at temperature ranging between 25 to 35^{0}C, illumination ranging between 1000 to 2000 lux, with photoperiod of 12 to 16 hours per day for total cultivation period ranging between 25 to 40 days to obtain a culture,
    b. Transferring the culture to seawater-based medium composition with initial optical density of about 0.1 at about 560 nm,
    c. growing the culture of step (b) at temperature ranging between 25 to 35^{0}C under 20 to 30 Klux illumination for a photoperiod ranging between 12 to 16 hours per day for a time duration that is required to reach the optical density of about 1.0 at about 560 nm,
    d. transferring the culture of optical density 1.0 to an open cement raceway filled with the seawater-based composition medium to make its optical density to about 0.1 at about 560 nm,
    e. agitating the culture of step (d) at the culture velocity ranging between 20-25 cms/sec, at temperature ranging between 25 to 35^{0}C, under illumination ranging between 30-45 Klux for time duration till the optical density of the culture reaches the value of about 2.0, and
    f. harvesting the culture at to obtain the mass produce of the Spirulina.
2. A method as claimed in claim 1, wherein the time duration of step (c) is about 6 to 12 days.
3. A method as claimed in claim 1, wherein the culture is agitated with paddle wheel.
4. A method as claimed in claim 1, wherein the culturing is in carboy.

5. A method as claimed in claim 1, wherein the method using seawater for culturing makes the method economical.

6. A method as claimed in claim 1, wherein the seawater can be both natural and synthetic.

7. A seawater based medium composition useful for mass production of spirulina, said composition comprising sodium bicarbonate of concentration ranging between 1.2 to 3.0 % w/v, nitrogen of concentration ranging between 0.1 to 0.3% w/v, phosphorus of concentration ranging between 0.1 to 0.3% w/v, potassium of concentration ranging between 0.1 to 0.3% w/v in seawater and having pH ranging between 6.5 to 8.0.

STATEMENT UNDER ARTICLE 19(1)

The Applicant respectfully submits that the claims are suitably amended to overcome the anticipation and obviousness rejections set by the Examiner. Further, the Applicant would like to draw the Examiner's attention to the fact that the method of instant invention facilitates mass production of spirulina. The reference to yield and quality on page 13 of the description with respect to conventional media was made to reflect that the spirulina produced by instant methodology is fit for consumption. It was a sole objective of statement made on page 13. Thus, the statement should be taken in right spirit and should not be used to undermine the high yield of the spirulina obtained in the instant invention.

Furthermore, claim 1 of the invention clearly shows that the method is indeed an efficient method of mass production of spirulina. As you would notice that the optical density of the spirulina culture rapidly increased from 0.1 to 1.0 and thereafter from 0.1 to 2.0 in no time. Thus, the novel composition of the instant application and the sequence of the steps of the methodology facilitate high yield of spirulina. However, none of the cited arts provide any motivation or clue to a person skilled in the art to arrive at the invention of the instant application.

Therefore, the Examiner respectfully requested to withdraw the rejections and issue a favorable report.

INTERNATIONAL SEARCH REPORT

International Application No
T/IB2004/002643

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FAUCHER O ET AL: "Utilization of seawater-urea as a culture medium for Spirulina maxima." June 1979 (1979-06), CANADIAN JOURNAL OF MICROBIOLOGY. JUN 1979, VOL. 25, NR. 6, PAGE(S) 752 - 759 , XP009040238 ISSN: 0008-4166	7
Y	the whole document	1-6,8
Y	COSTA JORGE ALBERTO VIEIRA ET AL: "Improving Spirulina platensis biomass yield using a fed-batch process." BIORESOURCE TECHNOLOGY. MAY 2004, vol. 92, no. 3, May 2004 (2004-05), pages 237-241, XP002306500 ISSN: 0960-8524 abstract page 238, paragraph 2.1-2.2; table 1	1-6,8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 2004	07/12/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kaas, V

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
Γ/IB2004/002643

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003/017558 A1 (DURAND-CHASTEL HUBERT ET AL) 23 January 2003 (2003-01-23) paragraphs '0049! - '0098!	1-8
A	EP 1 138 757 A (MICRO GAIA CO LTD) 4 October 2001 (2001-10-04) example 3	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
T/IB2004/002643

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 1138757 A1	04-10-2001
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