

Comparative studies on production of *Spirulina platensis* on the standard and newly formulated alternative medium



Amala. K and N. Ramanathan
Department of Microbiology, Annamalai University,
Annamalai Nagar-608 002, Tamilnadu-India.

Abstract:

Aim

This present study was aimed as formulation of new cost effective RME (rice mill effluent medium) for the cultivation of *Spirulina* single cell protein and compared with standard Zarrouk's medium to reduce *Spirulina* production cost.

Methods and results

Two strains of *Spirulina platensis* S3 and S4 were isolated from local temple ponds and characterized. The RME medium was formulated by using NaNO_3 as a nitrogen source and K_2HPO_4 as a phosphorus source. Effect of different concentration of NaNO_3 (1.00, 1.50, 2.00 and 2.50 gL^{-1}) and K_2HPO_4 (0.1, 0.3, 0.5 and 0.7 gL^{-1}) and biomass production in the RME medium was examined and found NaNO_3 at 2.00 gL^{-1} and K_2HPO_4 at 0.3 gL^{-1} recorded higher biomass.

Conclusion

The growth rate in terms of dry biomass, chlorophyll, protein and lipid content in RME medium was compared with Zarrouk's medium and recorded almost equal values.

Significance and impact of this study

From the scale up point of view, the RME medium was found to be highly economical, locally available, eco-friendly and cost effective medium, since it is cheaper than Zarrouk's medium.

Key words: *Spirulina*, RME medium, Biomass, Protein, Chlorophyll, Lipid.

Introduction

Spirulina platensis is a planktonic photosynthetic filamentous cyanobacterium that forms massive populations in tropical

water bodies which have a high alkaline pH value up to 11.0. The cyanobacterium *Spirulina* contains 74% dry weight of proteins, along with high concentrations of

minerals, pigments, unsaturated fatty-acids and vitamins (Cohen, 1997), because of which it is used as a dietary supplement, nutrient source in food, feed and pharmaceutical industries especially in developing countries. It can grow in a wide range of environments like soil, sand, marshes, brackish water, sea water and fresh water (Ciferri *et al.*, 1983). *Spirulina* is currently mass produced as a monoculture in the outdoor cultivation system (Venkataraman *et al.*, 1995). The first synthetic medium formulated for cultivation of *Spirulina* was Zarrouk's medium (Zarrouk, 1966) which is still used as standard medium. Subsequently many media were developed using sea water, sewage water, industrial effluent and clean water. Olguin *et al.* 2001 reported that *Spirulina* has potential to reduce BOD of high carbon containing waste water due to its mixotropic nature. Rice mill effluent (RME) is a rich source of starch and other nutrients can support profuse growth and aid in mass multiplication of *Spirulina*. In the present investigation was carried out to formulate a medium, based on locally available starch rich RME. A new cost effective RME medium was developed by reducing its BOD and supplemented with various concentration of N and P at different pH to generate valuable *Spirulina* single cell protein biomass compared to the Zarrouk's medium (ZM).

Materials and methods

Isolation of *Spirulina platensis* strains and characterized

The microalgae *Spirulina* was isolated from ponds in two different locations viz., Puducherry and Thiruvannamalai and designated S3 and S4. These strains were characterized based on parameters like average no. of spirals, direction of helix, distance between

spirals, diameter of spirals, width, length and shape of the spirals, pH tolerance, temperature tolerance and habitat. Morphological characters were observed under microscope by using micrometric method. *Spirulina* sample smear was prepared on the clean glass slide and viewed under compound microscope using. The object of the calibration was determined by the width in micrometers of each ocular scale division, calibrated against the stage micrometer scale and size of the object (*Spirulina*) was calculated. These S3 and S4 strains were grown in Zarrouk's medium under laboratory condition at 30°C in the light chamber for 30 days for further studies.

Formulation of cost effective medium (RME)

For laboratory experiment the rice mill effluent was collected from Parvathi rice mill Sethiyathopu in Cuddalore district, Tamilnadu. The physico-chemical characters of the effluent were analyzed. Dissolved oxygen, biological oxygen demand and chemical oxygen demand were determined by using the procedure of Manogari *et al.*, 2008. Electrical conductivity, salinity, total dissolved solids, and temperature and other micronutrient elements (sodium, calcium, potassium and magnesium) were analyzed by an atomic absorption spectrometer (AAS) Department of Soil Science, Annamalai University. The collected effluent was filtered through Whatmann no. 1 filter paper, to remove the dust particles. The high BOD level was decreased by Millipore (0.45µm pore diameter) filtration technique. It was stored in 4°C for further RME liquid medium formulation. For alkaline pH (9.5) was maintained by using 10gL⁻¹ NaHCO₃ was used. For formulating a new and cost-effective RME medium, in the first step was the filtered rice mill effluent was taken in eight Erlenmeyer conical flask and *Spirulina platensis* development were postulated by

changing in the various concentration of NaNO_3 (1.00, 1.50, 2.00 and 2.50 gL^{-1}) and K_2HPO_4 (0.1, 0.3, 0.5 and 0.7 gL^{-1}) for pH regimes (9.0, 9.5 and 10.0). It was sterilized in an autoclave at 121°C @ 15 lbs pressure. *Spirulina platensis* cultivation were carried out in two liter Erlenmeyer flasks containing 1000ml of RME medium, placed on the orbital shaker at 35°C . S3 and S4 strains was inoculated to the medium and incubated for 30 days in light chamber after incubation the dry weight was estimated. The dry weight was measured by the algal mat was collected by filtered through Whatman No.1 filter paper and dried for 1 hr. The filtered wet biomass was then washed with two volumes of distilled water, dried as above and weighed. The biomass concentrations in the cultures were determined through the cell weight measured by the method of Vonshak *et al.*, 1982.

***Spirulina platensis* single cell protein production in rice mill effluent (RME) medium compare with routine Zarrouk's (ZM) medium**

One liter of Rice Mill Medium (RME) and Zarrouk's Medium (ZM) were prepared and transferred in two liter Erlenmeyer conical flasks, standard inoculums (50 ml) of two strains (S3 and S4) were separately inoculated and maintained at room temperature in the light chamber for 30 days. After 30 days of growth the parameters such as dry biomass, chlorophyll content, protein content and lipid content were estimated. Chlorophyll was extracted by use of 90% methanol for 10 min in a water bath at 70°C . Its amount was determined by spectrophotometer at 665 nm and 750 nm (Youngman 1978). Quantitative estimation of protein was done using the procedure of Micro-Kjeldahl method against bovine serum albumin as a standard. Protein values are expressed as mg

ml^{-1} of culture suspension. Lipid content was evaluated using Folch's method (Folch and Lees, 1957) by extracting lipids in a 2:1 chloroform / methanol mixture and determining lipid content gravimetrically.

Result

The collected algal samples S3 and S4 were identified as *Spirulina platensis*. Each had a different morphology. The microscopic views of these two strains are shown in figure 1 and figure 2. The strain S3 had short filaments (0.2-0.25 mm long) contains 5-8 tightly coiled and right helix. Strain S4 consisted of very long filaments (1-5mm long) contain 5-7 loose coiled with right helix and all the morphological characters like distance between the spirals, diameter, width, length and shape of spirals were presented in Table 1. *Spirulina platensis* can grow at alkaline pH 9.5 and 35°C . The two *Spirulina platensis* strains S3 and S4 were grown in Zarrouk's medium for further analysis for 30 days in light chamber. These morphological differences influenced the harvesting performances.

The paddy soaked waste water was collected from Parvathy rice mill from Sethiyathopu at Cuddalore district, Tamilnadu, India. The waste water showed an acidic pH (7.5) with low concentration of DO (0.2 – 1.0), high BOD (530), COD (1650), nitrate (2.5mg), sodium (100.09 ppm), calcium (50.36 ppm), potassium (8 ppm), magnesium (43.78 ppm) were much higher than the recommended standard set by ISI (1977). The high BOD level was decreased by Millipore (0.45 μm pore diameter) filtration technique. Moreover the waste water was rich in sodium, Calcium and magnesium.

Spirulina platensis strains were inoculated in the RME liquid medium. In nitrogen nutrient used RME liquid medium, the higher biomass productivity (P_{max}) was observed in S3 followed by S4 strains at

2gL^{-1} NaNO_3 . In S3 strain after 30 days the biomass productivity (P_{max}) was observed 2.500gL^{-1} at pH 9.5. In S4 strain after 30 days the biomass productivity (P_{max}) was observed and 2.325gL^{-1} at pH 9.5. These datas are given in Table 2. (Fig 3).

The biomass productivity (P_{max}) of phosphorus nutrient used RME medium the highest biomass was observed in 0.3gL^{-1} K_2HPO_4 . In S3 strain the biomass productivity (P_{max}) was estimated 1.500gL^{-1} at pH 9.5. In S4 strain biomass was 1.490gL^{-1} at pH 9.5. these datas are shown in Table 3. (Fig 4). The increased biomass productivity (P_{max}) was observed in RME medium in nitrogen (2gL^{-1} NaNO_3) and phosphorus nutrient (0.3gL^{-1} K_2HPO_4), based on high productivity these two concentration of nutrient was used for RME medium development for mass cultivation of SCP production.

The low cost RME medium was standardized and compared with regular ZM medium composition was shown in Table 4. The developed RME medium was compared with standard medium (Zarrouk's medium), all the growth parameters were higher in S3 followed by S4. In RME medium In S3 strains dry biomass, protein, chlorophyll and lipid content were 3.245gL^{-1} , 1.900, 0.350 and 0.260mgml^{-1} . In S4 strains dry weight, protein, chlorophyll and lipid content were and 3.012gL^{-1} , 1.630, 0.312 and 0.230mg ml^{-1} respectively. In Zarrouk's medium the growth parameters of S3 strains, dry biomass, protein, chlorophyll and lipid content were 2.920gL^{-1} , 1.500, 0.265 and 0.218mg ml^{-1} respectively. In S4 strains dry biomass, protein, chlorophyll, and lipid content were 2.420gL^{-1} , 1.220, 0.211 and 0.075mg ml^{-1} respectively. These data are presented in Table 5. All the growth parameters were almost equal value in RME medium when compared with ZM medium.

Table.1. General Characteristics of *Spirulina platensis* strains

| Characters | S3 | S4 |
|--|----------------------------------|----------------------------------|
| Average number of spirals | 5-8 | 5-7 |
| Direction of helix | Right | Right |
| Distance between spirals (μm) | 0.5 | 0.2 |
| Diameter of spirals (mm) | 0.2-0.25 | 1-5 |
| Width of spirals (μm) | 45 | 18 |
| Shape of spirals | Tight | Loose |
| pH tolerance | Alkaline | Alkaline |
| Temperature tolerance | Mesophile (35°C) | Mesophile (35°C) |

Table.2. Changes in the biomass of *Spirulina platensis* in different NaNO_3 concentration at various pH

| NaNO_3 (gL^{-1}) | Biomass productivity (P_{max}) of <i>Spirulina platensis</i> (After 30 days) (gL^{-1}) | | | | | |
|--------------------------------------|--|-------|-------|-------|-------|-------|
| | S3 | | | S4 | | |
| pH | 9 | 9.5 | 10 | 9 | 9.5 | 10 |
| 1.00 | 1.435 | 1.665 | 1.523 | 1.357 | 1.523 | 1.452 |
| 1.50 | 1.625 | 1.825 | 1.700 | 1.523 | 1.753 | 1.520 |
| 2.00 | 1.690 | 2.500 | 1.983 | 1.553 | 2.325 | 1.825 |
| 2.50 | 1.425 | 1.900 | 1.823 | 1.421 | 1.823 | 1.601 |

| | | | | | | |
|----------------|------------|------------|------------|------------|------------|------------|
| Mean | 1.5 437 | 1.9 725 | 1.7 572 | 1.4 635 | 1.8 56 | 1.5 995 |
| SED | 0.0 670 | 0.1 825 | 0.0 972 | 0.0 453 | 0.1 689 | 0.0 811 |
| CD (p=0.05) | 0.1 340 | 0.3 65 | 0.1 944 | 0.0 960 | 0.3 378 | 0.1 622 |

* Base medium contains 1000g^L⁻¹ rice mill effluent, 10 g^L⁻¹ NaHCO₃ and pH 9.5.

Table.3. Changes in the biomass of *Spirulina platensis* in different K₂HPO₄ Concentration at various pH

| K ₂ HPO ₄ (g ^L ⁻¹) | Biomass productivity (P _{max}) of <i>Spirulina platensis</i> (After 30 days) (g ^L ⁻¹) | | | | | |
|---|---|-----------|-----------|-----------|-----------|-----------|
| | S3 | | | S4 | | |
| pH | 9 | 9.5 | 10 | 9 | 9.5 | 10 |
| 0.1 | 0.4 00 | 1.4 20 | 0.3 25 | 0.3 85 | 1.3 05 | 0.3 20 |
| 0.3 | 1.4 60 | 1.5 00 | 1.4 83 | 1.3 23 | 1.4 90 | 1.4 00 |
| 0.5 | 1.4 32 | 1.4 90 | 1.3 56 | 1.3 20 | 1.4 75 | 1.2 51 |
| 0.7 | 1.3 50 | 1.4 50 | 1.4 03 | 1.0 98 | 1.3 25 | 1.0 00 |
| Mean | 1.1 60 | 1.2 15 | 1.1 41 | 1.2 48 | 1.3 98 | 1.2 69 |
| SED | 0.2 54 | 0.2 65 | 0.2 73 | 0.0 52 | 0.0 48 | 0.0 97 |
| CD (p=0.05) | 0.5 08 | 0.5 30 | 0.5 46 | 0.1 04 | 0.0 96 | 0.1 94 |

* Base medium contains 1000g^L⁻¹ rice mill effluent, 10 g^L⁻¹ NaHCO₃ and pH 9.5.

Table.4. Comparison of standard (ZM) and newly formulated low cost (RME) medium

| Zarrouk's medium (ZM) | Composition (g ^L ⁻¹) | Rice mill effluent medium (RME) | Composition (g ^L ⁻¹) |
|--------------------------------------|---|--------------------------------------|---|
| NaHCO ₃ | 16.8 | NaHCO ₃ | 10 |
| NaNO ₃ | 2.5 | NaNO ₃ | 2.0 |
| K ₂ HPO ₄ | 0.5 | K ₂ HPO ₄ | 0.3 |
| K ₂ SO ₄ | 1.0 | K ₂ SO ₄ | - |
| NaCl | 1.0 | NaCl | - |
| MgSO ₄ .7H ₂ O | 0.2 | MgSO ₄ .7H ₂ O | - |
| CaCl ₂ .2H ₂ O | 0.04 | CaCl ₂ .2H ₂ O | - |
| FeSO ₄ .7H ₂ O | 0.01 | FeSO ₄ .7H ₂ O | - |
| EDTA | 0.08 | EDTA | - |
| pH | 9.5 | pH | 9.5 |
| Distilled water | 1000ml | Rice mill effluent | 1000ml |

Table.5. Response of *Spirulina platensis* on Rice mill effluent medium (RME) compared with standard medium (ZM) under laboratory condition.

| Strains | Parameters | (RME) Rice mill effluent medium | (ZM) Zarrouk's medium |
|---------|------------|---------------------------------|-----------------------|
|---------|------------|---------------------------------|-----------------------|

| | | | |
|----|--|-------|-------|
| S3 | Dry biomass (P_{max}) ($g L^{-1}$) | 3.245 | 2.920 |
| | Protein ($mg ml^{-1}$) | 1.900 | 1.500 |
| | Chlorophyll ($mg ml^{-1}$) | 0.350 | 0.265 |
| | Lipid ($mg ml^{-1}$) | 0.260 | 0.218 |
| S4 | Dry biomass (P_{max}) ($g L^{-1}$) | 3.012 | 2.420 |
| | Protein ($mg ml^{-1}$) | 1.630 | 1.220 |
| | Chlorophyll ($mg ml^{-1}$) | 0.312 | 0.211 |
| | Lipid ($mg ml^{-1}$) | 0.230 | 0.175 |

Fig.1.Strain S3



Fig.3.Biomass productivity (P_{max}) of *Spirulina platensis* (After 30 days)

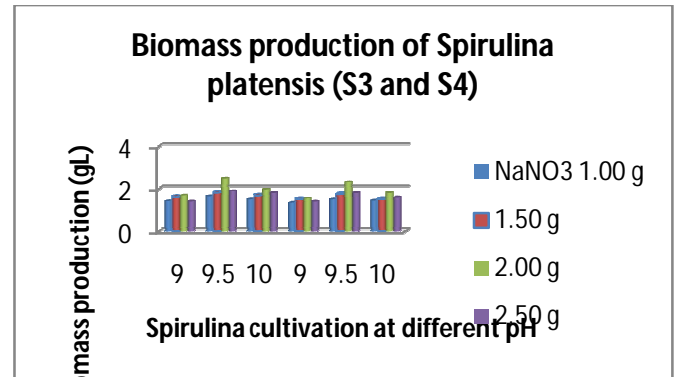
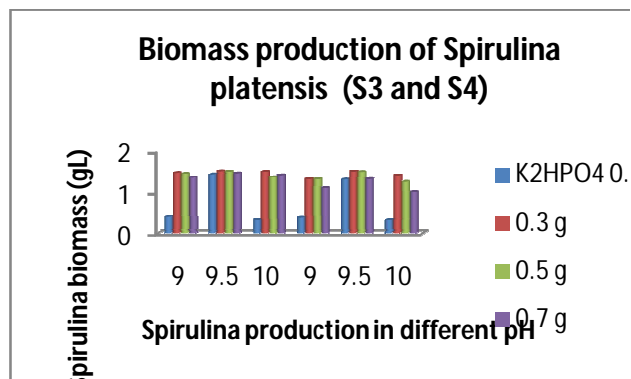


Fig.2.Strain S4
Fig.4.Biomass productivity (P_{max}) of *Spirulina platensis* (After 30 days)



Discussion

This microalga presented in naturally alkaline rich regions. In this present study the strains microalgae *Spirulina* was isolated from temple ponds in two natural temple ponds located in different local regions viz., Puducherry and Thiruvannamalai. Five cultures of *S. platensis* were used for the present study (CS-1, SM-2, S-10, S-20 and Sp). Three *S. platensis* cultures viz, SM-2, S-10 and S-20 were isolated from field soils of paddy breeding station and lands of TNAU, Coimbatore and the biomass production and biochemical constituents were compared with standard cultures CS-1 and SP obtained from algal laboratory, Madurai soundarapandian, 2008.

Large-scale production of *Spirulina platensis* very complicated one and their successful growth, the environment needs to be conditioned to meet as many of the essential requirement of the organisms. In tropical countries, especially developing countries such as India, emphasis is placed more on the production cost. Therefore, the present investigation was aimed towards the formulation of a cheaper medium for the growth of *Spirulina platensis*, by stepwise adding nutrients (of Zarrouk's medium) using locally available starch rice mill waste water. The rice mill waste water contains lot of nutrients for algal growth. Rice mill effluent does not contain toxic compounds

or pathogenic bacteria; but it can contain the traces of pesticide overdose, more so in the third world countries. Discharge into soil or water bodies on a continuous basis causes major environmental problems. The stagnant water emits off-odor. Off-odor during soaking can be generated due to fermentative changes. The growth of natural flora is effected due to discharge of effluent into the soil. Since it is rich in nutrients it triggers the growth of algae in water bodies. Literature reports on physico-chemical analysis of industrial effluents reveal that rice mill effluents possess low BOD, COD and organic matter in comparison to effluents generated by other industries, however, in quantitative terms it compels for treatment before disposal. For this reason the rice mill waste water was collected and supplemented with various nitrogen and phosphorus nutrient and formulated a new RME medium for cultivation of *Spirulina platensis*. This rice mill waste water was available in local mini rice mills. The RME medium formulation first step involved, the RME base medium was prepared by Table 2 and 3. The various concentration of NaNO₃ (1.00, 1.50, 2.00 and 2.50 gL⁻¹) and K₂HPO₄ (0.1, 0.3, 0.5 and 0.7 gL⁻¹) was added in RME base medium for select which concentration of NaNO₃ and K₂HPO₄ was suitable for RME medium formulation. Nitrogen is required for synthesis of the amino acids, which make up proteins and other cellular components such as phycocyanin. However, at 30°C, nitrogen uptake seems to be limited because the experiments with higher concentrations of sodium nitrate (1.875 and 2.500 gL⁻¹) showed no increase in the level of protein, while at 35°C an increase was observed. With regard to lipids, higher concentrations of sodium nitrate resulted in an increase in lipids, similar to that which was obtained by Manabe *et al.*, (1992), who

demonstrated higher total lipid in *Spirulina* grown in media containing up to 25mM of ammonium chloride. However, Piorreck *et al.*, (1984) found that the concentration of nitrogen had little influence on the total lipid and fatty acid composition of some cyanobacteria, the influence of nitrogen being more marked in eukaryotic algae. Piorreck *et al.* (1984) also found that for cyanobacteria, total lipid content remained constant at all nitrogen concentrations studied (0.001–0.1% of potassium nitrate), with only a slight increase occurring at the highest nitrogen concentration tested (0.1%). Olguín *et al.* (2001) observed a higher content of total lipids in *Spirulina* growing in Zarrouk's medium as compared to *Spirulina* cultivated under conditions of nitrogen starvation. In this study 0.3 gL⁻¹ K₂HPO₄ was select for RME medium formulation, in this concentration was found higher biomass 1.5000 gL⁻¹. The optimum phosphate concentration at 0.5gL⁻¹ could be due to improvement of biomass production by *Spirulina platensis* (Costa *et al.*, 2002; Radmann *et al.*, 2007). Phosphorus is a major nutrient required for the growth of alga and determines its primary productivity. Mostert and Grobbelaar, 1981 have indicated the essential role of phosphorus in maintaining high production rates of microalgae mass cultures.

Rafiqul *et al.*, 2005, reported that *Spirulina platensis* production in Zarrouk's medium the dry biomass was reached 2.7gL⁻¹ at day 20. *Spirulina platensis* biomass was 1.2 gL⁻¹ in rice mill effluent supplemented with NaHCO₃ and NaNO₃ (Amala and Ramanathan, 2012). In this present study the dry biomass was found in RME medium was 3.245gL⁻¹ at 30th days.

Spirulina platensis has a high bicarbonate requirement, which acts not only as a carbon source but also helps to maintain alkaline conditions, and increase the growth of *Spirulina platensis*. Since

laboratory grade sodium bicarbonate is costly in the Indian context, in RME medium 10 gL⁻¹ compared with ZM medium 16.8 gL⁻¹. Therefore, the significant of the RME medium are clearly emphasized, not only as a low-cost alternative but also as a highly productive input, which can be profitably used by the rural population for large-scale biomass production of protein-rich *Spirulina platensis*. In tropical countries, especially developing countries such as India, emphasis is placed more on the production costs.

Conclusion

This investigation was taken up with the basic aim of providing a simple, locally available, eco-friendly and cost effective medium and the results clearly indicate that RME medium is compared with Zarrouk's medium with regards on the performance of *Spirulina platensis*, when evaluated the growth parameters like dry weight, chlorophyll content, protein content and lipid content were almost equal value. Therefore the present investigation was aimed towards the formulation of a new cheaper, cost effective RME medium for the growth of cyanobacterium *Spirulina platensis* (a rich source of proteins), using locally available rice mill waste water and create eco-friendly environment.

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Reference

1. Amala,K., Ramanathan, N., 2012. Mass cultivation of *Spirulina platensis* in Sea water and Rice mill effluent for the production of Single cell protein. Research journal of biological science. pp, 16-22.
2. Ciferri. O., 1983. *Spirulina* the edible microorganism. Microbiol Rev 47: 551- 578.
3. Cohen.Z., 1997. The chemicals of *Spirulina*. In: Vonshak, A(Ed), *Spirulina platensis* (Arthospira) physiology, cell biology and bio technology. pp, 175 – 204.
4. Costa, J.A.V., Colla, L.M., Filho, P.D., Kabke, K., Weber, A., 2002. Modeling of *Spirulina platensis* growth in fresh water using response surface methodology. World J.Microbiol. Biotechnol 18, 603–607.
5. Folch, J., and Lees, M., 1957. A simple method for isolation and purification of total lipids from animal tissues. Journal of Biological chemistry 226, 497 -509.
6. ISI (Indian Standards Institution), 1977. Tolerance limit for discharged of industrial effluents on land for irrigation. No. 3307, New Delhi.
7. Manabe, E., Hirano, M., Takano, H., Ishikawa-Doi, N., Sode, K., Matsunaga, T., 1992. Influence of ammonium chloride on growth and fatty acid production by *Spirulina platensis*. Applied Biochemistry and Biotechnology 34/35, 273–281.
8. Manogari. R., Daniel, D., A. Krastanov., 2008. Biodegradation of rice mill effluent by immobilized *pseudomonas* sp. Cells. Ecological engineering and environment protection, No 1, pp, 30-35.
9. Mostert,EW, Grobbelaar, JU., 1981. Protein manipulation of mass cultured algae. University of the Orange Free State Publications Series C3, 86–90.
10. Olguin. J., Sonia Galicia., Ofelia Angulo Guerrero., Elizabeth Hernandez., 2001. The effect of low light and nitrogen deficiency on the chemical composition of *Spirulina* sp. (*Arothrospira*) grown on digested pig waste, Bioresource Technology, 77, 19-24.
11. Piorreck, M., Baasch, K.H., Pohl, P., 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue green algae under different nitrogen regimes. Phytochemistry 23 (2), 207–216.
12. Radmann, E.M., Reinehr, C.O., Costa, J.A.V., 2007. Optimization of the repeated batch cultivation of microalga *Spirulina platensis* in open raceway ponds. Aquaculture 265 (1-4), 118–126.
13. Rafiqul, I.M.; Jalal, K.C.A.; Alam, M.Z. 2005. Environmental factors for optimization of *Spirulina* biomass in laboratory culture. Biotechnology, 4(1), 19-22.
14. Venkataraman LV., Bhagyalakshmi, N., Ravishankar, G.A., 1995.Commercial production of micro and macro algae problems and potentials. Indian Journal of Microbiology.35,1–19.
15. Vonshak, A., Richmond, A., 1982. Mass production of blue–green alga *Spirulina*-an overview. Biomass. 15, 233–47.
16. Youngman, R.E., 1978. Measurement of chlorophyll-a. Water research center, Tech. Rap. Tr. 82.

17. Zarrouk C. Contribution a l'étude d'une cyanobactérie: influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulinamaxima* (Setchell et Gardner) Geitler. Ph.D. thesis, University of Paris, France, 1966.