BACTERIAL ASSOCIATION AND THEIR ROLE IN CULTURE AND PRESERVATION OF *SPIRULINA* IN BANGLADESH.

A DISSERTATION SUBMITTED
FOR THE DOCTOR OF PHILOSOPHY
BY

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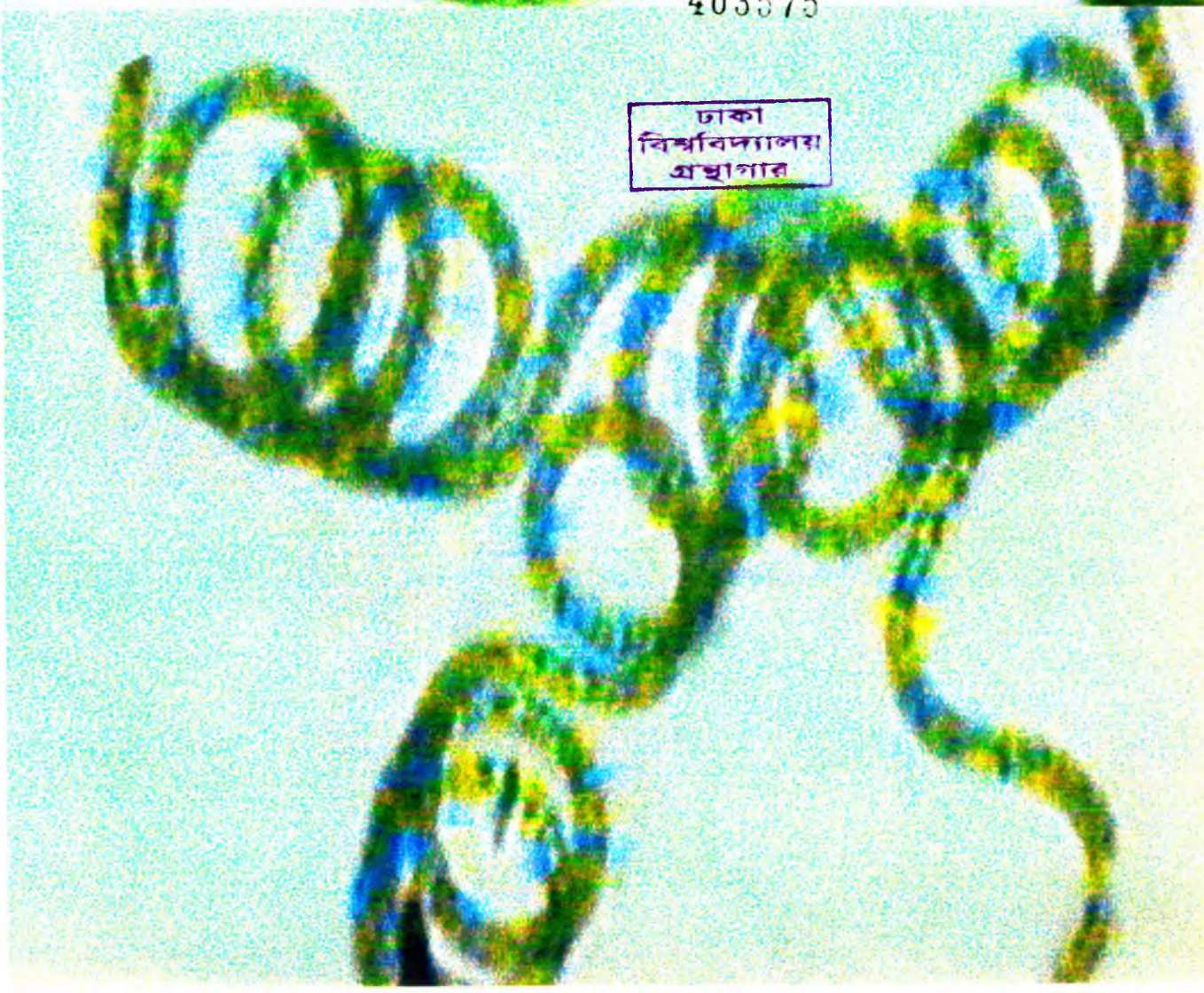
June, 2006



Certificate

This is to certify that the research work embodied in this thesis titled **"Bacterial association and their role in culture and preservation of *Spirulina* in Bangladesh"** submitted by Nasima Akhtar was carried out under our supervision in the laboratory of Microbiology, Department of Botany, University of Dhaka and Biological Research Division, BCSIR Laboratories, Dhaka. It is further to certify that the research work presented here is original and suitable for submission for the award of Ph. D degree. To the best of our knowledge this work has not been submitted elsewhere for any other degree.

18/6/06

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Dedicated

To

The most influential persons in my life.

My Parents, my Husband
And
my Children

Acknowledgement

Author wishes to express her profound sense of gratitude to the Almighty Allah for enabling her to carryout this work successfully.

The author would also like to express her sincere and heartfelt gratitude to her honorable supervisors Professor M. R. Khan, Department of Botany, University of Dhaka and Dr. F. Z. Majid, Ex –Chairman, Bangladesh Council of Scientific and Industrial Research (BCSIR), under whose joint guidance and supervision the present work was carried out.

The author is deeply indebted to the Ministry of Science and Information and Communication Technology for financial support, which helped her during the study.

The author is also indebted to Dr. M. Kabirullah (Member Developement), BCSIR and Dr. Borun Kumar Shaha, Nasima Begum, Senior Scientific Officers, Food microbiology division, IFST, BCSIR, Dhaka, for their kind help in carrying out analysis of protein and beta-carotene during this study.

Sincere thanks are also due to Dr. Fauzia Begum and Mazeda Begum, Principal Scientific Officers of IFST, BCSIR Dhaka, for their valuable suggestions.

Thanks are also due to Dr. Mihirlal Shaha, Department of Botany, Md Saiful Islam ex-student of Microbiology laboratory, Tabbasum, Mumtaz Afroza, Rosy, M. Phil students of the Department of Botany, University of Dhaka, for their help during the microbiological study.

The author would also like to express her heartfelt thanks to her colleagues Dr. Rahima Khatun, Principal Scientific Officer, Md. Motahar Hossain and John Liton Munshi, Senior Scientific Officers, Md. Motin Sharker Scientific Officer of BCSIR Labs., Dhaka, for their inspiration and cooperation during the study.

Thanks also extended to Hasina Akhter, Asma Sultana and Adus Samad of the same lab.

Author hereby extends her heartfelt thanks to her colleague Dr. Parvin Noor for helping her to prepare and arrange the manuscript of the thesis systematically and would also like to express her great appreciation to her colleague Dr. Miskat Ara Akhter Jahan for her sincere support in the graphic and photographic arrangement of this study.

Sincere thanks are also due to Mr. Nasiruddin, Asstt. Secretary, of BCSIR. for his sincere cooperation in Statistical analysis of this study.

Thanks are also due to Dr. Samir K. Sha, Professor, Department of. microbiology, Bangladesh Institute of Child Health Dhaka Shisu Hospital, for his kind cooperation during the microbiological study.

The author is grateful to the BCSIR authority for providing necessary facilities during this work.

The author would like to thank the Chairman, Department of Botany, University of Dhaka, for providing facilities for this study.

Finally the author expresses her great appreciation to her husband, two children and all her family members for their inspiration and encouragement during the study.

Nasima Akhtar.

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Abstract

Spirulina platensis culture was started in Bangladesh in 1989. Since then *Spirulina platensis* has been mainly cultured in BCSIR pilot plant ponds and local industrial ponds. During the period of study it was found that *Spirulina platensis* (cultured under local conditions) contains 65% protein, 0.24% fat, 1.25% fibre, 6.6% ash, 0.20% beta-carotene, 15.89% carbohydrate, and 11% moisture.

In Bangladesh optimum pH for the growth of *Spirulina platensis* is 10.0. Summer was observed to be the optimum growth period for the culture of *Spirulina platensis* in Bangladesh, followed by winter and then monsoon. It was also observed that under favorable conditions growth of *Spirulina platensis* was doubled within 2-3 days.

95% Pb was found to be adsorbed by the sun-dried biomass of *Spirulina platensis* (when it was pre treated with alcohol). It was also observed that 85% Cd was adsorbed by the sun-dried biomass of the same (when it was pre treated with alkali).

While observing the bacterial association of *Spirulina platensis* culture, 31 bacterial strains were isolated from BCSIR pilot plant pond and different commercial *Spirulina platensis* culture ponds in Bangladesh. The isolated bacterial strains were provisionally identified. Out of 31 bacterial strains, one belonged to the genus *Brochothrix,* five belonged to the genus *Listeria* and 25 strains were members of the genus *Bacillus* i.e., *B. alcalophilus*(1), *B. pumilus*(5), *B. firmus*(1), *B. badius*(1), *B. pantothenticus*(1), *B. alvei*(1), *B. polymyxa*(3), *B. lentus*(3), *B. licheniformis*(1), *B. megaterium*(1), *B. macerans*(2), *B. subtilis*(2), *B. circulans*(1), *B. sphaericus*(1), *B. brevis*(1).

In the laboratory, reduction of bacterial load in *Spirulina platensis* culture was 73%, when the culture was treated with 50ppm of 37% formaldehyde for 10 minutes and it was 70% when the culture was treated with

50ppm concentrated acetic acid for 5 hours. The reduction of bacterial load was 51.8% when the *Spirulina platensis* culture was treated with 200ppm of 95% alcohol for 5 hours.

It was also noted that reduction of bacteria in *Spirulina platensis* culture was 99% when the culture was treated with150gm/l NaCl for one hour. The bacterial load reduction was 95%, when the *Spirulina platensis* culture was treated with 10gm/l sterilized Sugar for three hours. The reduction of bacterial load was 99.3%, when *Spirulina platensis* culture was treated with 6gm/l Doxycyclin for 1h treatment; while the reduction of bacteria was 89.0% when the culture was treated with 60gm/l Amoxicillin for 24hrs.

Bacterial load in *Spirulina platensis* slurry was reduced by 99% through washing with non sterilized and sterilized BCSIR tap water.

Protein content of sun-dried *Spirulina platensis* biomass after one year preservation was found to be 60% and beta-carotene content was 0.0047%. The load of bacterial strains within one year preservation period was $1x10^6$ - $8x10^6$ CFU/ml.

It was possible to regenerate *Spirulina platensis* 10,070/ml to 13, 275/ml from its sun-dried biomass (one week, one year and two years old).

LIST OF ABBREVIATIONS

The following abbreviations have been used throughout the thesis

%	Percentage.
1st	First.
2nd	Second.
3rd	Third.
AOAC	Association of official analytical chemicals.
BCSIR	Bangladesh Council of Scientific and Industrial Research.
BD_1	Bangladesh medium one.
BDH	British Drug House.
^0C	Degree Celsius.
Conc.	Concentration.
Cd	Cadmium.
CFU	Colony Forming Unit.
Cm	Centimeter.
d	day.
e.g.	For example.
ed	Edition
etc.	Etcetera.
Fig.	Figure.
gm	Gram.
H(rs)	Hour (s).
ml	Milliliter.
mg	Milligram
No	Number.
nm	Nanometer
N	Normality.
O.D.	Optical density.
ppm	Parts per million.
pH	Negative logarithm of hydrogen ion (H)
pb	Lead.
TN	Total Nitrogen.

Appen.	Appendix.
i.e.,	Id est (That is)
IFP	Institute of France Petroleum.
Vita.	Vitamin.
Yrs	Years.
Wt.	Weight.
PBL	Project Builders Limited.
sec.	Second
M.R.	Methyl Red
V.P.	Voges Proskauer
sp.	Species
viz.	Namely
AIDS	Acquired Immuno Deficiency Syndrome.

Chapter 1

Introduction

Spirulina is a photosynthetic, microscopic, filamentous blue-green algae. In 1827, Turpin isolated *Spirulina* from a fresh water stream and from variety of environments, such as soil, sand, marshes, brackish water, sea water and fresh water (Ciferri 1983). A small quantity of the unprocessed dry material contains significant nutritional benefits. It is a blue-green micro algae, belonging to the family Oscillatoriaceae. It is considered to be closely related to the first form of true plant life that appeared in earth over three billion years ago. It is one of the first photosynthetic organisms. *Spirulina* consists of a simple microscopic multi-cellular filament in a helix (Fig.1). The filament is less than a millimeter long (Switzer 1982) and may be with 5 to 7 coils. The diameter of the filaments is about 10μ. It may show much morphological variations under the different cultural conditions (Buacille 1990). Species of *Spirulina* have been isolated from tropical water to the North sea, thermal springs, salt ponds, warm water, from power plants, fresh ponds etc. The plant appears to be capable of adaptation to very different habitats. Under normal water condition *Spirulina* may be one of many algal species, but the more alkaline and salty the water becomes more in hospitable to the other life forms, allowing *Spirulina* to flourished as a single species. Population of *Spirulina platensis* obtained in certain alkaline lakes in Africa and *S. maxima* of lake Taxcoco in Mexico. In some of these lakes *Spirulina* grows as a quasi-monoculture (Ciferri 1983). In 1940 French psychologist Dangeard first mentioned the use of *Spirulina* as a human food (Henrikson 1989).

Spirulina is the richest source of vitamin B_{12} higher than beef liver, *Chlorella* and sea-vegetables. B_{12} is the most rare vitamin to get from plant sources. 10 gm of *Spirulina* contains 20-30 micrograms of B_{12} according to the standard microbiological assay method. Recently an alternative method, Radio assay was used to measured the B_{12} actually bio-available to humans.

Radio assay finds much lower levels of available B_{12} in all foods and supplements and shows *Spirulina* having only 20% of original B_{12}. Even using this level, it remains the best non-animal source of B_{12} (Henrikson 1989). *Spirulina* is the only vegetable source of vitamin B_{12} and the highest known (Seshadri, MCRC 1977). *Spirulina* is rich in vitamin B complex, Vitamin A, minerals including iron and protein (Venkataraman 1982)

Healthy *Lactobacillus* in the intestine provides human being with three major benefits: - better digestion and absorption, protection from infection and stimulation of the immune system. *Spirulina* builds up *Lactobacilli*, as research in Japan showed, it increased *Lactobacilli* in rats by three times over control group. Feeding rats a diet with 5% *Spirulina* for 100 days revealed, (i) weight of the caecum increased 13%, (ii) *Lactobacilli* increased 32.7% and (iii) vitamin B_1 inside the caecum increased 43%. *Spirulina* did not supply additional B_1 but improved overall B_1 absorption. The study suggested, eating *Spirulina* may increased the efficiency of absorption of B_1 and other vitamins from the diet (Henrikson 1989).

Among the intestinal flora, lack of *Lactobacilli* in intestine encourages growth of pathogen in human intestine. Rat feeding trial in Japan indicated three times growth of *Lactobacilli* in *Spirulina* fed rats, in comparison to control (Bucaille 1990).

Outdoor cultures of *Spirulina* had an initial bacterial load of 2.0×10^3 CFU/ml, which increased to 9×10^4 CFU/ml after 15 days. The types of bacteria present in *Spirulina* culture were identified as: *Bacillus megaterium*, *B. licheniformis*, *B. firmus*, *B. polymyxa*, *B. alvei*, *B. circulaus* and *B. maceraus*, no pathogen like *Salmonella* or *Staphylococcus aurius* were found either in the culture or in the *Spirulina* powder (Venkataraman 1983).

A two year study on laboratory animals conducted by a U. N. toxicologist, testing *Spirulina* for acute and chronic toxicity as well as for any

cause of cancer or birth defects, certified *Spirulina* as free from such problems (Switzer 1982).

Microbiological and toxicity data provided by *Spirulina* producers indicate that *Spirulina* culture is not affected by *Salmonella* or *E. coli* (Switzer 1982).

Spirulina biomass is free from phycotoxins and no other toxins are present in the samples investigated (Venkataraman 1983).

In the present day world *Spirulina* farming is part of new stage of ecological agriculture. It presents one of the solutions required to produce food. (Henrikson 1989). Modern consumers use *Spirulina* for disease purposes, as a vitamin source (Vitamin A-14000 I.U., Vitamin B_{12}-30 microgram per 10 gm. dry *Spirulina*), as concentrated food resource (protein content 60–70%) and as a source of iron (10.6 mg./10 gm of dry *Spirulina*). Athletes and body builders also use it for body development and stamina, clinical tests revealed its effectiveness against ulcer, anemia, diabetes, cancer, night-blindness, obesity, baldness etc. [Murugappa Chettiar Research Center (MCRC)]. It is also used as a health food in U.S.A, Europe, Japan, S. E. Asia, India and now days also in Bangladesh. *Spirulina* is used as ingredient for cosmetics in France and Japan.

New clinical research reveals health benefits of *Spirulina*, which contains 15% to 20% carbohydrates. The primary forms of carbohydrates are rhamnose and glycogen, two polysaccharides, which are easily absorbed by the body, with minimum insulin inversion (Henrikson 1989).

Spirulina offers quick energy, with taxing the pancreas (Henrikson 1989). Gamma Linolenic acid (GLA), an essential fatty acid, seems to control many essential body functions. Dietary intake of GLA can help arthritis, heart disease, and obesity and zinc deficiency (Henrikson 1989, Jassby 1983, Nichols *et al.* 1986). *Spirulina* is rich in beta-carotene, a precursor of vitamin A, which can control night blindness. Over a period of twenty years cancer health authorities have published number of studies showing beta-carotene

reduces the risk of all kind of cancer (Henrikson 1989). The natural products branch of National Cancer Institute (NCI), USA, announced that sulpholipid from *Spirulina* and other blue-green algae were found to be active against the AIDS virus. *Spirulina* contains 5.8% lipids of which 2.5% are sulpholipids (Henrikson 1989). Enzymes, catalysts for chemical changes in the body are present in *Spirulina* in quite a good numbers. *Spirulina* has super oxide dismutase (SOD), which is important to reduce the number of free radicals. Free radicals damage the cells, leading to cancer. 10 gm of *Spirulina* has SOD enzyme activity from 10000 to 37000 units (Henrikson 1989).

Natural sulfated polysaccharides, calcium spirulan, isolated from *Spirulina platensis* is a potential antiviral agent against both HIV-1 and HSV-1 (Hayashi *et al.* 1996). Antiviral activity of *Spirulina maxima* against simplex virus type 2 has been reported (Hernandez Corona Armida *et al.* 2002). Inhibition of tumor invasion and metastasis by calcium spirulin, a novel sulfated polysaccharides, derived from *Spirulina,* was also reported (Mishima – Takaaki *et al.* 1998). Inhibitory effect of Phycocyanin from *Spirulina platensis* on the growth of human leukemia K562 cells was also reported (Liu-yufeng *et al.* 2000).

The statement, *Spirulina* grows as a quasi-monoculture, does not mean that other microorganisms do not grow in *Spirulina* culture. It has been found that all spp. of *Spirulina* so far isolated even from the most alkaline lakes are always associated with bacteria. The bacterial floras are associated with the culture of *Spirulina.* It is not known that whether mutuality relation exists between these bacteria and *Spirulina.*

It has been reported that due to the alkalinity of the growth medium, the microbial load of *Spirulina platensis* culture is lower than that of eucaryotic algae, such as *Scenedesmus,* which grows in acid media.

Spirulina is commercially cultivated in several countries like Mexico, U.S.A., Taiwan, Thailand, Japan and Israel, with a total production of few hundred tons per annum. (Dillon 1995, Khatun and Majid 2004).

In 1989 George Bonnin of France introduced *Spirulina platensis* culture in Bangladesh. Initially inoculum of *Spirulina platensis* obtained from C.M.M. Murugappa Chattier Research Madras and NCRC Mysore, India. The biologist of the Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka first adapted the culture system to suit the climatic and economic condition of the country. Commercial production of *Spirulina platensis* has already been started by the producer Vie. Project builders limited, Nature food products, Wonder herbs, Ever green enterprise and Eureka enterprise, in the country under the supervision of the biologists of BCSIR. And it has been marketed as a health food in the form of capsules, tablets, powder, drinks and bakery items (Fig.2a and 2b). People of Bangladesh are consuming *Spirulina platensis* since last thirteen years. However, it may be mentioned here that no survey has been conducted on bacterial association in *Spirulina platensis* culture in Bangladesh. Various other aspects such as estimation of protein, fat beta-carotene, ash and carbohydrate content of *Spirulina platensis* are yet to be studied and reported. The present work was undertaken to study the following:

1. Chemical composition of *Spirulina platensis* cultured under local conditions
2. Effect of pH on *Spirulina platensis* culture
3. Biomass production of *Spirulina platensis* in different seasons of Bangladesh
4. Role of sun-dried *Spirulina platensis* biomass as biosorbent, at room temperature.
5. Isolation of associated bacteria from *Spirulina platensis* culture
6. Identification of the isolated bacteria

7. Effect of sun-dried *Spirulina platensis* extracts on the isolated bacteria
8. Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by different treatments
9. Preservation of sun-dried *Spirulina platensis* at room temperature
10. Regeneration of *Spirulina platensis* from sun-dried materials

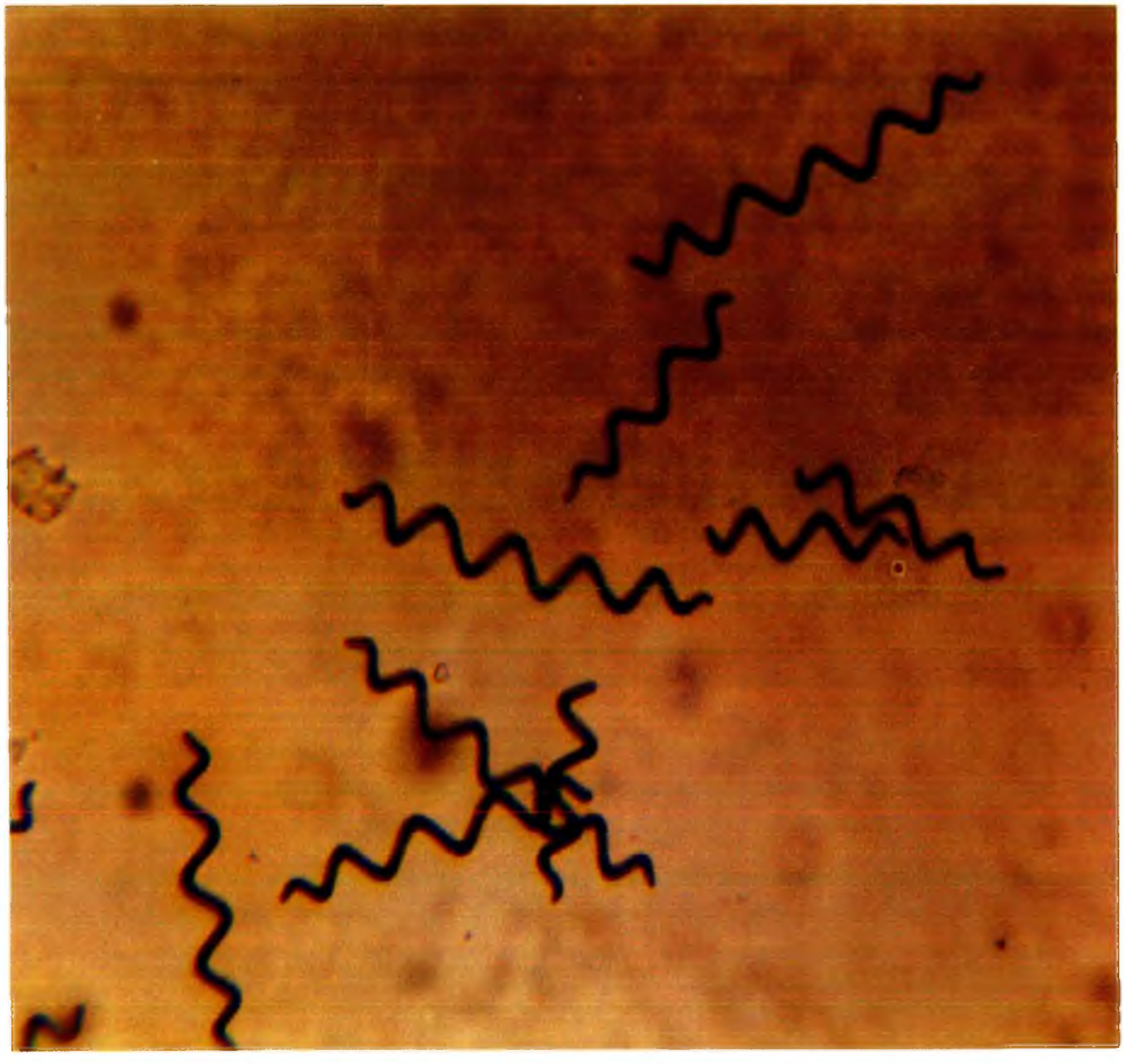


Fig. 1: *Spirulina platensis* under Microscope (100X).

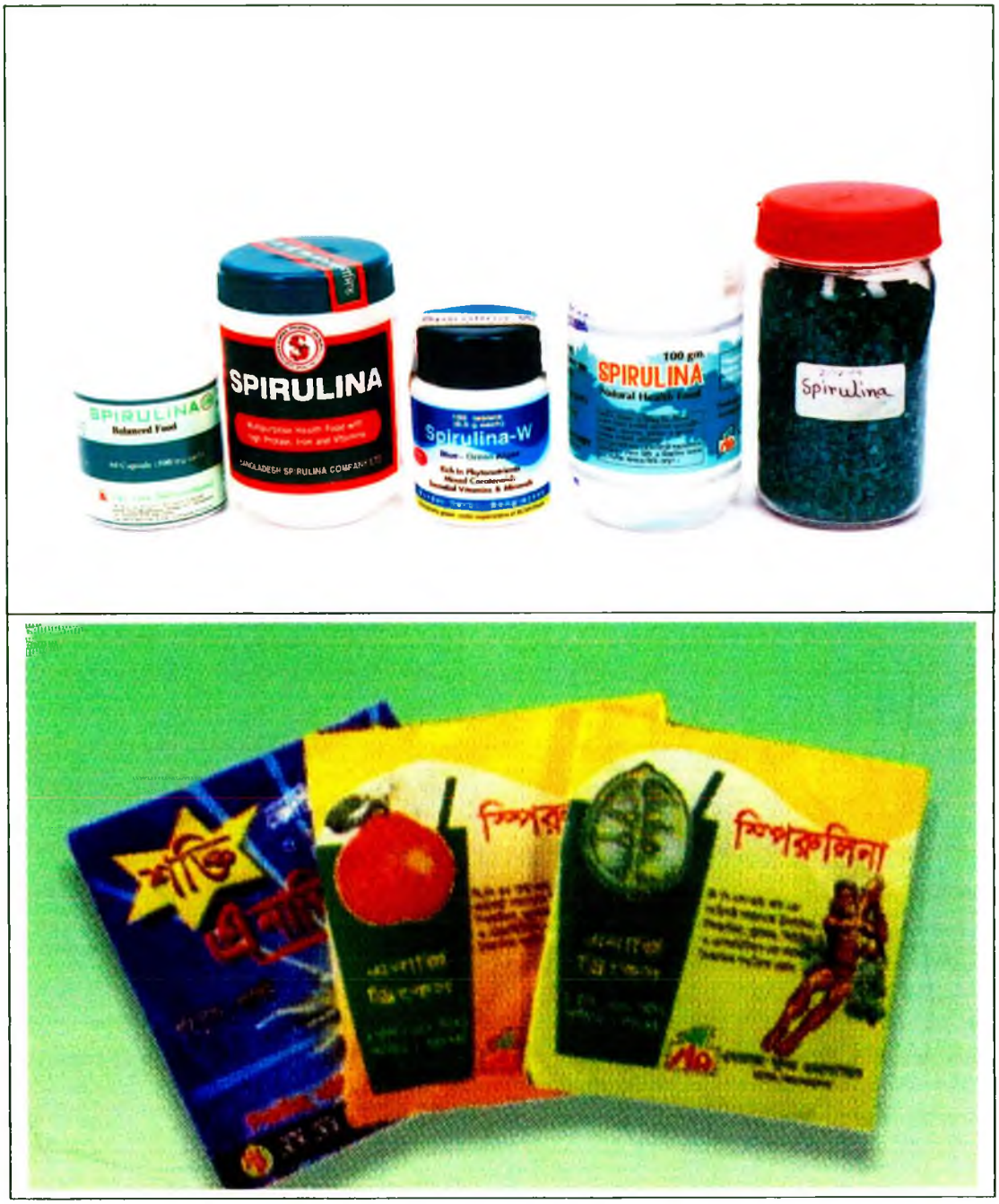


Fig. 2a: Photographs showing *Spirulina* capsule, tablets, powder and drinks, available in local markets.

Fig. 2b: Photographs showing Spirulina enriched food articles.

Chapter 2

Review of literature

According to Henrikson (1989) *Spirulina* is the immortal descendent of the first photosynthetic life form. Beginning three and one–half billion years ago, blue-green algae created our oxygen atmosphere so other life could evolve. Since that time, algae helped in regulation of the planet biosphere.

A fundamental aspect of *Spirulina* biology is its life cycle due to the taxonomic, physiologic and cultivation implications (Ciferri 1983 and Richmond 1984). This period is summarized in three fundamental stages: trichomes fragmentations, hormogonia cells enlargement and maturation processes and trichomes elongation. The mature trichomes are divided into several small filaments or hormogonia through previous formation of specialized cells, necridium cells, in which the cell material is reabsorbed allowing fragmentation. The number of cells in the hormogonias is increased by binary fission. For this process, the trichomes grow lengthwise and takes their helical form (Balloni *et al.* 1980).

Blue-green algae are amongst the first plants on earth, these fossils of 3.5 billion years have been found. Algae have been used as human food in some parts of the world for centuries. The blue-green algae *Spirulina* was eaten by the Aztecs in Mexico who called it 'Tecuitlati'. The same alga also formed a part of the food of the Kanebou tribe in north of Lake Chad, in Central Africa, who made it into a Sauce called 'Dihe (Venkataraman 1983).

Cyanobacteria or blue-green algae are photoautotrophic microorganisms widely distributed in nature. They have been used as human food for centuries. *Spirulina sp.* is the known genus because of its nutritional value. It contains 18 of the 20 known amino acids, high quality proteins, more calcium than milk, more vitamin B_{12} than cow liver, vitamins A, B_2, B_6, E, and K, and all essential minerals, trace elements and enzymes (Fox 1986).

Ciferri (1983) sited that *Spirulina* alga have a higher content of beta-carotene and other carotenoids than any other plant source. *Spirulina* algae are found in highly alkaline lakes of Africa, Mexico, and Asia, where the natives included this item in their daily diets for centuries.

Dillon (1995) reported that *Spirulina* are cyanobacteria living in high salt alkaline water in subtropical areas. These algae are filamentous and measure upto 250 micrometer in length. Under the microscope, they appear as blue-green filaments.

According to Chamorro (1996) *Spirulina* is a unicellular filamentous blue-green alga, consumed by man since ancient times in Mexico and central Africa. It is currently grown in many countries by synthetic methods. Initially the interest in *Spirulin*a was on its nutritive value as it was found almost equal to other plant proteins. More recently, some pre clinical testing suggests it has several therapeutic properties such as hypocholesterolemia, immunological antiviral and antimutagenic.

Huh-Nam Yan (1997) reported that *Spirulina platensis,* one of the oldest alga species on earth, has a history of about 3 billion years. Various algae have been used as human food, and it is well known that some of them contain components possessing biological activities. In particular, the blue–green micro algae *Spirulina platensis* has been used for regulation of the immune response, and still occupies important place in traditional Chinese medicine.

Blue-green micro algae *(Spirulina)* used in daily diets by natives in Africa and America was found to be a good source of carotenoids. micronutrient and beta- carotene (Miranda *et al.* 1998).

Hayashi *et al.* (1994) cited that *Spirulina,* a cyanobacterium grows naturally in lakes and has been used for over a thousand years as a source of food because of its high protein content and good amino acid composition.

Spirulina platensis is especially rich in proteins. The proteins with the highest economic potential are the biliproteins (e.g., Phycocyanin and

allophycocyanin), which are water- soluble blue pigments. The protein fraction may have a Phycocyanin content of up to 20%. Fatty acid composition is largely influenced by environmental conditions. *Spirulina platensis* can be characterized by about 45 to 50% saturated and 50 to 55% unsaturated fatty acids. up to 30% of its fatty acids is Gamma Linolenic Acid, a rare polyunsaturated fatty acid claimed to have medicinal properties (Cohen 1997, Vonshak 1997a).

Since 1970, *Spirulina* has been analyzed chemically. It has been shown to be an excellent source of proteins, vitamins and minerals (Switzer 1980).

According to Venkataraman (1983) dry *Spirulina* biomass contains 55-65% protein, 2-6% lipid, 10-15% carbohydrates, 1-4% crude fibre, 6-15% ash and moisture 5-10%

Venkataraman (1984) stated that dry *Spirulina* contains 62.5% protein, 3% lipid 8.5% carbohydrates.

According to Henrikson (1989) dry *Spirulina* contains 68% protein, 5% fat, 7% minerals 3% moisture and 20% carbohydrate. Protein is composed of both essential and non-essential amino acids. Essential amino acids cannot be manufactured in the human body and must be supplied in the diet. Non-essential amino acids can be synthesized in body. A protein is considered complete if it has all the essential amino acids. *Spirulina* is just that. Henrikson also reported that *Spirulina* has the highest protein of any natural food (65%) far more than animal and fish (15-25%), soybeans (35%), dried milk (35%), peanut (25%), egg (12%), grains (8-14%) or whole milk (3%). *Spirulina* is the richest iron food, 20 times higher than common iron rich foods. Ten gram of *Spirulina* supplies 15mg of iron.

According to Bonnin (1992) chemical composition of dry *Spirulina* is 60 to 70% protein fat 6 to 7%, ash 6.4-9% and carbohydrate 14%.

Dillon (1995) reported that *Spirulina* on the basis of dry weight contains protein 60-70%, fat 5-7%, carbohydrate 10-15%.

According to Mathew (1995) chemical composition of dry *Spirulina* is 9% water, 55% protein, 0.9% crud fibre and 9% ash.

Vonshak (1997) reported that the dried biomass of *Spirulina* contains 3-7% moisture, 55-60% protein, 6-8% lipids, 12-20% carbohydrates, 10% ash, 8-10% fibre and 1-1.5% chlorophyll and wide range of vitamins.

According to Eykelenburge (1977), due to absence of cellulose in its cell walls, *Spirulina* dose not require chemical or physical processing steps to make it digestible.

Spirulina has no cellulose in its cell walls, being composed of soft mucopolysaccharides. Hence its protein is easily digested and assimilated in the human body (Henrikson 1989).

Spirulina does not have cellulose in its cell wall, a feature that makes it an appropriate and important foodstuff for people with problems of poor intestinal absorption and geriatric patients (Richmond 1992).

Due to the absence of cellulose cell walls *Spirulina* does not require chemical or processing steps in order to become digestible (Dillon *et al.* 1995).

Anusuya Devi (1981) reported that fresh algal samples were more digestible (80%) than sun-dried or freezes dried sample (70%).

Venkataraman (1989) reported that the fresh algal sample had the highest digestibility of 85%, while sun-dried and freeze-dried had a digestibility of about 70%.

Spirulina has only 4 to7 % lipids or fats, and most of these are essential fatty acids. Ten grams have an average of 225mg of essential fatty acids in the form of Gamma Linolenic Acid (GLA) an essential fatty acid, a precursor for the body's prostaglandin's, master hormones that seem to control many essential body functions. Dietary intake of GLA can help arthritis, heart disease, and obesity and zinc deficiency (Henrikson 1989, Jassby 1983 and Nichola *et al.* 1986).

A study in Japan showed cosmetic packs containing *Spirulina* and its enzymatic hydrolyzates promoted skin metabolism and reduced scars. (Yoshida 1977).

In take of four grams of *Spirulina* after each meal, in 30 days, showed blood hemoglobin content increase by 21% from 10.9 to 13.2, a satisfactory level no longer considered anemic (Takeuchi 1978).

Venkataraman (1982) reported that *Spirulina* is rich in vita. B complex, vitamin A, minerals including iron and protein. Its easy digestibility makes it an ideal natural food .The use of the algae has also been suggested for maintaining the lower blood sugar levels. Plant foods are not generally considered source of vitamin B_{12}. Thus its presence in *Spirulina* is surprising. It is assumed that the algae do not have the ability to synthesis the B_{12} but it is provided by closely associated bacteria. This is adsorbed and concentrated by the algae.

Beta-carotene is one of the most well known natural anti cancer substances, vegetables rich in beta-carotene reduces the risk of all kinds of cancer. *Spirulina* is richest in beta-carotene, with a ten times higher concentration than carrots, ten grams of *Spirulina* provide a remarkable 2300IU(14%) of beta-carotene. High doses of vitamin A supplements may be toxic, but beta-carotene in *Spirulina* and other vegetables is always safe because human bodies only convert beta-carotene to vitamin A as needed (Henrikson 1989).

Among food, *Spirulina* has a relative high pro vitamin-A concentration (Belay 1997).

Ciferri (1983) reported that *Spirulina* algae have a higher content of beta-carotene and other carotenoids than any other plant source. *Spirulina* algae are found in highly alkaline lakes of Africa, Mexico, and Asia, were the natives have been including this item in their daily diets for centuries.

Iron in some nutritional complements is not appropriately absorbed. Iron in *Spirulina* is 60% better absorbed than ferrous sulfate and other complements. Consequently, it could represent an adequate source of iron in anemic pregnant women (Puyfoulhoux *et al.* 2001).

According to Switzer (1982) *Spirulina* is used as livestock feed, fish feed and ingredients for cosmetics in different countries of the world.

According to Henrikson (1989) *Spirulina* fat content is only 5% lower than almost all other protein sources. This means *Spirulina* is a low –fat, low – calories cholesterol- free source of protein and is not loaded with the fat grease, calories and cholesterol of meat and dairy protein.

Studies have shown that *Spirulina* consumption during 4 weeks reduces serum cholesterol levels in human beings by 4.5% (Henrikson 1994)

Henrikson (1989) reported that pharmaceutical compounds containing *Spirulina* as an active ingredient have been shown to accelerate wound healing. Treatments consisted of creams, ointment, solutions and suspensions. Studies in Japan showed cosmetics pack containing *Spirulina,* promoted skin metabolisms. Additional research showed extracts of *Spirulina* inhibited the growth of yeast and fungi; the antibiotic substances in those extracts may have medical applications.

Other benefits are attributed to *Spirulina*: anti-arthritic effect due to anti-inflammatory and antioxidative properties of phycocyanin (Ramirez *et al.* 2002), tumor burden inhibition (Desgupta *et al.* 2001), chemo protective and radio-protective effect (Zang *et al.* 2001) and antioxidant activity on lead-induced toxicity in rats (Upasani *et al.* 2001).

Henrickson (1989) and Hayakaway (1996) cited that *Spirulina* is a highly protinacius and vitamin us plant and for this reason *Spirulina* has good advantage to feed malnourished children. *Spirulina* is also rich in a protein complex pigment, phycocyanin, which may stimulate immune system.

Fariduddin and Misbahuddin (2004) observed that *Spirulina* was found to be effective in reducing the amount of arsenic accumulation in all the tissue of rats. Oral administration of *Spirulina* caused 16.8-52.2% reduction of arsenic accumulation in different organs.

According to Sha *et al.* (2005) hexane extract of *Spirulina* causes highly significant removal of arsenic. So it can be assumed that the active compound (s) of *Spirulina* is present mostly in its hexane extract.

Azmat (1991) stated that *Spirulina* has potent hypoglycemic effects in experimentally induced diabetic state. This hypoglycemic effect of *Spirulina* may also be useful in human diabetics.

Administration of *Spirulina* in normal rats in a dose of 100mg/kg body weight orally once daily for 21consecutive days did not produce any significant change in serum chol. triglyceride and HDL-chol. levels except LDL- chol. level which was reduced significantly (Jahan 1991).

Begum. *et al.* (1993) cited that *Spirulina* in the dose of 200mg/kg weight for 28 days decreased serum lipid levels in normal rats but not significantly. In case of LDL-chol. it is decreased significantly in the study.

Remirez *et al.* (2000) reported that Phycocyanin, a biliprotein found in blue-green micro algae *Spirulina* extracts has anti-inflammatory effects in some animal models of inflammation.

A sulfated polysaccharide named calcium spirulan (Ca-SP) has been isolated from a sea alga, *Spirulina platensis*, as an antiviral component, effective against anti-human immunodeficiency virus type 1(HIV-1) and anti-herpes simplex virus type 1 (HSV-1) (Hayashi *et. al.*1996).

Spirulina has been enormous impacts as curative measure on arsenicosis patients (Jahan *et. al.* 2002).

Yoghurt and other fermented milk products contribute to health with natural nutrient and enrich the intestinal flora with lactic acid bacteria (LAB). They provide humans with major benefits: protection from infection (Nader de

Macias *et al.* 1993), stimulation of the immune system (Perdigon *et al.* 1992), as well better digestion and absorption of lactose and minerals (Hove *et al.* 1999).

Parada *et al.* (1998) reported that (LAB) in synthetic media was promoted by *Spirulina platensis* extra cellular products.

Gloria *et al.* (2000) reported that the stimulatory effect of aqueous suspension of *Spirulina platensis* dry biomass extracted at pH 6.8 and 5.5 was studied on four lactic acid bacteria (LAB) grown in milk. The addition of dry *Spirulina platensis* to milk (6mg/ml) stimulated growth of *Lactococcus lactis* by 27%. The growth of other strains was also promoted.

Spirulina reduced kidney nephrotoxicity from mercury and three pharmaceutical drugs in laboratory rats (Yamane 1988).

The effects of Phycocyanin from *Spirulina platensis* on the growth of human chronic myelogenous leukemia- blast crisis k 562 cells was studied by semi-solid agar assay and cell viability measurement Phycocyanin significantly inhibited the growth of k 562 cells in a dose –dependent manner, Liu –Yufeng (2000).

Ayehunie *et al.* (1998) reported that an aqueous extracts of the blue-green filamentous algae *Arthrospira platensis* (previously called *Spirulina platensis)* inhibited HIV replication in human.

National cancer institute (NCI) announced that chemicals from blue-green algae were found to be active against the AIDS virus Gustafson (1989).

Average cell mass productivity of *Spirulina* reached the maximum in summer followed by autumn and spring (E l- Fouly 1998).

According to Noor *et al.* (1999) red, green, and blue all the three lights supported better growth of *Spirulina* than sunlight. They also reported that effect of red light was found to be more pronounced in winter than in summer.

Pareek (2001) cited that the optimum photoperiod for the growth of *Spirulina platensis* is 16hs/day.

Spirulina grows quickly and produces more protein then other crops. In agriculture, the harvest is obtained after several months of cultivation's, while *Spirulina* is produced continually (Switzer 1980).

Akhtar *et al.* (1996) reported that rice husk ash might be more economic than $NaHCO_3$ for rural technology-of *Spirulina* culture.

Spirulina grew fairly well in Bd_1 medium. Growth rate of the algae was 8-20/m^2/day, depending on the weather condition, in that cloudy weather reduced the growth rate, while it was enhanced by bright sun -shine. Contamination was less than that in IFP and it could be more easily controlled in Bd_1 medium (Jahan *et al.* 1994).

Begum *et al.* (1998) cited that five culture media, developed in BCSIR Laboratories, Dhaka, were designated as Bd_1, Bd_2, Bd_3 Bd_4, and Bd_5. Bd_1 is used in Bangladesh for commercial production of *Spirulina.* They also cited that Bd_4 medium was found to be more suitable for domestic production of *Spirulina* in comparison to the others.

Jahan *et al.* (1999) reported that different media as well as different ash had varied effects on the coil characteristics of *Spirulina*. In that the effect of Bd_4 medium was best, followed by Bd_1. Banana ash was found to be superior to the other ashes.

Switzer (1982) reported that *Spirulina* commercial ponds require constant supervision to assure the degree of purity of the culture. Contamination of the ponds by wild micro-algae remains the most serious problems.

According to Venkataraman (1989) contamination of *Spirulina* with other algae is limited to very few forms (chlorella, diatoms). This is mainly due to the heavy specific culture conditions of this algae (high pH), which are not favorable for most other algae. He also reported that large-scale algal cultures are always contaminated with certain type of bacteria. Heterotrophic bacteria are grown autotrophically in organic media, because algal cells exude several

organic compounds into the medium, which are likely to promote bacterial growth.

The microbial load in the out door cultures has been found to be $2.3x10^4$ CFU/ml in 90 days old culture. Generally spore forming aerobic bacillus lives symbiotically in *Spirulina* cultures and dry products, which were found to be free from Staphylococcus, Salmonella and Coliforms (Venkataraman 1992).

Nahar *et al.* (1994) cited that *Spirulina* culture is possible in Bangladesh in spite of unpredictable climatic conditions. Heavy growth of Chlorella, Amoeba and Rotifer may hamper *Spirulina* culture during unfavorable weather conditions. However, low growth of Chlorella and Amoeba had no adverse effect on *Spirulina* culture during favorable weather conditions.

Noor *et al.* (1994) reported that common contamination's in *Spirulina* culture Amoeba, Rotifer and Zooplanktons were controlled with ammonium sulphate.

Ciferri (1983) cited that if the trichomes are washed repeatedly with sterile physiological solution, the bacterial contribution to the total biomass becomes negligible.

According to Bonnin (1992) algal slurry has to be washed to eliminate undesirable chemicals, this operation also eliminates a large proportion of the micro- organisms including bacteria, which have not been eliminated by filtration in the culture medium.

Venkataraman (1983) reported that *Spirulina* biomass is free from phycotoxins and that no other toxins are present in the samples investigated. So the algal culture can be assumed as safe for use as food. He also cited that the various feeding trial performed by several authors with different algal samples have not revealed any pathological symptoms due to biogenic toxins.

The bacterial forms occurring in cultures and dried powder were identified as aerobic spore formers. No pathogenic forms affecting the products safety were identified (Mahadevaswamy *et al.* 1989)

According to Switzer (1982) like all protein food, protein rich *Spirulina* when wet, at room temperature and exposed to the air, can be spoiled quickly (like cheese, milk, meat and fish). Even refrigerated *Spirulina* are very sensitive to spoilage. He also reported that dry dark and cool place should be selected for its storage using airtight container, to prevent bacterial growth or vitamin loss.

Dried *Spirulina* flakes with 8-10% moisture content can be stored in polyethylene bags. This does not lower the nutrient quality of the product, and also does not develop problem of rancidity for at least twelve months. Studies on bacterial contamination of the dried product have shown no increase of contamination level with incremental storage time (Venkataraman 1983).

Venkataraman and Becker (1989) reported that bacterial contamination of the dried products have shown no increase of contamination level with incremental storage time.

Bonnin (1992) cited that once dried, the product is not subjected to fermentation, and can be stored for duration as long as 2 years, without significant changes in biochemical composition or losses in nutritional properties. Further, long-term storage seems to have a positive influence on the decreases of the possible contaminating microbiological flora. He also cited that special care should be taken in order to allow best preservation. The algal powder must be kept away from direct light.

Biologically active (living) or inactive (non living) algae cells can reversibly bind significant quantities of metals ion from aqueous solution (Ferguson and Babula 1974, Crist *et al.* 1981, Wood and Wang 1983, Greeme *et al.* 1987, Krambeer1987, Volesky 1987, Darnall *et al.* 1988, Gee and Dudeney 1988, Knyucak and Volesky 1988).

Belay and Vonshak (1997a, 1997b) stated that since1970, *Spirulina platensis* has been marketed and consumed as a safe human food and has been provided for human nutrition by many governments, health agencies, and association of some 80 countries, including the United States and Hungary. Based on 30 yrs of safety and quality research, several countries and organizations have established *Spirulina* quality safety standards. More than 70% of the current *Spirulina* market is for human consumption, mainly as health food.

Chapter 3

Materials and methods

3.1 Chemical composition of *Spirulina platensis* cultured under local conditions.

Estimation of protein, fat, fibre, ash, carbohydrate and beta-carotene of sun-dried *Spirulina platensis*.

3.1.1 Estimation of protein, fat, fibre, ash, and beta-carotene.

The sample of *Spirulina platensis* was harvested from BCSIR pilot plant pond, washed and dried under direct sunlight, a constant dry weight was thus obtained.

Principle of protein estimation: For protein estimation total nitrogen was determined by Kjeldahl method (Cocks and Van Rede 1966). Protein content was calculated by multiplying the total, nitrogen content by 6.25. The sample was digested with pure concentrated sulphuric acid, when the nitrogen present was converted to ammonium sulphate. The solution was then made alkaline, the liberated ammonia was distilled off, total nitrogen was then collected in dilute hydrochloric acid and excess acid was titrated.

Reagents used

(1) Digestion mixture: 98 parts K_2SO_4+ 2 parts $CuSO_4$.

(2) Concentrated sulphuric acid.

(3) 40% aquous sodium hydroxide.

(4) N/10 hydrochloric acid and N/10 sodium hydroxide solution.

(5) Titrating agent:

5.1 Meythyl red indicator: 0.1gm of the indicator dissolved in 60ml. of alcohol and water added to make 100ml.

5.2 Phenolphthalein.

Procedure of protein estimation: 0.2386gm. of sun-dried *Spirulina platensis* sample was taken in a 100ml. kjeldahl flask and 2gm. of digestion mixture and 20ml. of conc. sulphuric acid was added (glass beads were added

to prevent the bumping). The contents were digested in a digestion chamber, digestion was continued till the sample became clear solution. After cooling the solution was made alkaline with 40% sodium hydroxide using 2-3 drops phenolphthalein as indicator. The ammonia thus liberated was immediately distilled and the distillate was collected in a standard acid solution (10ml. of 0.1 N hydrochloric acid) using methyl red as indicator. When about 20ml. of the distillate had been collected, the distillation was complete and the excess acid was titrated with standard sodium hydroxide solution. A blank titration was carried without with out sample as described above.

Calculation :

% of total nitrogen (TN) = { (X-Y) x N x M x 100} / (W x 1000)

Where, X = Vol. of NaOH solution for blank.

Y = Vol. of NaOH solution for sample.

M = Molecular weight of nitrogen.

W = Weight of sample.

% of protein = % T N x 6.25

3.1.2 Estimation of fat.

Proedure: Fat was estimated as crude ether extractive of the dry material. The sundry sample (16.8905gm) was weighed accurately into a thimble and plugged with the fat free cotton. The thimble was then placed in a Soxhlet apparatus and extracted with anhydrous ether for about 16 hours. The ether extract was filtered into a weighed conical flask. The flask containing the original ether extract was washed 4 to 5 times with small quantities of ether and the washings were also transferred to the filter. The ether in the conical flask was then removed by evaporation and the flask with the residue was dried in an oven (80° to 100°C), cooled in a desecrator and weighed.

Calculation:

% Of fat content = (Weight of ether extractive x 100)/Weight of sample taken.

3.1.3 Estimation of crude fibre.

Procedure: AOAC, (1984) method was followed for estimation of crude fibre 5.1422 gm of moisture and fat free sample was weighed into a 500 ml beaker, and 200 ml of boiling 0.255N (1.25 % w/v) sulphuric acid was added. The mixture was boiled for 30 minutes keeping the volume constant by the addition of water at frequent intervals (a glass rod was inserted in the beaker to help smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water till free from acid. The material was then transferred to the same beaker and 200 ml of boiling 0.313 N (1.25%) NaOH was added. After boiling 30 min. (keeping the volume constant as before) the mixture was filtered through a muslin cloth. The residue was washed with hot water till free from alkali followed by washing with alcohol and ether. It was then transferred to a crucible (which has been weighed before), dried over night at 80°C-100°C and weighed. The crucible was heated in muffle furnace at 600°C for 2 to 3 hours, cooled and weighed again. The difference in the weights represents the weight of crude fiber.

Calculation:

% Of Crude fibre = Wt. of ether extractives x 100 / Wt. of the sample taken (Moisture and fat free).

3.1.4 Estimation of ash.

Procedure: For estimation of ash, AOAC (1984) method was followed; 0.07575gm of the sun-dry sample was weighed accurately in a platinum crucible (which has previously been heated to about 600°C and cooled). 1-2 drops of concentrated nitric acid was added for oxidation.

The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 3-5 hours at about 600°C. It was then cooled in a desecrator and weighed. The heating was repeated till two consecutive weights were same and the ash was almost white or grayish white in color.

% Of Ash content = (Wt. of the ash x 100) / Wt. of the sample taken

3.1.5 Estimation of beta-carotene.

Procedure: Modified Holden (1981) method was followed for estimation of beta-carotene. 0.1712gm of the sun-dried *Spirulina platensis* sample was ground rapidly using mortar and pestle with 1-2gm sand, 5ml acetone and 5ml of petroleum ether. The liquid was transferred into a 25 ml volumetric flask and the extraction process was repeated three times by addition of 5ml of petroleum ether on each occasion. The extract was made upto the volume (25ml) with petroleum ether and the flask was stoppered and stored in the dark until analysis, beta-carotene was extracted from the *Spirulina* extract using a glass column (2.5 x 40cm) with a sintered glass disk at the bottom holding 8cm of an absorbent of Alumina (activated Aluminium oxide BDH). 10ml of the extract was placed on the top of the column bed. A deep yellow band containing beta-carotene traveled down. Petroleum ether was added until the elute turned color-less. The elute was made upto a final volume of 20ml with petroleum ether. The elute was recorded by spectrophotometer at 450nm using a 1cm cell.

Reagents used :

(i) Acetone (Analar grade, BDH)

(ii) Peptone (Analar grade b.p. 40C-60C BDH)

3.1.6 Estimation of carbohydrate.

Procedure: The content of the available carbohydrate was determined by difference = 100 – (moisture + protein +fat + ash).

1.7 Estimation of moisture.

Moisture content of *Spirulina platensis* was estimated by AOAC (1984) method. About 10gm of fresh sun dried *Spirulina* was taken in a weighed moisture box and dried in an oven at 100° C for 24 hrs, it was then cooled and weighed. Heating and cooling was repeated till a constant dry weight was obtained. Percentage of moisture content was calculated from the weight loss.

% of moisture = Initial weight - Final weight x 100/ Weight of the sample

3.2 Effect of pH on *Spirulina platensis* culture.

The study was conducted in BCSIR laboratories, Dhaka, using culture of *Spirulina platensis*. The culture was maintained in BD_1 medium (Appen.1). Beakers (500ml) were used as culture vessels, which were kept in the southern verandah of Biological research Division of BCSIR Laboratories, Dhaka.

The experiment was conducted as follows:

1. pH was adjusted once a day and temperature was recorded.
2. pH was not adjusted, but temperature and pH were recorded.

Each treatment had 05 replicas.

All the samples were observed under compound microscope (100x) twice a week.

3.3 Biomass production of *Spirulina platensis* in different season of Bangladesh.

The study was conducted in BCSIR labs, Dhaka in different seasons (winter, summer and monsoon) using culture of *Spirulina platensis* originally obtained from Shri A.M.M. Murugappa Chettiar Center, Madraz, and MCRC Mysore, India.

The culture was maintained in the Biological Research Division in BCSIR Laboratories Dhaka, since 1989. Small rectangular aquariums (measurement) were used as culture vessels, which were kept in the southern verandah of Biological Research Division of BCSIR Laboratories, Dhaka. Depth of the culture and the pH was initially recorded and adjusted once a day. Optimum density of the culture was observed with the help of spectrophotometer once a day (560 wave length). The experiment conducted in each season and had 5 replicas. The seasonal data on rainfall, temperature and relative humidity were collected from Bangladesh Meteorological Climatic Division, Agargaon, Dhaka.

3.4 Role of sun-dried *Spirulina platensis* biomass as biosorbent at room temperature.

The *Spirulina* biomass as a biosorbent was studied by the procedure as suggested by Paknikar and Agate (1995).

Biomass preparation: Wet biomass collected directly from BCSIR pilot plant ponds. The biomass was washed five times with clean tap water and then it was dried under the sun. The sun-dried biomass was powdered manually and sieved by siever (having 1.5mm pores). Autoclaving at 15Ibs pressure and 121°C sterilized the *Spirulina* granules. The sterilized biomass was then pre-treated for 30 minutes as following steps.

Batch I = Acid treated (1N HCL)

Batch II = Alcohol treated (100% ethanol)

Batch III = Alkali treated (1N NaOH)

All batches of treated biomass was washed five times with distilled water and finally washed with 4pH distilled water. The washed biomass was dried at 60°C and stored in clean glass bottles under dry condition. Using the 3 batches of biomass the following experiments were conducted.

3.4.1 Rate of metal uptake (Pb) by the *Spirulina platensis* biomass.

3.4.1.1 Rate of metal uptake at different pH: To determine the lead biosorption, all the 3 batches of biomass were subjected to different pH levels (2, 3, 4, 5 and 6) of lead nitrate solution (a Merck analytical grade product), pH was adjusted with 1N HNO_3 and 1N NaOH. 1gm/100 ml treated biomass was incubated for 30 minutes in 50ppm lead nitrate solution. The cell mixture was shaken from time to time manually during the incubation period. The mixture was then sieved by filter paper (Whatman 1) to remove the biomass and the concentration of the solution was determined by atomic absorption spectrophotometer at 217nm wavelength. Each batch had 5 replicas. Control sample had no biomass.

3.4.1.2 Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH:

For this experiment the pH selected as the previous experiment was maintained. Concentration of lead nitrate was 50ppm and 1gm/100ml biomass was used. The incubation period ranged from (0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, 90 min and 100 min). The cell mixture was shaken and filtered as before. Atomic absorption spectrophotometer was also used in this study.

3.4.1.3 Rate of metal uptake by the *Spirulina platensis* biomass with different concentration of lead nitrate solution at selected pH and selected incubation period.

Lead nitrate solution having concentration of 50ppm, 100ppm, 150ppm, 200ppm, 250ppm and 300ppm 1gm/100ml were used in this experiment. Biomass of different batches was used in this trial and the experimental procedure was same as before.

3.4.2 Rate of metal uptake (Cd) by the *Spirulina platensis* biomass.

3.4.2.1 Rate of metal uptake at different pH:

3.4.2.2 Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH:

3.4.2.3 Rate of metal uptake by the *Spirulina platensis* biomass with different concentration of cadmium solution at selected pH and selected incubation period.

For this study the rate of cadmium uptake by *Spirulina platensis* biomass was studied, the previously mentioned procedures were followed.

3.5 Isolation of associated bacteria from *Spirulina platensis* culture.

Samples were collected form pilot plant ponds of BCSIR Laboratories. Dhaka, and four commercial ponds producing *Spirulina platensis* in Dhaka district (Table-1 and Fig. 3).

For isolation, dilution plate technique and nutrient agar medium were adopted. After initial selection the selected isolates were purified by repeated streaking on agar plates.

3.5.1 Identification of the isolated bacteria.

Thirty one bacterial strains were selected and studied for identification.

3.5.2 Maintenance and preservation of selected isolates.

Purified bacterial strains were maintained on nutrient agar slants during the course of research (Appen. 2). The culture slant tubes in polyethylene bags were preserved as stock culture in a refrigerator at 5-10°C. Sub-culture of strains were made periodically (15-20 days interval).

Table 1. **Sampling sites.**

Sample no.	Month of collection	Name of the *Spirulina platensis* ponds	Location of producing ponds
1-14	August	Pilot plant ponds of BCSIR, Dhaka	Dr. Qudrat- E-Khuda road Dhaka (Fig. 3a)
15-19	August	Commercial pond of PBL	Katchpur Langolbon (Fig. 3b)
20-22	August	Commercial pond of Natural Food products	Dhamrai (Fig. 3b)
23-28	August	Commercial pond of Evergreen enterprise	Savar (Fig.3c)
29-31	August	Commercial pond of Wonder herbs	Katchpur (Fig. 3c)

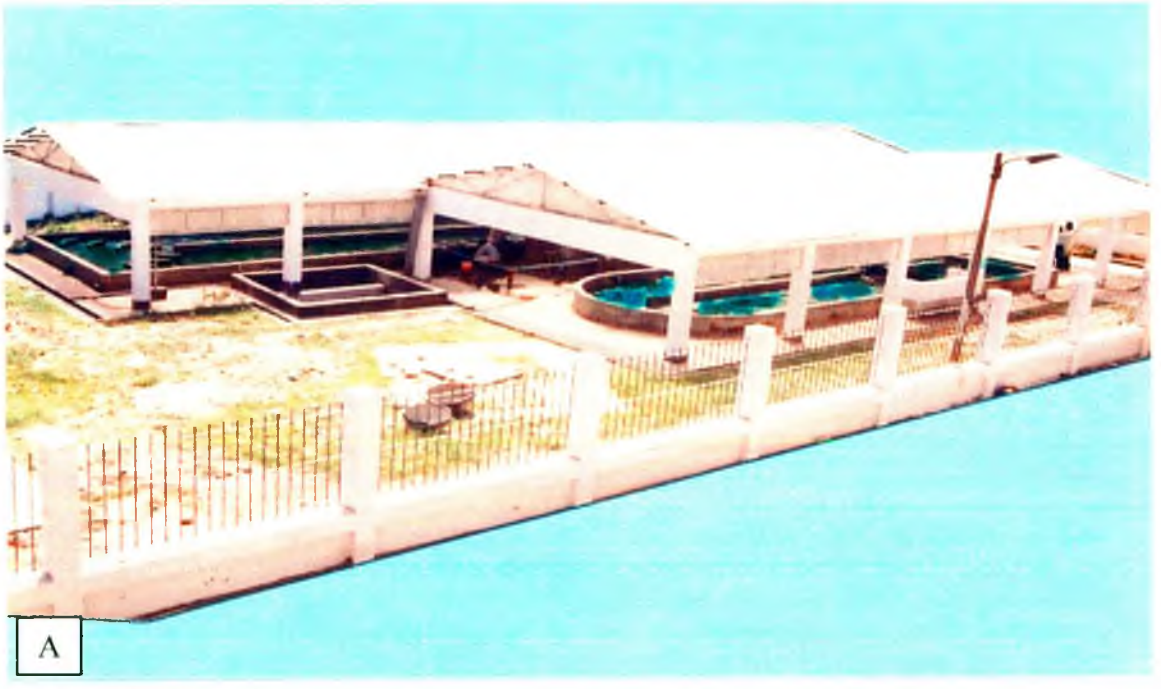


Fig. 3a: (A) Pilot plant pond in BCSIR.

Fig. 3b: (B) Commercial pond in Wonder herbs,
(C) Commercial pond in Project builders limited.

Fig. 3c: (D) Commercial pond in Natural food product,
(E) Commercial pond in Evergreen enterprise.

3.5.3 Morphological studies of the selected isolates:

3.5.3.1 Colony characteristics of selected isolates:

Morphological characteristics of the selected bacterial colonies, including shape, edge, elevation, opacity and color were studied.

3.5.3.2 Cultural characteristics of the selected strain on agar slant and nutrient broth:

The pattern of growth was studied on agar slant and also in nutrient broth.

3.5.3.3 Vegetative cells and spores of bacteria:

The size and shape of vegetative cells of selected strains were determined. The arrangement of cells (single, in chain or cluster) was recorded. Sporulation characteristics were also studied. For the measurement of cells, spores and sporangia and stage micrometer scale (having one small division = 10 micron) was photographed, using similar lens combination. The measurement was done using proportionately magnified photographs of the organisms.

3.5.3.4 Preparation for light microscopic examination:

For microscopic examination of bacterial strains, thin smears were prepared with distilled water, heat fixed and stained by two different methods: simple staining and gram straining.

3.5.3.4.1 Simple staining:

Manual of Microbiological methods (SAB 1957) was followed for simple staining. Basic dye Crystal violet was used (Appen. 3).

3.5.3.4.2 Gram staining, simple staining, spore and sporangium

For Gram staining method of Claus (1995) was followed. Fixed smear was treated as follows. Crystal violet 30sec, I_2 solution 60sec, ethyl-alcohol 15-25sec (until free color was washed off) and basic fuchsin 20sec. After application of each solution, the slide was gently rinsed with fresh tap water for a few seconds and then drained. The slide was then blotted dry and observed under compound microscope (1000X).

3.5.3.4.3 Spore and sporangium:

Spore and sporangium were studied under phase contrast microscope using wet mount method.

3.5.4 Biochemical and physiological studies of the selected isolates:

Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986), Pure culture study of bacteria (Bryan 1950), Manual of Microbiological Methods (Society of American Bacteriologists 1957), Laboratory Manual for General Microbiology (Ekland and Lankford 1967), Microbiological Methods (Collins and Lyne 1984) and Understanding Microbes (Claus 1995) were followed for the physiological and biochemical tests of bacterial strains.

3.5.4.1 Catalase test (Claus 1995).

For this test nutrient agar slant tubes were inoculated with 24 hours old culture and incubated at 37^0C for 24 hours. After incubation fresh cells were placed on a clear dry slide and a few drops of 30% H_2O_2 was directly added on the cells. Production of oxygen bubbles indicated the positive reaction.

3.5.4.2 Motility test (Eklund and Laukford 1967, Claus 1995, Appen. 4).

For motility test tubes of sloppy agar (0.5%) medium was stabbed with sterilized inoculation needle containing fresh bacterial culture. The tubes were incubated at 37°C for 48hrs for bacterial growth, diffusing through the medium indicated the motility of organism, while non-motile organisms were confined to the stabbed line. Each strain had three replicas.

Motility of the strain was also observed directly under phase contrast microscope. Wet mount method was used. The edges of the cover slip were sealed with nail polish.

3.5.4.3 Fermentation test (Claus 1995, Appen. 5).

Fermentation test is of considerable significance in the identification and classification of bacteria. Microorganisms differ in their ability to ferment different carbohydrates. Some of them produce both acid and gas from carbohydrate. Others produce only acid and no gas, some produce only alkali and still others can not ferment carbohydrates at all.

In this study fermentation tests were conducted using the following carbohydrates and Sugar alcohol.

Monosaccharides:

1. Pentose:L-arabinose and D-xylose
2. Hexose:D-glucose
3. Sugar alcohol: D-mannitol

Bromothymal blue was used as indicator. One Durham tube was introduced in fermentation tubes only in case of D-glucose test. The tubes of all the above tests were inoculated with bacterial cell suspension and were incubated for 48hours at 37^0C.

The change of color of the indicator to yellow indicated the production of acid, presence of bubbles in the Durham tubes indicated the production of gas.

3.5.4.4 Hydrolysis of starches (Claus 1995, Appen. 6).

Some organisms are capable of hydrolyzing starch (Polysaccharide) to form monosaccharides or disaccharides with the enzyme amylase. By this test the presence or absence of the enzyme amylase in the organism may be ascertained. For this test, starch agar plates were inoculated with 24hrs fresh bacterial culture by streak method and the plates were incubated at 37^0C for 24hrs. After incubation Lugol's iodine solution was added to each of the plates,

which turned the medium blue. Development of a clear zone around the bacterial growth indicated positive results.

3.5.4.5 Hydrolysis of gelatin (Collins and Lyne 1984, Appen. 7).

Many organisms possess the enzyme gelatinase and are capable of hydrolyzing gelatin resulting in its loss of property to gel. By this test it can be proved whether the organisms have the enzyme or not. Nutrient gelatin agar plates were inoculated with bacteria by streak method and incubated at 37^0C for 24 hours. After incubation, the inoculated plates were covered with acidified mercuric chloride ($HgCl_2$) solution witch formed a white opaque precipitation on the gelatin. Clear zone surrounding the bacterial growth indicated hydrolysis of gelatin by the enzyme gelatinase.

3.5.4.6 Hydrolysis of casein (Collins and lyne 1984).

For this test 1ml of sterilized skim milk was taken in sterilized petridish and then melted, nutrient agar was poured into each petridish, mixed thoroughly and allowed to solidity. Inoculation was done with 24hrs old culture by streak method and incubated at 37^0C for 48 hours. Clear transparent zone around the bacterial growth indicated hydrolysis of casein.

3.5.4.7 MR test (Bryan 1950, Appen. 8).

For this test MR/VP broth medium was inoculated with 24 hours fresh bacterial culture and incubated at 37°C for 72hours. After incubation a few drops of methyl red indicator was added in each culture tube. Red color indicated positive reaction.

3.5.4.8 Voges- Proskauer test (Bryan 1950, Appen. 8).

For this test MR/VP medium was inoculated with 24 hours old fresh bacterial culture and incubated at 37°C for 3-5 days. When sufficient growth was observed, α-napthol and KOH creatine indicator was added to the culture tube in 3:1 ratio. It was shaken vigorously for 1 minute. Within 5-10 minutes a crimson ruby color developed which indicated positive result.

3.5.4.9 Test for nitrate reduction (Bryan 1950, Appen. 9).

Nitrate is reduced to nitrite by certain microorganisms proving that they contain the enzyme nitrate reductase.

The following reagents were required for this test.

Reagents:

1. 8 gm sulphanilic acid in 1 litre of 5N acetic acid-Solution A
2. 6 ml of dimithyl α-Napthylamine in 1 litre of 5N acetic acid. Solution B

Tubes containing nitrate broth medium were inoculated with pure young bacterial culture (24hrs) from solid medium and incubated at 37°C for 24 hours. After incubation a few drops of solution A and equal volume of solution B was added to each inoculated tube, then the tubes were shaken well. The formation of reddish pink or red color indicated the reduction of nitrate. Reddish pink or red color indicated the presence of nitrite. In the tubes having light-pink and yellow color, a very small amount of Zinc dust was added. Production of red color indicated the presence of nitrate i.e. absence of nitrate reductase enzyme. No color development after addition of Zinc dust, indicated the decomposition beyond nitrite stage, i.e., Presence of nitrate reductase enzyme.

3.5.4. 10 Production of indole (Bryan 1950, Appen. 10).

For this test Kovac's (1926) method was used. Tubes containing tryptone broth were inoculated with 24hrs old bacterial cultures and incubated at 37°C for 3-5 days.

After proper growth 3ml of kovac's reagent was added to 5ml of culture medium (Appen.10) the tubes were shaken well, a rose pink color indicated the production of indole.

3.5.4.11 Utilization of citrate (Claus 1995, Appen. 11).

Simmon's eitrate agar slants were inoculated with 24 hours fresh bacterial culture and incubated for 2-3 days at 37°C. Development of blue color developed indicated the utilization of citrate, while no change in color indicated negative reaction.

3.5.4.12 Deamination of phenylalamine (Sneath *et at.* 1986, Appen. 12).

To demonstrate deamination of pheneyalanine to phenyl pyruvic acid, phynylalamine agar slants were inoculated and incubated for 7 days 4/5 drops of 10% (w/v) $FeCl_3$ solution was then pipetted out over the growth.

Production of a green colour beneath the growth indicated the presence of phenylpryuvic acid while no colour represented negative reaction.

3.5.4.13 Degradation of tyrosine (Sneath *et at.* 1986, Appen. 13).

Tyrosine agar plates were inoculated with 24hrs fresh bacterial cultures by single streak method and incubated at 37°C for 3-5(15) days. Degradation of tyrosine was revealed by the clearing of tyrosin zone around and below the bacterial growth.

3.5.4.14 Growth response of the selected bacterial strains at different temperature.

For this purpose, tubes of nutrient agar medium were inoculated with 24hrs old bacterial cultures and incubated at different temperature viz. 10, 20, 30, 40, 50, 55, 60°C for 24hrs. Each strain had three replicas.

3.5.4.15 Growth response at different concentrations of NaCl.

To test the salt tolerance of the strains, nutrient broth with different concentrations of NaCl viz. 2, 5, 7 and 10% were used. Test tubes were inoculated with 24hrs.

Bacterial culture and were incubated at 37°C for 48hrs. Optical density was measured by using spectrophotometer at 550nm wavelength.

3.5.4.16 Observation of bacterial growth at selected pH.

To observe the bacterial growth at selected pH, nutrient broth medium (Appen.14) was used. The selected pH was 5.7 and 6.8 by using different phosphate buffering agent. pH of the medium was adjusted. Tubes were inoculated with 24hrs fresh bacterial culture and incubated at 37°C for 24 hours. Results were reordered by spectrophotometer at 550nm wavelength.

3.6 Identification of selected bacteria.

The isolated bacterial strains were provisionally identified according to Begey's Manual Sneath *et al.* 1996 and Buchanan and Gibbons 1974.

3.7. Effect of sun-dried *Spirulina platensis* extracts on the isolated bacteria.

Source of material: Algal materials: For this study *Spirulina platensis* was collected from mixed culture of *Spirulina platensis* and *Spirulina fusiformis* in the pilot plant pond in Biological Research Division, in BCSIR Dhaka. The *Spirulina platensis* culture was maintained in BD_1 medium (Appen. 1).

Bacterial materials: Thirty-one strains of bacteria were isolated from pilot plant *Spirulina platensis* culture medium pond of BCSIR Dhaka and 5 commercial ponds of Dhaka.

Composition of culture medium used for isolated bacteria: For the culture of bacteria nutrient agar medium was used (Appen. 2).

Algal plates: Nutrient agar plates were used for testing the interaction of *Spirulina platensis* extracts with isolated bacteria.

Maintenance of bacterial stock culture: Culture slants were kept in polyethylene bag, properly tightened and preserved as stocked culture at 10±1°C. For the purpose of keeping the culture in active condition with character unimpaired, bacterial stains were sub-cultured at an interval of 15 days.

Preparation of paper discs: The paper discs were prepared according to the method of Bauer *et al.* (1966). Filter paper discs of 7.0mm diameter were made with the help of a punching machine. These discs were placed inside empty test tubes, plugged and then autoclaved. The sterilized discs were soaked with extracts, dried for 24 hours and then used in experiment. Sterilized filter paper discs soaked with inspective solvents were used as control. In the present study three different solvents namely water, methanol and ethanol were used.

Preparation of bacterial suspension: One loop of bacterial culture was transferred to 1ml sterilized distilled water contained in petridish and was mixed well. The bacterial suspensions were then used for the experiment.

Preparation of algal extracts: *Spirulina platensis* was collected from pilot plant pond of Biological Research Division in BCSIR laboratories Dhaka. The extracts were prepared according to the method of Campos Takaki *et al.* (1988) with some modifications. The *Spirulina platensis* sample was washed thoroughly with tap water and finally with distilled water. The sample was then dried in shade for 4 to 5 days at room temperature (25°C) and ground into coarse powder with a mortar and pestle. The coarse powder was mixed well with different solvents in the ratio of 0.2: 10 W/V. the extracts were kept for 6 hours at room temperature and after then stored in deep freeze for further study. The different solvents namely water; methanol and ethanol were used for the preparation of crude extract of *Spirulina platensis.*

3.7.1 Effect of algal extracts on the growth of isolated bacteria.

Interaction between the algal extracts and isolated bacteria (1-32) was studied. The cultures were maintained in nutrient agar medium. Bioassay was conducted by the agar diffusion technique (Pesando and Caram 1984) where sterile (7.0 mm diameter, whatman no-1) filter paper discs were soaked in different extracts of *Spirulina.* These discs were then kept at room temperature for 24 hours for evaporation of the solvents. Sterilized filter paper discs soaked

in the respective solvents, served as control. For the purpose of test bacterial materials agar plates were seeded using suspension. After preparing the plates, paper discs impregnated with extract along with control discs were placed gently on the agar surface and incubated for 24 hours at 37°C. Each experiment had 3 replicas. After incubation the degree of sensitivity of the test bacteria was determined by measuring the zone of inhibition or stimulation around the paper discs.

3.8 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by different treatments.

The study was conducted in BCSIR laboratories, Dhaka, using culture of *Spirulina platensis*, collected from BCSIR pilot plant ponds. The culture was maintained in Bd_1 medium (Appen.1).

3.8.1 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by antibiotic treatment (Doxycyclin and Amoxicillin).

To study the effect of antibiotics on bacterial count of *Spirulina platensis* culture different concentrations (50mg/l-60gm/l) of Doxycyclin, and Amoxicillin, were individually used. The period of treatment ranged from 10 minutes to 24 hrs (10 min, 20 min, 30 min, 40 min, 50 min, 1hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, and 24 hrs). Nutrient agar plate (pH9) was then inoculated with above-mentioned treated samples by pour plate technique. After 72 hours incubation at room temperature (22°C-25°C), the bacterial colonies on nutrient agar plates were counted. Each treatment had 5 replicas. The study was conducted in BCSIR laboratories Dhaka, using *Spirulina platensis* collected from BCSIR pilot plant pond. The culture was maintained in BD_1 medium (Appen. 1). The findings were compared with the control.

3.8.2 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by chemical treatments (Formaldehyde, Acetic acid and Alcohol).

Effect of different concentrations (50, 100, 200 and 300ppm) of 37% Formaldehyde, Concentrated Acetic acid and 95% Alcohol, were studied on bacterial count of *Spirulina platensis* culture.

The period of treatment ranged from 2 minutes to 24 hours (2 min, 4 min, 6 min, 8 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 2 h, 3 h, 4 h, 5 h and 24 hours). Nutrient agar plate (pH-9) was then inoculated with above treated samples by pour plate technique. After 72 hours incubation at room temperature (22°C-27°C), the bacterial colonies on Nutrient agar plates were counted. Each treatment had 5 replicas. Control of bacteria in *Spirulina platensis* culture under optimum treatment conditions were compared.

The study was conducted in BCSIR laboratories Dhaka, using *Spirulina platensis* collected from BCSIR pilot plant ponds. The culture was maintained in BD_1 medium. (Appen. 1).

3.8.3. Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by chemical treatments (Sodium chloride/Sugar).

Effect of different concentrations (10gm/l and 150gm/l) of sodium chloride and sugar were studied on bacterial count of *Spirulina platensis* culture. The period of treatment ranged from one hour to 24 hours (one hour, two hours, three hours, four hours, five hours, six hours and 24 hours). Nutrient agar plate (pH-9) was inoculated with the treated samples by pour plate technique. After 72 hours incubation at room temperature ($28^{\circ}C$-$32^{\circ}C$) bacterial colonies on nutrient agar plates were counted. Each treatment had 5 replicas. Control of bacteria in *Spirulina platensis* culture under optimum treatment conditions were compared.

3.8.4. Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture through washing.

The reduction of bacterial load was observed through washing with non-sterilized and sterilized tap water of BCSIR laboratories Dhaka. For the study 500ml *Spirulina platensis* culture was collected from pilot plant pond of BCSIR laboratories Dhaka. CFU/ml was determined by dilution technique at room temperature when pH was 9.

The culture was then sieved using sterilized cloth. 50% slurry was washed 5times with the non-sterilized water and the remaining 50% with the sterilized water. Washed slurry was then diluted with 250ml non-sterilized water and 250 ml sterilized water respectively. From both sets the CFU/ml was determined according to plate technique.

3.9 Preservation of sun-dried *Spirulina platensis* at room temperature.

3.9.1 Effect of storage period on protein content of sun-dried *Spirulina platensis* biomass.

A study was conducted on effect of storage period on the protein content of sun-dried *Spirulina platensis* biomass. The sun-dried sample was stored in12 sealed double polyethylene bags at room temperature. The storage period was one year and protein content of the storage sample was recorded during this period, once every month.

Preparation of sun dried sample, principle, reagent used, procedure and calculation were one by one the same procedure of protein analysis Kjeldahl method (Cocks and Van Rede 1966). The seasonal data on rainfall, temperature and relative humidity were collected from Bangladesh Meteorological climatic Division, Agargaon, Dhaka.

3.9.2 Effect of storage period on beta carotene content of sun-dried *Spirulina platensis* biomass.

The biomass was stored in double polyethylene bags, which were placed in a black polyethylene and then stored at room temperature. The storage period of this study was one year. To study the effect storage period, beta-carotene percentage of the samples were recorded during this period, twice a month.

Preparation of sun-dried sample, principal reagent, procedure, and calculation determination methodology was adapted as before beta-carotene analysis.

The seasonal data on rainfall, temperature and relative humidity were collected from Bangladesh Meteorological climatic Division, Agargaon, Dhaka.

3.9.3 Effect of storage period on bacterial load and moisture content of sun-dried *Spirulina platensis* biomass.

A study was conducted on effect of the storage period on bacterial load and moisture content of sun-dried *Spirulina platensis* biomass.

Four sun-dried samples were collected from four different commercial companies. Each sample was used as four replicas and was enclosed in double polyethylene bags. The above samples were stored for one year at room temperature. Initial bacterial load and percentage of moisture content were recorded. During the storage period, bacterial load and percentage of moisture content were duly observed and recorded once every four months.

For observation of bacterial load dilution plate method technique was followed and for the percentage of moisture content AOAC (Association of Official Analytical Chemicals) method was followed. The seasonal data on rain fall, temperature and relative humidity were collected from Bangladesh Meteorological climatic Division, Agargaon, Dhaka.

3.10 Regeneration of *Spirulina* from sun-dried, *Spirulina platensis* biomass (1 week, one year and two years old).

The study was conducted in Biological Research Division of BCSIR Laboratories, *Spirulina platensis* was harvested from pilot plant ponds of BCSIR washed and dried under direct sunlight. Sun-dried biomass was stored in sealed double polyethylene bags at room temperature. The storage period was one week, one year and two years. Moisture content of one week old biomass was 11.2 %, that of one year old sample was 13.0 % and in two years old sample it was 14.1%.).

Procedure: Dry *Spirulina platensis* biomass (one week, one year and two years old) was taken in 500 ml beakers containing 300 ml freshly prepared IFP medium (Appen.15). Inoculum size of the dried biomass was 0.03%, 0.04%, 0.05% and 0.1%. The beakers were kept in the southern verandah of Biological Research Division of Dhaka. Depth of the culture medium was 10cm. Cultures were observed periodically under compound microscope (100X). Regeneration of *Spirulina platensis* was observed upto one month. Regenerated *Spirulina platensis* filaments were counted by dilution method.

Chapter 4

Results and discussion

4.1 Chemical composition of sun-dried *Spirulina*, cultured under local condition

Spirulina culture under local conditions was harvested, sun-dried and used for the analysis of protein, fat, fibre, ash, beta-carotene and carbohydrate. The moisture percentage of the sun-dried *Spirulina* was 11. The results are presented in (Appen.16 and Fig. 4).

4.1.1 Protein content

In the present study the percentage of protein was found to be 65 in the sun-dried *Spirulina* biomass (Appen.16 and Fig.4).

According to Venkataraman (1983) *Spirulina platensis* contained 55-65% protein of dry weight. Becker and Venkataraman (1984) reported that dried biomass of *Spirulina* contained 62.5% protein. According to Henrikson (1989) *Spirulina platensis* contained high percentage of protein, 55-70% of dry weight. Bucaille (1990) reported that percentage of protein in *Spirulina platensis* was 64.7 –71% of dry weight. Bonnin (1992) reported that *Spirulina platensis* contained 62-70% protein of dry weight. Mathew *et al.* (1995) reported that average percentage of protein in *Spirulina* was 55% of dry weight. According to Dillon *et al.* (1995) 60-70% protein was present in *Spirulina platensis* of dry weight. According to Majid *et al.* (1996) *Spirulina* contained 55-70% protein of dry weight. Varga *et al.* (2002) reported that dried *Spirulina* typically contained 50-60% protein. Present author found 60-65% protein in sun-dried biomass of *Spirulina*. The present finding more or less agrees with the above reports

4.1.2 Fat content

The percentage of fat was 0.24 in the present study (Appen.16 and Fig. 4). According to Henrikson (1989) *Spirulina* contained 5% fat of dry weight. Becker and Venkataraman (1984) reported that 3% fat was present in *Spirulina* biomass of dry weight. Dillon *et al.* (1995) reported that 5.7% fat was present in *Spirulina.* Venkataraman (1983) reported that 2-6% fat was present in *Spirulina* of dry weight. In the present study it was found that 0.24 % fat was present in sun-dried biomass of *Spirulina*. This finding differed all the above findings but it agreed with the finding of BSTI (Bangladesh Standard and Testing Institute, Appen.17).

4.1.3 Fibre content

In this study the percentage of fibre was 1.27 (Appen. 16 and Fig. 4)

According to Venkataraman (1983) 1-4% crude fiber was present in *Spirulina* of dry weight. According to Varga *et al.* (2002), 1-1.5% crude fibre was present in *Spirulina* of dry weight. Mathew *et al.* (1995) reported that 0.9% crude fibre was present *Spirulina* biomass of dry weight. Present author found 1.27 % crud fiber in sun-dried biomass of *Spirulina.* This finding agrees with above findings excepting the maximum fibre content reported by Venkataraman (1983).

4.1.4 Ash content

The percentage of ash was found to be 6.60 (Appen. 16 and Fig. 4).

According to Mathew *et al.* (1995) 9% ash was present in *Spirulina* of dry weight. In the present study, author found 6.6 % ash in sun-dried biomass of *Spirulina*, this finding disagreed with the above finding. Venkataraman (1983) reported that 6-15% ash was present in *Spirulina* of dry weight. According to Verga *et al.* (2002) 7-10% ash was present in *Spirulina* biomass of dry weight.

The present author found 6.6 % ash in sun-dried *Spirulina* biomass. which agreed with the minimum range of the above findings agree with the present finding.

4.1.5 Beta carotene content

The percentage of beta-carotene was 0.20 (Appen. 16).

According to Dillon *et al.* (1995) 0.15 -0.20% beta-carotene was present in *Spirulina* of dry weight. According to Annapurna *et al.* (1991) freshly harvested *Spirulina* contained 2mg beta-carotene per gram algal powder (i.e., 0.2%). Present author found 0.2% beta-carotene in sun-dried *Spirulina* biomass. This finding agrees with the above findings.

According to Faggi (1980) 840mg/kg (i.e., 0.084%) beta-carotene was present in *Spirulina* of dry weight. Venkataraman (1983) reported that, 500mg/kg (i.e., 0.05%) beta-carotene was present in *Spirulina* of dry weight.

According to Henrikson (1989) 10gm *Spirulina* contained 23000 I.U. (14mg i.e., 0.14%) of dry weight. Becker and Venkataraman (1984) reported that 300mg/kg (i.e., 0.03%) beta-carotene was present in *Spirulina* of dry weight. The above findings disagree with the present finding.

According to Venkataraman (1983) chemical composition of algae vary to some extent depending on the growth condition such as temperature, pH availability of nutrients etc. It is for this reason that different chemical analysis of algae results in different data. Therefore, the above report suggests that the difference of chemical analysis of *Spirulina,* as mentioned before, may be due to the difference of nutrients in the culture media (Appen. 1 and 15).

4.1.6 Carbohydrate content

According to Becker and Venkataraman (1989) 13.20% carbohydrate was found in *Spirulina* biomass of dry weight. According to Bonnin (1996) 14% carbohydrate was present in *Spirulina* of dry weight. The present author

found 15.89% carbohydrate in sun-dried biomass of *Spirulina.* Which disagrees with the above findings.

According to Henrikson (1989) 20% carbohydrate was present in *Spirulina of* dry weight, which disagrees with the present finding. According Verga *et al.* (2002) *Spirulina* contained 12-20% carbohydrate of dry weight. The present author's finding is similar to the lower range of the above finding. Dillon *et al.* (1995) reported that 10-15% carbohydrate was present in *Spirulina* of dry weight. The present finding agrees with the above findings.

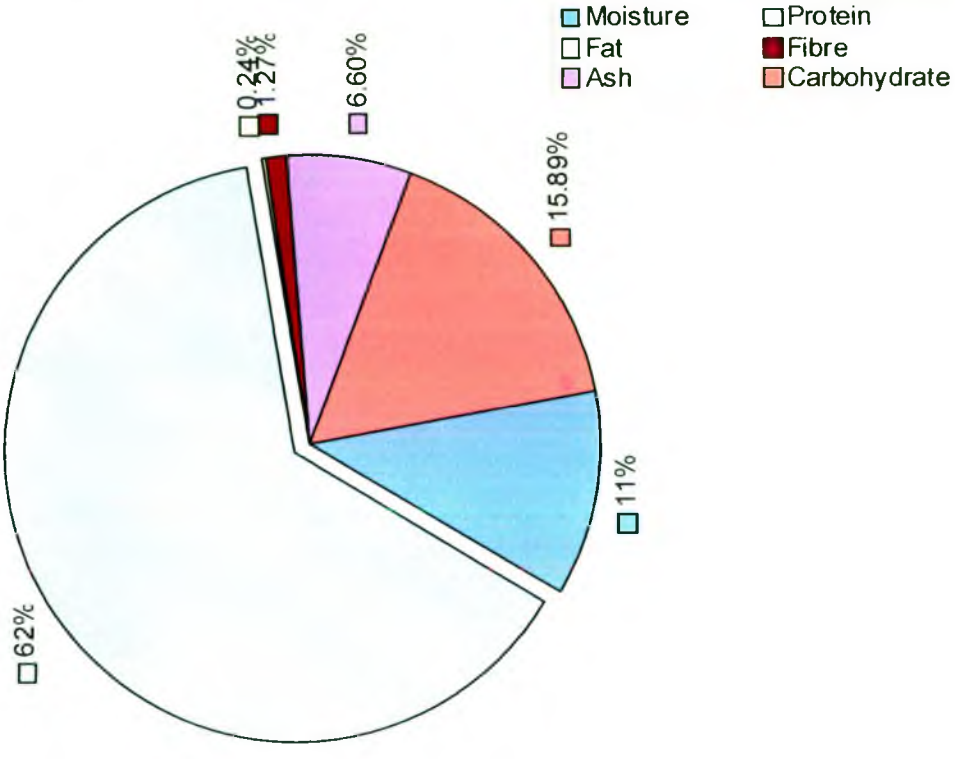


Fig. 4: Chemical composition of *Spirulina platensis* under local conditions.

4.2 Effect of pH on *Spirulina platensis* culture

Effect of different pH on cultures of *Spirulina platensis* are presented in Appen. 18 and 19. The optimum pH for growth of *Spirulina platensis* varied from 7-10, maximum O. D. was found to be 1.21-1.24 and 1.52-1.53 at 9-10 pH (Fig. 5, 7 and Appen.18 and 19). Growth of *Spirulina* was found to be affected adversely at lower and higher pH (Fig. 5, 7 and Appen. 18, 19), at pH 6 and 11, the O. D. of *Spirulina platensis* came down to 02. In pH 6 and 11 50% to 60% *Spirulina* filaments were broken within 3days (Table. 2a). On the other hand, even after 7days, filament conditions were found to be good at the pH label 7 to 10 (Table. 2b).

During the period of study it was noticed that when pH was adjusted daily at different levels (6, 7, 8, 9, 10 and11), after 7days pH of all the cultures, except pH 6, reached at pH 10.2-10.3 (Fig. 6 and Appen.18). When pH was not adjusted pH levels of all the cultures (including that of pH 6) reached pH 10 within 7 days (Fig. 8 and Appen.19).

From the above results it may be concluded that optimum pH for the growth of *Spirulina platensis* is 10 and maximum growth of *Spirulina platensis* was found at pH range 9-10.

Optimum range of pH was reported to be 8-10 by Bonnin (1996) and the maximum pH tolerated by the culture was reported to be 11.3. According to Venkataraman (1983) *Spirulina* grows well at pH value between 9 and 11. According to Monaselidze *et al.* (2002) maximum growth of *Spirulina* was at pH range 9.3-10.3. Venkataraman and Becke (1982) reported that *Spirulina* grows well at pH values between 9 and 11.

In the present study the optimum pH was found to be10 and maximum growth of *Spirulina platensis* was found at pH range 9-10. Maximum pH tolerated by the culture was 11.1. The present study agrees with the findings of the above authors.

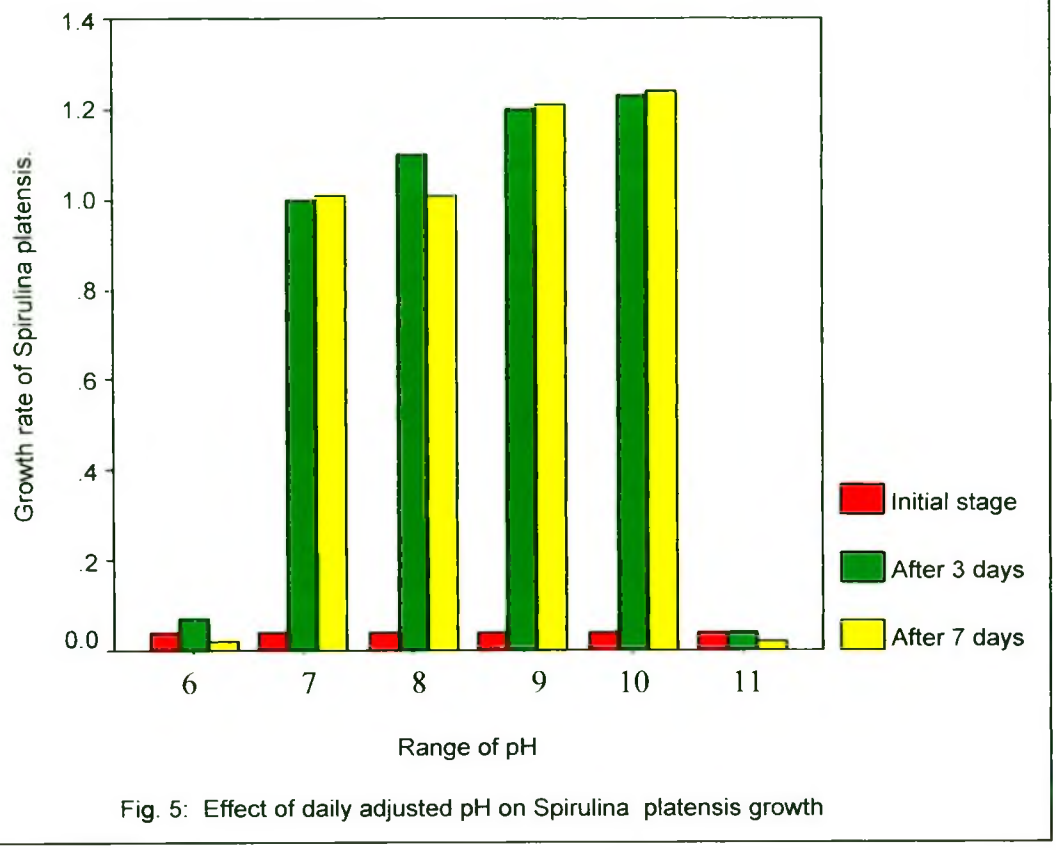


Fig. 5: Effect of daily adjusted pH on Spirulina platensis growth

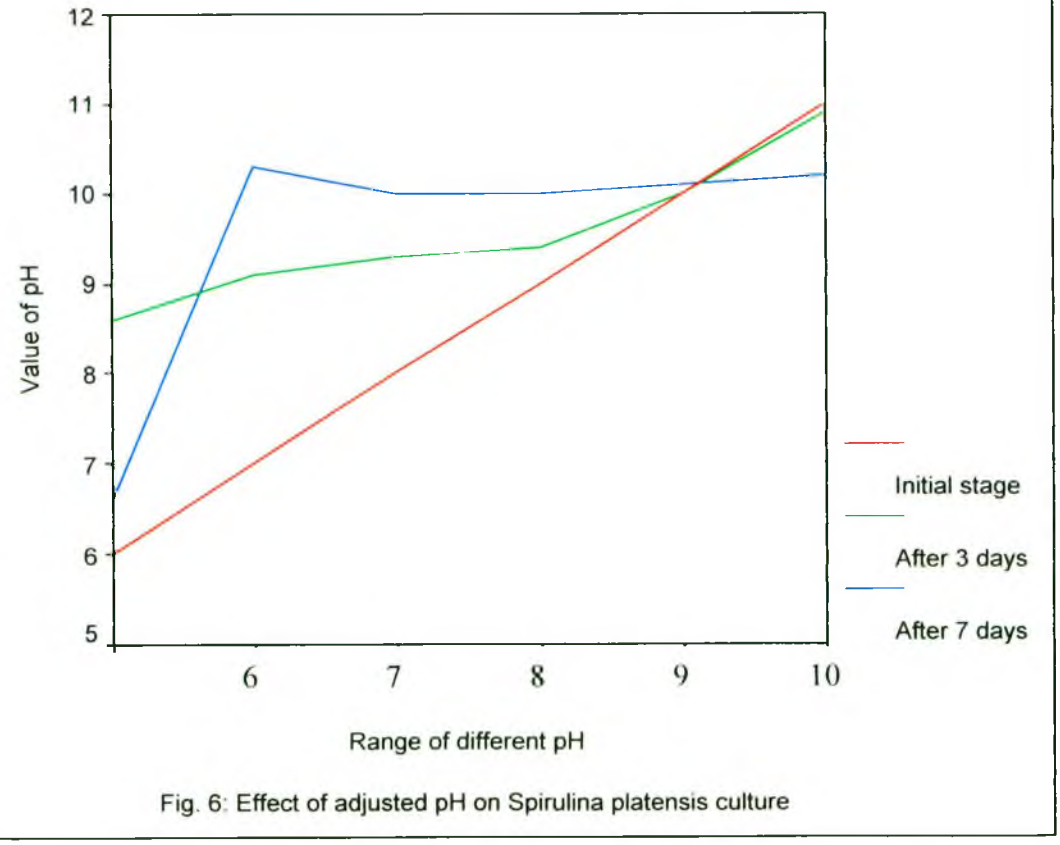


Fig. 6: Effect of adjusted pH on Spirulina platensis culture

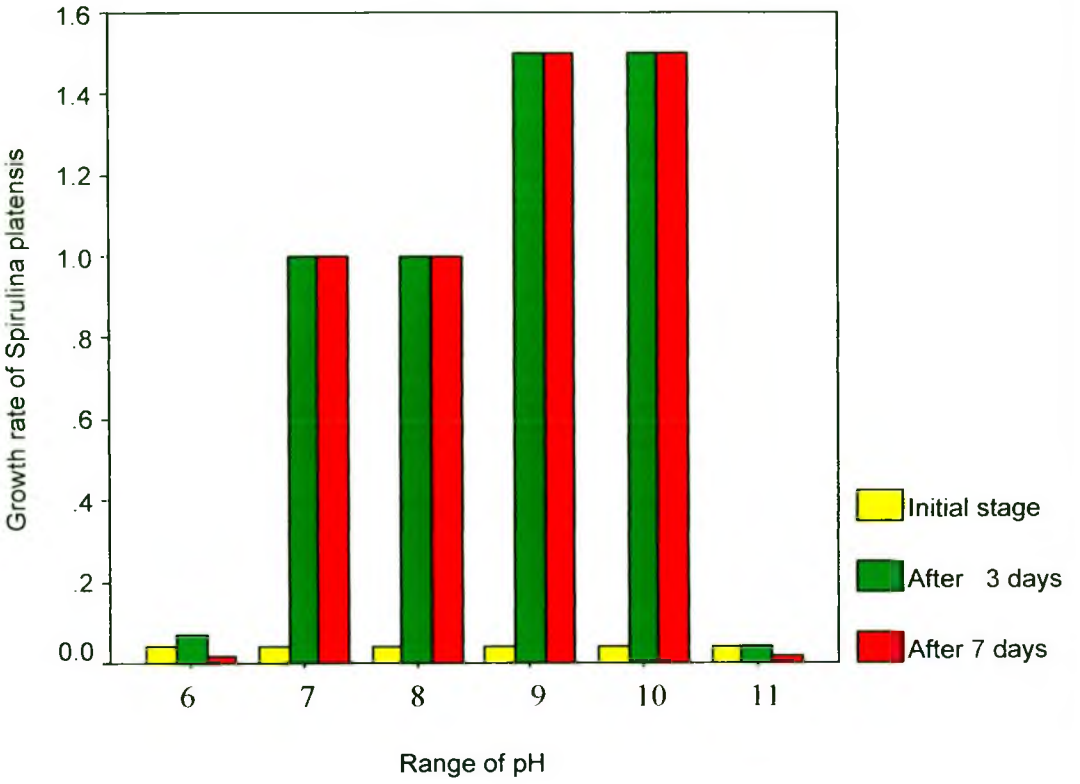


Fig. 7: Effect of non-adjusted pH on Spirulina platensis growth

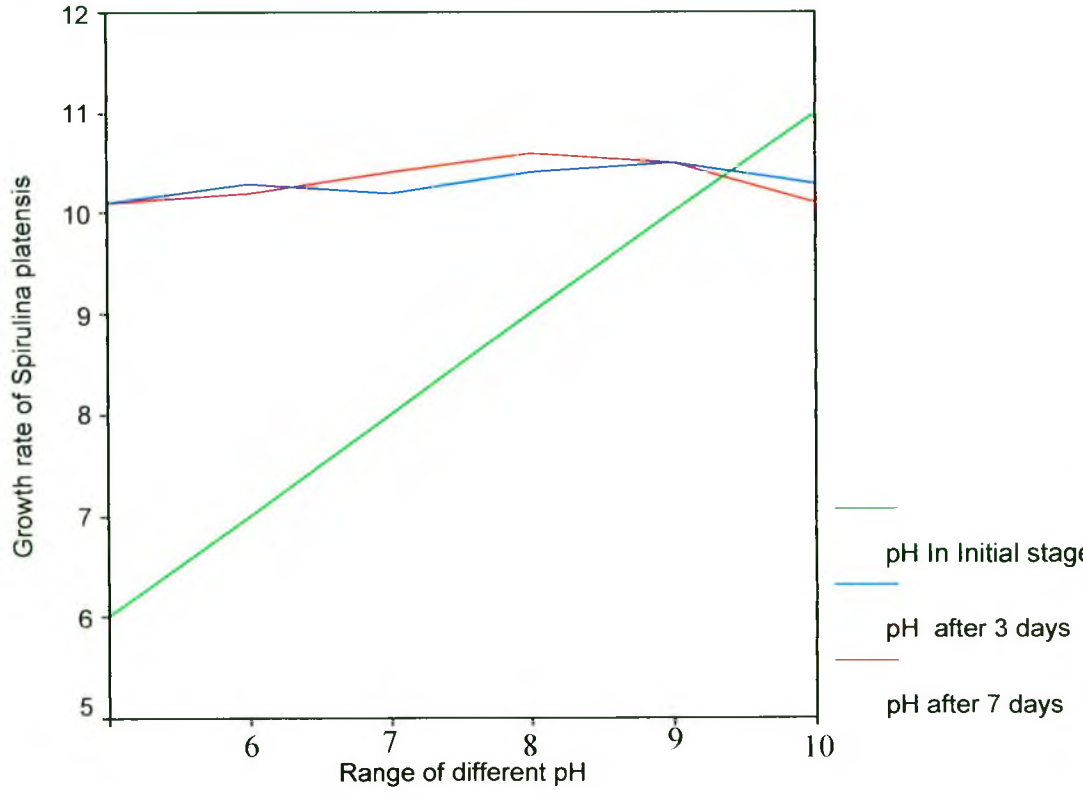


Fig. 8: Effect of non adjusted pH on Spirulina platensis culture

Table 2a. **Effect of pH (once a day adjusted) on *Spirulina platensis* culture**

Sample no.	Initial pH	Initial microscopic observation	Microscopic observation (After 3days)	Microscopic observation (After 7days)
1	6	Filaments were normal and healthy, few were broken.	50% broken filaments were present.	Very few filaments alive.
2	7	Filaments were normal and healthy, few were broken.	Filaments were good, very few broken filaments were present.	Filaments were good.
3	8	Filaments were normal and healthy, few were broken.	Filaments were good.	Filaments were good.
4	9	Filaments were normal and healthy, few were broken.	Filaments were good.	Filaments were good.
5	10	Filaments were normal and healthy, few were broken.	Filaments were good.	Filaments were good
6	11	Filaments were normal and healthy, few were broken.	Filaments were not good 60% filaments were broken.	Very few filaments were present.

Table 2b. Effect of pH (not adjusted) on *Spirulina platensis* culture.

Sample no	Initial pH	Initial microscopic observation	Microscopic observation (After 3days)	Microscopic observation (After 7days)
1	6	Filaments were normal and healthy, few were broken.	Filaments conditions were same.	Presence of filaments was less.
2	7	Filaments were normal and healthy, few were broken.	Filaments were very good.	Filaments were good.
3	8	Filaments were normal and healthy, few were broken.	Filaments were very good.	Filaments were very good.
4	9	Filaments were normal and healthy, few were broken.	Filaments were very good.	Filaments were very good.
5	10	Filaments were normal and healthy, few were broken.	Filaments were very good.	Filaments were very good.
6	11	Filaments were normal and healthy, few were broken.	Filaments were not good.	Few filaments present.

4.3. Biomass production of *Spirulina platensis* in different seasons of Bangladesh.

Table. 3 showed that rate of biomass production varied from 8 to 20gm/m^2/day (on dry basis) in winter. The average growth per week was 11.99±3.96 gm/m^2/day. The rate of biomass production of *Spirulina platensis* was found to increase in summer 10.6 to 19.8gm/m^2/day in comparison to winter (Table. 4). The average biomass per week was 13.36±3.61gm/m^2/day in summer. The growth rate of *Spirulina platensis* was found to be minimum, 5.8gm/m^2/day in monsoon. The average growth per week was 7.99±2.15gm/m^2/day (Table. 5).

It was found that per day growth of *Spirulina platensis* was maximum in summer 13.36gm/m^2, minimum in monsoon, 7.99gm/m^2 and 11.99gm/m^2 in winter (Appen.20).

The present study revealed that summer is the optimum growing period for *Spirulina platensis* in Bangladesh followed by winter. Growth rate was found to be minimum in monsoon (Fig. 9).

According to Fouly *et al.* (1998) average biomass productivity of *Spirulina* reached the maximum in summer. The findings of the present study agree with the above.

Venkataraman (1983) mentioned that the growth of *Spirulina was* reduced during the monsoon due to limiting light intensities caused by cloudy weather. This finding also agrees with the present study.

The seasonal study also showed that in winter and summer under optimum conditions, growth of *Spirulina platensis* was double within 2-3 days (Table- 3, 4 and 5).

According to Majid *et al.* (1996) growth of *Spirulina platensis* may be double within two to three days, when abundant nutrient is available. Henrikson (1989) also stated that this *Spirulina* can double its biomass every 2 to 5days.

Table 3. **Biomass production of *Spirulina platensis* in winter.**

Number of observation	Initial O.D.	Initial volume (m^3)	Final O.D.	Final volume (m^3)	Initial biomass (kg)	Final biomass (kg)	Biomass /day (kg)	Biomass/ day (gm)	Dry wt. of biomass ($gm/m^2/day$)
1	0.418	0.0639	0.590	0.0639	0.02136	0.03016	0.0088	8.80	11.99 ±3.96
2	0.590		0.799		0.03016	0.04084	0.01068	10.68	
3	0.799		0.990		0.04089	0.05060	0.00971	9.71	
4	0.990		1.225		0.05060	0.06080	0.0102	10.20	
5	1.225		1.585		0.06080	0.08102	0.0202	20.20	
6	1.585		1.859		0.08102	0.0950	0.0140	14.01	
7	1.859		2.061		0.0950	0.10539	0.01035	10.35	

Table 4. **Biomass production of *Spirulina platensis* in summer.**

Number of observation	Initial OD	Initial volume (m^3)	Final OD	Final volume (m^3)	Initial biomass (kg)	Final biomass (kg)	Biomass /day (kg)	Biomass/ day (gm)	Dry wt of biomass ($gm/m^2/day$)
1	0.324	0.0639	0.535	0.0639	0.0165	0.0273	0.01084	10.8	13.36±3.61
2	0.535		0.752		0.0273	0.0384	0.0114	11.4	
3	0.752		1.140		0.0389	0.0582	0.0198	19.8	
4	1.140		1.357		0.0582	0.0693	0.0111	11.1	
5	1.357		1.689		0.0692	0.0863	0.0170	17.04	
6	1.689		1.939		0.0863	0.0992	0.0128	12.8	
7	1.939		2.144		0.0992	0.1090	0.0106	10.6	

Table 5. **Biomass production of *Spirulina platensis* in monsoon.**

Number of observation	Initial O.D.	Initial volume (m^3)	Final O.D.	Final volume (m^3)	Initial biomass (kg)	Final biomass (kg)	Biomass /day (kg)	Biomass/ day (gm)	Dry wt of biomass ($gm/m^2/day$)
1	0.244	0.0639	0.358	0.0639	0.01247	0.01830	0.00583	5.8	7.99 ± 2.15
2	0.358		0.552		0.01830	0.0282	0.0099	9.9	
3	0.552		0.699		0.0282	0.0357	0.0075	7.5	
4	0.699		0.858		0.0357	0.0438	0.0081	8.16	
5	0.858		1.082		0.0438	0.0553	0.0115	11.5	
6	1.082		1.188		0.0553	0.0607	0.0059	5.4	
7	1.188		1.326		0.0607	0.0677	0.0070	7.68	

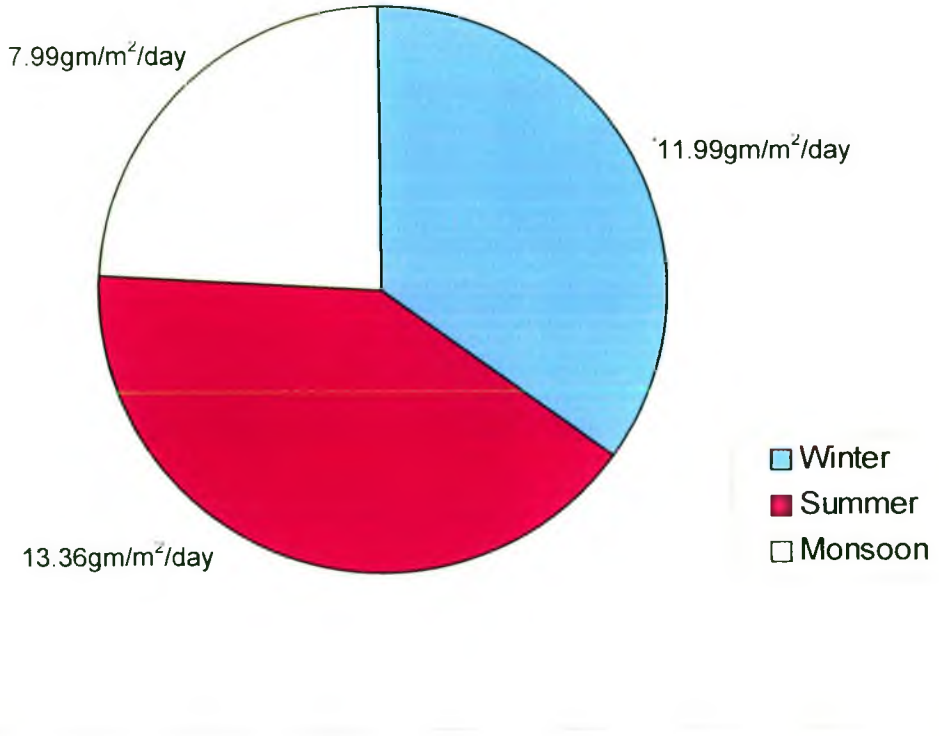


Fig. 9: Biomass production of *Spirulina platensis* in different season in Bangladesh.

4.4 Role of sun-dried *Spirulina platensis* biomass as biosorbent, at room temperature.

4.4.1 Rate of metal uptake (Pb) by the *Spirulina platensis* biomass.

4.4.1.1 Rate of metal uptake in different pH (Pb).

The kinetics of the biosorption in three batches of biomass gradually increased at higher pH (2<3<4<6). The biomass, which was treated by alcohol, showed maximum efficiency as biosorbent for Pb followed by alkali treated and then acid treated biomass. Maximum efficiency of the biomass by biosorbent was recorded at pH 6 in all cases (Fig. 10 and Appen.21)

4.4.1.2 Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH.

In this study kinetics of the biosorption gradually increased during 10 to 70 minutes incubation period, then decreased. In all the three batches of biomass, 70 minutes incubation period was therefore selected in this study (Fig. 11 and Appen. 22)

4.4.1.3 Rate of metal uptake in different concentration of $PbNO_3$ solution at selected pH and selected incubation period.

This study acid treated cells (batch-I) didn't give satisfactory results, maximum absorption 20% was obtained in 100ppm concentration and minimum 3.2% in 50ppm concentration. Alcohol (batch-II) and alkali (batch-III) treated cells yielded good result, biosorption in these two batches followed similar pattern. Here biosorption decreased gradually when concentration of the solution increased. In batch-II and III maximum absorption was in 50ppm concentration and minimum in 300ppm concentration. In batch-II maximum

adsorption was 95% and minimum 32% in batch iii maximum was 86% and minimum 25% (Fig.12 and Appen. 23).

The study indicated the potential of *Spirulina platensis* as an agent for biosorption of lead maximum adsorption being 95% when alcohol treated biomass was used. Darnall and Greene examined and proved that under specific conditions *Spirulina platensis* biosorped significant quantities of lead, but metallic binding was found to depend on pH, temperature and presence of competing ions. The present study agrees with the findings of Darnall and Greene (1988).

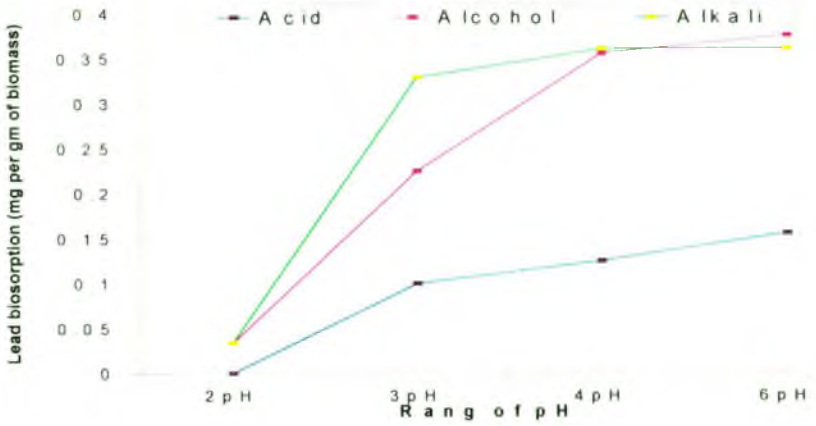


Fig. 10: Rate of metal (lead) uptake in different pH.

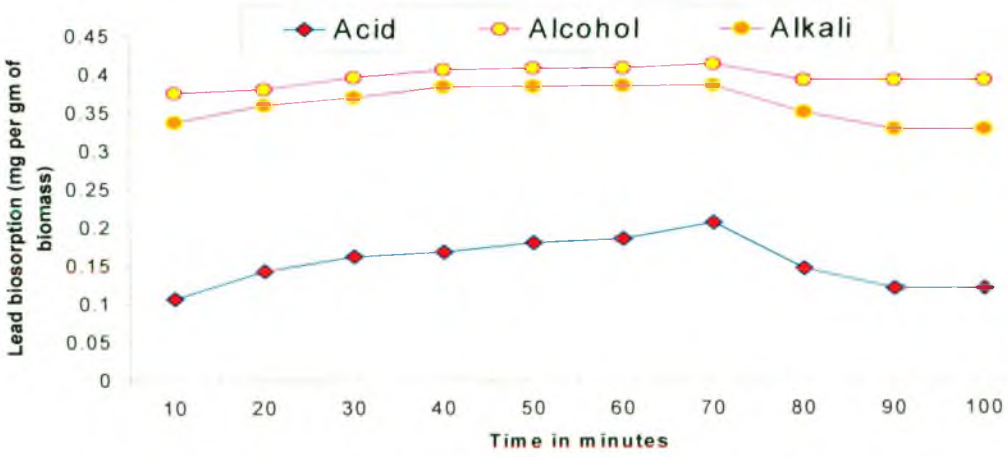


Fig.11: Rate of metal (lead) uptake in different incubation period.

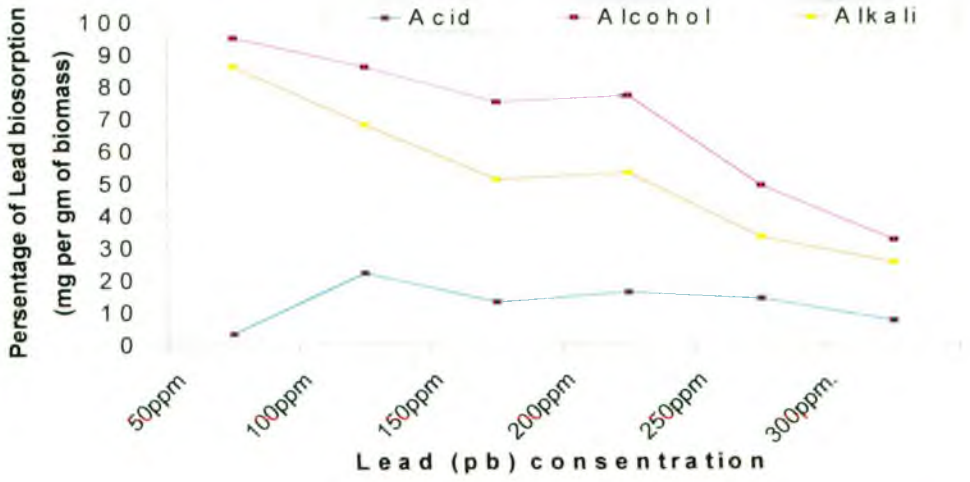


Fig.12: Rate of metal (lead) uptake in different concentration.

4.4.2 Rate of metal uptake (Cd) by the *Spirulina platensis* biomass.

4.4.2.1 Rate of metal uptake with different pH (Cd).

Kinetics of the biosorption all the batches of biomass gradually increased at higher pH (2<3<4<6). The biomass, which was treated by alkali showed maximum efficiency as biosorbent for Cd followed by alcohol treated then acid treated biomass (Fig. 13 and Appen. 24).

Maximum efficiency of the biomass by biosorbent as recorded at pH 6 in all the cases.

4.4.2.2 Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH.

In this study the kinetics of the biosorption gradually increased during 10 minutes to 100 minutes incubation period in the all the batches of biomass, 100 minutes incubation was therefore selected in this study (Fig.14 and Appen. 25).

4.4.2.3 Rate of metal uptake in different concentration of $CdSO_4$, solution at selected pH and selected incubation period.

In this study acid treated cells (Batch-I) did not give satisfactory results, maximum absorption 17.3% was obtained in 50ppm concentration and minimum 2% in 150 ppm concentration.

Alkali (Batch III) and alcohol (Batch II) treated cell yield good results, biosorption in this two batches pattern was not same. In this study alkali (Batch-III) showed maximum biosorption 85% in 50ppm concentration and minimum 13% in 300ppm. In alcohol (batch -II) maximum biosorption 59% in 50ppm and minimum 0% in 150ppm, here the pattern of biosorption was gradually decreased from low concentration to high concentration upto 150ppm and again increased in 200ppm and gradually deceased upto 300ppm. In batch

III maximum absorption was 84% and minimum 13.5%, in batch II maximum absorption was 59% and minimum 0% (Fig. 15 and Appen.26).

Present author found that maximum (85%) adsorption of Cd by sun-dried *Spirulina platensis* biomass was recorded (from aqueous solution) under specific conditions.

Review of literature revealed no specific information on Cd adsorption but according to Ferguson and Babel 1974, Crist *et al.*1981, Karambeer 1987, Volesky 1987, Darnall *et al.* 1988, Gee and Dudeney 1988, Knyucak and Volesky 1988 biologically active (living) or in active (non living) algal cells can reversibly bind significant quantities of metal ion from aqueous solution.

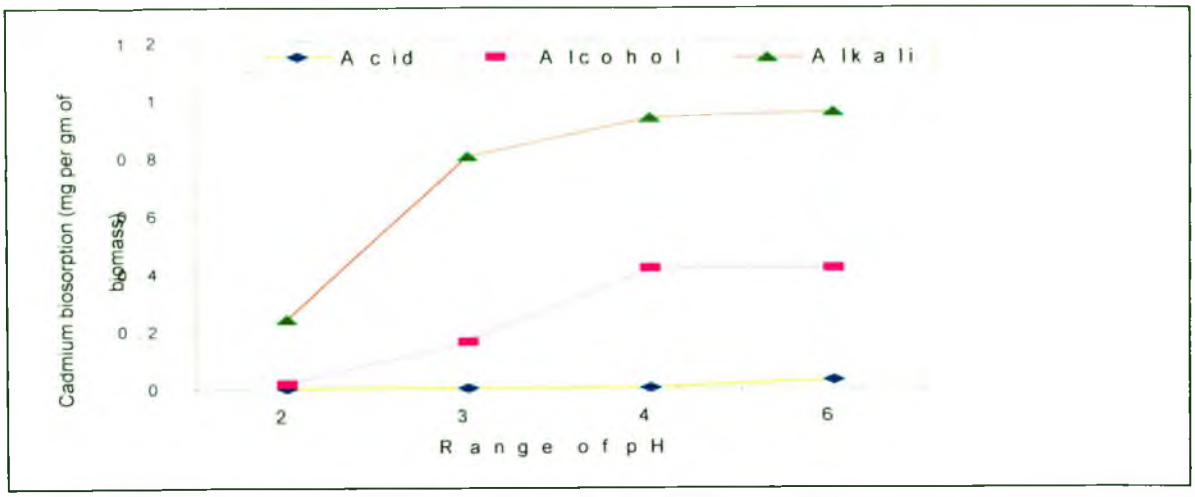


Fig. 13: Rate of metal (cadmium) uptake in different pH.

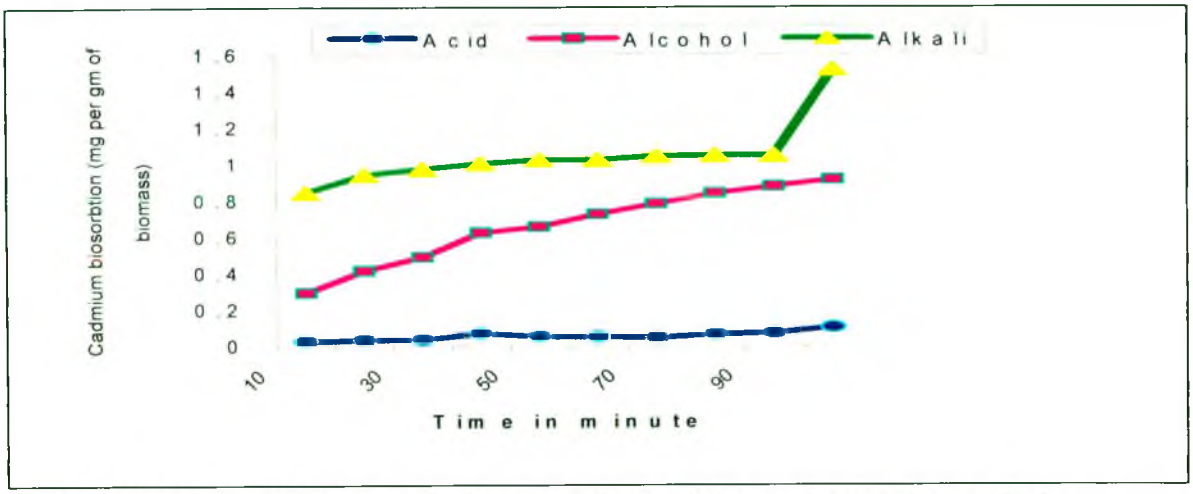


Fig. 14: Rate of Metal (cadmium) uptake in different incubation period.

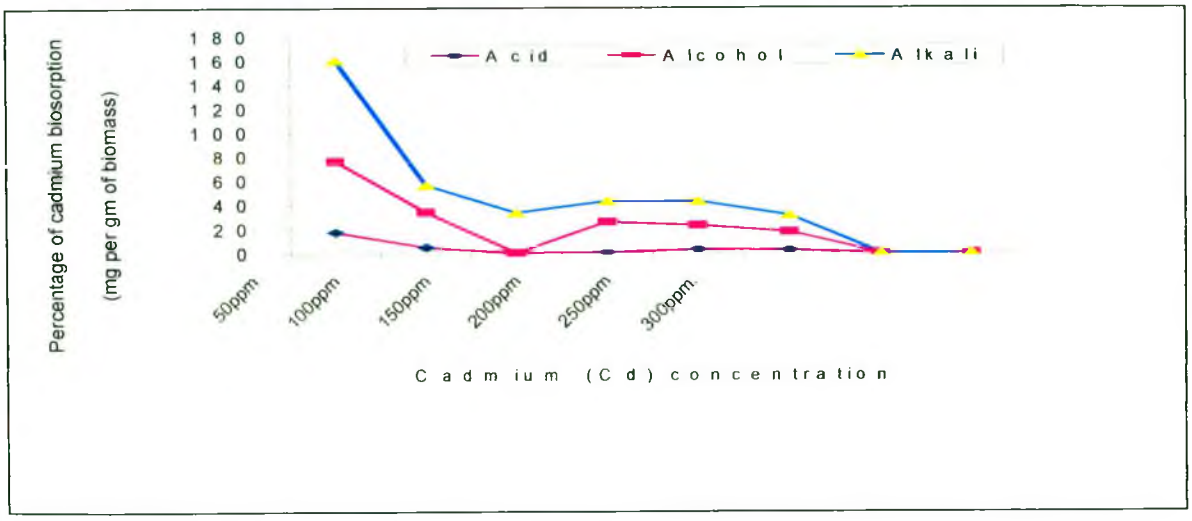


Fig.15: Rate of Metal (cadmium) uptake in different concentration.

4.5 Isolation of associated bacteria from *Spirulina platensis* culture.

31 bacterial strains were selected from pilot plant ponds of BCSIR and four commercial ponds. Purified isolates were taken for identification (Fig.16).

4.5.1 Identification of the isolated bacteria

31 bacterial strains are provisionally identified.

4.5.2 Maintenance and preservation of selected isolates.

Preserved 31 bacterial strains were studied for identification. The results of the study involving colony characteristics, cultural characteristics, microscopic observations (morphological study), biochemical and physiological characteristics of the selected strains are presented in table. 6-9.

4.5.3 Morphological studies of the selected isolates:

4.5.3.1Colony characteristics of the selected strains.

Colonial characteristics of the selected strains are presented in the Table.6. The colonies were circular or irregular. Maximum colonies were cream coloured, however, some where light yellow, orange or white in color. Most of the colonies were opaque in nature. Margin of the colonies were undulate, entire or lobate, surface of the colonies were smooth. Diameters of the studied colonies were 0.5-6.0mm.

4.5.3.2 Cultural characteristics of the selected strain.

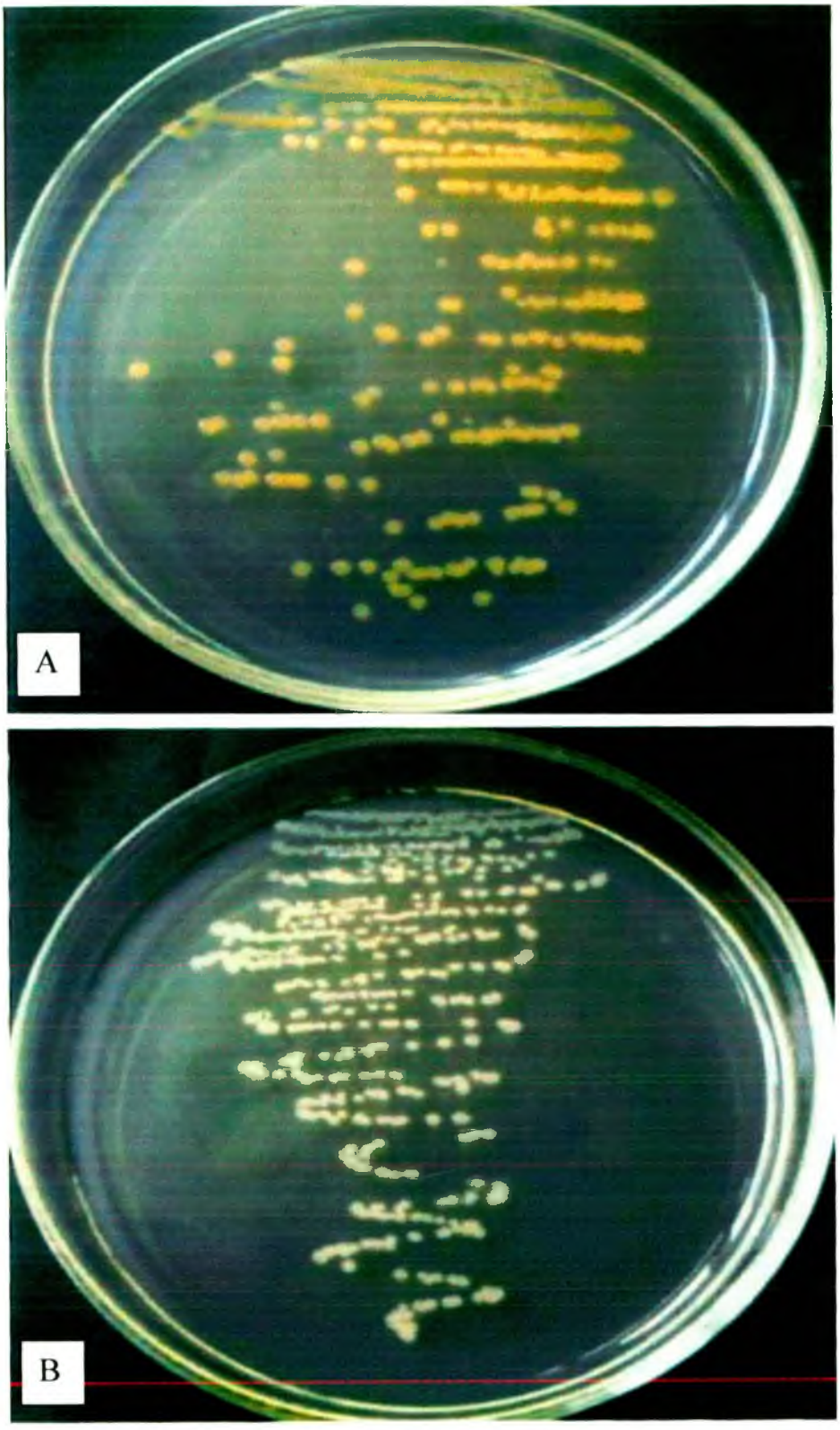
The growth pattern of the selected bacterial strains on nutrient agar slants and in nutrient broth is presented in table. 7.

4.5.3.3 Vegetative cells and spores of bacteria:

All bacterial strains were rod shaped (long rod, short rod and very short rod).

4.5.3.4 Preparation for light microscopic examination:

The result of microscopic observation of the selected strains is presented in the table. 8. Cell shape, arrangement of cells along with their Gram reaction as well as the shape and position of sporangia and spores are shown in fig.17.



F. 16: (A-B) Photographs showing the purification of selected bacterial strains.

Table 6. Colony characteristics of the selected strains on nutrient agar medium.

Strain no.	Form	Elevation (side view)	Margin	Opacity	Surface appearance	Diameter (mm)
1	Irregular	Raised	Undulate	Opaque	Smooth	1.50
2	Irregular	Convex	Undulate	Opaque	Smooth	1.75
3	Irregular	Raised	Entire	Opaque	Smooth	2.50
4	Circular	Raised	Entire	Opaque	Smooth	1.00
5	Circular	Convex	Entire	Opaque	Smooth	0.50
6	Irregular	Raised	Undulate	Opaque	Smooth	2.00
7	Irregular	Raised	Entire	Opaque	Smooth	3.00
8	Irregular	Raised	Lobate	Opaque	Smooth	3.00
9	Circular	Convex	Entire	Opaque	Smooth	0.50
10	Circular	Convex	Entire	Opaque	Smooth	1.50
11	Irregular	Raised	Undulate	Opaque	Smooth	1.00
12	Circular	Convex	Entire	Opaque	Smooth	0.50
13	Irregular	Flat	Entire	Translucent	Smooth	1.25
14	Circular	Convex	Entire	Opaque	Smooth	2.50
15	Circular	Raised	Entire	Translucent	Smooth	3.00

(continued)

Table 6. Colony characteristics of the selected strains on nutrient agar medium

Strain no.	Form	Elevation (side view)	Margin	Opacity	Surface appearance	Diameter (mm)
16	Irregular	Raised	Lobate	Opaque	Smooth	4.00
17	Circular	Convex	Entire	Opaque	Smooth	2.00
18	Circular	Convex	Entire	Opaque	Smooth	3.00
19	Circular	Convex	Entire	Opaque	Smooth	5.00
20	Irregular	Flat	Lobate	Opaque	Smooth	2.50
21	Circular	Convex	Entire	Translucent	Smooth	2.00
22	Circular	Flat	Entire	Opaque	Smooth	3.00
23	Circular	Convex	Entire	Opaque	Smooth	1.50
24	Circular	Convex	Entire	Translucent	Smooth	1.50
25	Circular	Convex	Entire	Opaque	Smooth	2.00
26	Circular	Flat	Entire	Opaque	Smooth	1.00
27	Circular	Convex	Entire	Opaque	Smooth	4.50
28	Circular	Convex	Entire	Opaque	Smooth	1.75
29	Circular	Convex	Entire	Opaque	Smooth	1.50
30	Circular	Flat	Entire	Opaque	Smooth	2.00
31	Circular	Convex	Entire	Opaque	Smooth	6.00

Table 7. Cultural characteristics of selected strains.

Strain no	Growth on nutrient agar slant	Growth on nutrient broth	Strain no	Growth on nutrient broth	Growth on nutrient agar slant
1	Filiform	Ring	17	Ring	Beaded
2	Filiform	Ring	18	Ring	Beaded
3	Filiform	Flocculent	19	Flocculent	Filiform
4	Filiform	Ring	20	Ring	Rhizoid
5	Filiform	Ring	21	Flocculent	Rhizoid
6	Filiform	Flocculent	22	Ring +Beaded	Filiform
7	Filiform	Flocculent	23	Beaded	Filiform
8	Filiform	Pellicle	24	Beaded	Filiform
9	Filiform	Ring	25	Beaded	Filiform
10	Filiform	Ring	26	Ring	Filiform
11	Filiform	Ring	27	Beaded	Filiform
10	Filiform	Ring	28	Flocculent	Filiform
13	Filiform	Ring	29	At the bottom	Filiform
14	Beaded	Flocculent	30	Ring	Rhizoid
15	Filiform	At the top	31	Flocculent	Rhizoid
16	Rhizoid	Ring	-	-	-

4.5.3.4.1 Simple staining:

Fig.18 shows the crystal violet stained selected bacteria cells.

4.5.3.4.2 Gram staining:

All strains showed Gram-positive reaction (Table. 8)

4.5.3.4.3 Spore and sporangium:

Maximum selected strains were spore formers (Fig.17). Maximum spores were terminal or sub terminal (Table. 8). Strain no. 17 had elliptical spore central in position, strain no. 29 had round spore and terminal in position.

4.5.4 Biochemical and physiological characteristics of the selected strains

Selected bacterial strains were biochemically and physiologically studied for identification. The results of the studies are presented into tables 9-15 and described as follows.

4.5.4.1 Test for catalase and motility

It was found that in all the selected strains produced catalase. Strains 1, 2, 9, 10, 19, 23, 25 and 28 were motile, strains 4, 11, 15, 21, 22, 24, 30, 31 were highly motile and strains 3, 5, 6, 7, 8, 12, 13, 14, 16, 17, 18 20, 26, 27, 29, were non-motile (Table -9 and Fig. 19).

4.5.4.2 Fermentation of carbohydrates

In this test, D-glucose, L-arabinose, D-mannitol and D-xylose were studied. It was found that majority of the strains utilized glucose during the oxidative fermentation test. Acid was not produced in stain numbers 27, 29 and 30. In case of L-arabinose, with the exception of 7, 8, 22, 24 and 31, all other strains utilized L-arabinose during the test. Except stains 11, 16, 18, 20 and 25, D-xylose was not utilized by the other stains during the study. In case of D-Mannitol all strains utilized mannitol and produced acid from mannitol with the exception of strains 6, 7, 8, 18, 23, 24 and 31. Gas was not produced during any of the above tests (Table. 10, Fig. 20a and 20b).

Table 8. Microscopic studies and Gram reaction of the selected strains.

Strain no.	Vegetative cell	Length of the cell (μm)	Width of the cell (μm)	Sporangium	Shape and position of spore	Gram reaction
1	Short rod occurring single with round ends	2.0-2.45	0.6-0.7	Not swollen	Elliptical sub-terminal	+
2	Short rod occurring single with round ends	1.5-2.8	0.5-0.6	Not swollen	Elliptical sub-terminal	+
3	Long rod, single and short chain with rounded ends	2.8-3.2	0.6-1.0	Swollen	Elliptical sub-terminal	+
4	Short rod, single and short chain	1.7-2.8	0.5-0.8	Non spore former		+
5	Long rod, single with rounded ends	2.5-3.0	0.6-0.8	Not swollen	Oval sub-terminal	+
6	Long rod, single and long chain with flat ends	2.0-3.2	1.0	Not swollen	Elliptical sub-terminal	+
7	Long rod, single and long chain with flat ends	2.5-3.0	1.0-1.1	Not swollen	Elliptical terminal	+
8	Short rod, single and round ends	2.0-3.0	0.5-0.8	Swollen	Elliptical sub-terminal	+
9	Short rod, single with rounded ends	1.5-2.6	0.5-0.6	Swollen	Elliptical sub-terminal	+
10	Short rod, single rounded ends	1.5-1.8	0.5-0.6	Not swollen	Oval terminal	+
11	Short rod, single with rounded ends	1.8-2.2	0.5-0.6	Swollen	Elliptical sub-terminal	+

(Continued)

Table 8. Microscopic studies and Gram reaction of the selected strains.

Strain no.	Vegetative cell	Length of the cell (μm)	Width of the cell (μm)	Sporangium	Shape and position of spore	Gram reaction
12	Short rod, single with flat ends	1.5-1.8	0.5-0.6	Not swollen	Elliptical terminal	+
13	Short rod, single with rounded ends	2.0-3.0	0.6-0.7	Not swollen	Elliptical sub-terminal	+
14	Thin long rod	2.0-3.5	0.45-0.6	Non spore former		+
15	Very short rod	1.0-1.0	1.0	Non spore former		+
16	Long rod, single and long chain with flat ends	2.5-3.1	1.0-1.1	Not swollen	Oval sub-terminal	+
17	Short rod, single with flat ends	1.6-3.1	0.6-0.7	Not swollen	Elliptical central	+
18	Very short rod	1.2-1.5	1.1-1.2	Not swollen	Oval sub-terminal	+
19	Thin short rod, single	1.8-2.5	0.5-0.6	Swollen	Oval terminal	+
20	Short rod	0.9-2.0	0.5-0.6	Swollen	Oval terminal	+
21	Rod with long chain	0.5-3.0	0.5-0.6	Spore former Oval terminal.		+
22	Short rod	1.0-1.0	0.7-0.9	Non spore former		+

(Continued)

Table 8. Microscopic studies and Gram reaction of the selected strains.

Strain no.	Vegetative cell	Length of the cell (μm)	Width of the cell (μm)	Sporangium	Shape and position of spore	Gram reaction
23	Very short rod	1.2 -1.5	0.7 -0.8	Non spore former		+
24	Short rod, single and long chain	1.5 - 2.5	0.5-0.6	Non spore former		+
25	Very short rod	1.5 -1.6	0.6-0.8	Swollen	Oval central	+
26	Short rod	2.5-3.0	0.5-0.6	Swollen	Oval sub-terminal	+
27	Short rod, single	1.2-2.0	0.5-0.6	Spore former		+
28	Long rod, single and short chain	2.0-3.0	0.4-0.5	Swollen	Oval terminal	+
29	Long rod, single and short chain	2.5-3.5	0.8-1.0	Swollen	round terminal	+
30	Short rod, single	1.8-2.5	0.8-0.9	Swollen	Oval terminal	+
31	Long rod with long chain	2.5-3.25	0.8-0.9	Not swollen	Oval terminal	+

(+) Denotes the positive results; (-) denotes the negative results.

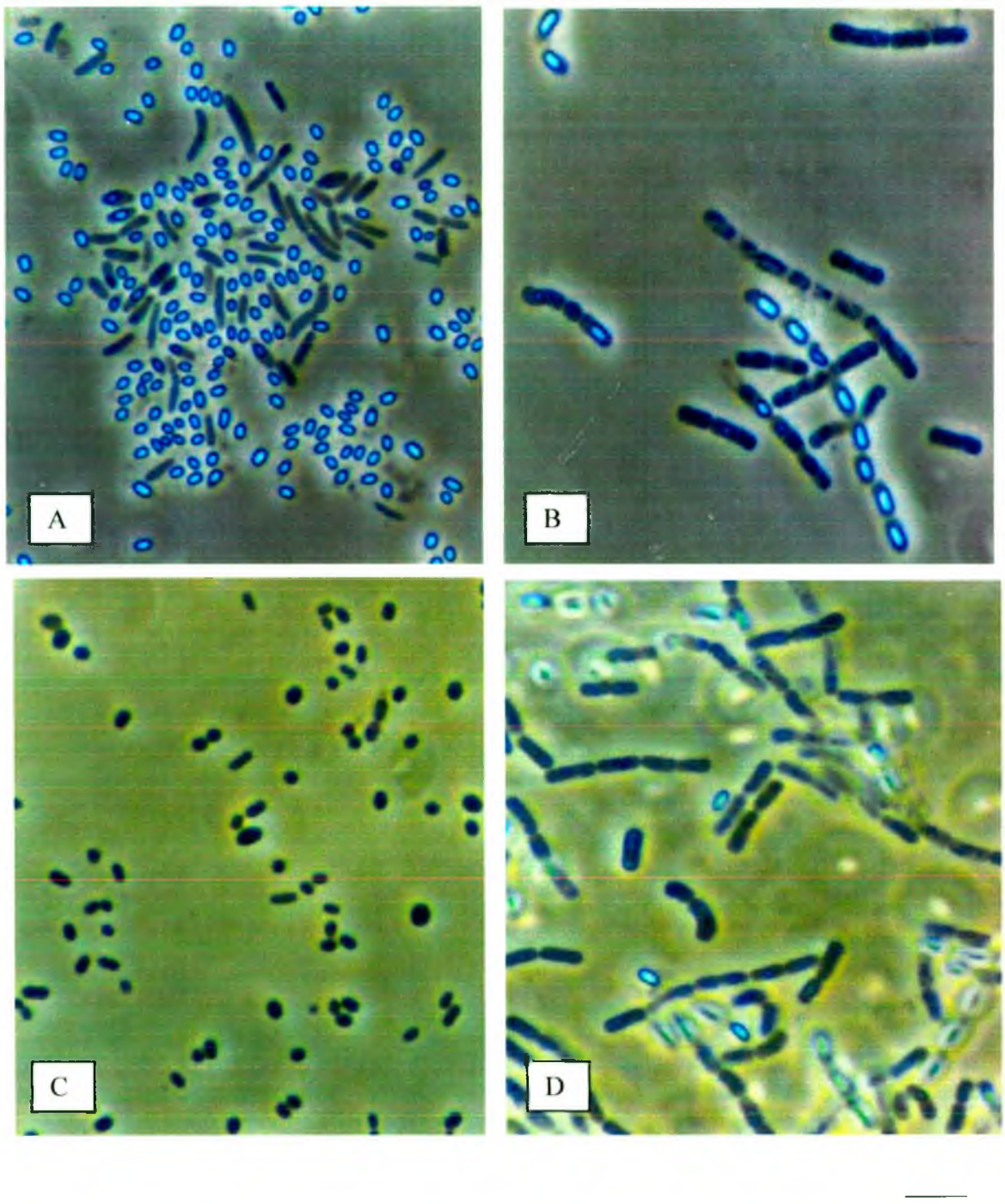


Fig. 17: (A-D) Photomicrographs showing spores and sporangium of the selected bacterial strains under phase contrast Microscope
(A) Strain no-8, (B) Strain no-7,
(C) Strain no-25, (D) Strain no-6.
Bar = 10μm

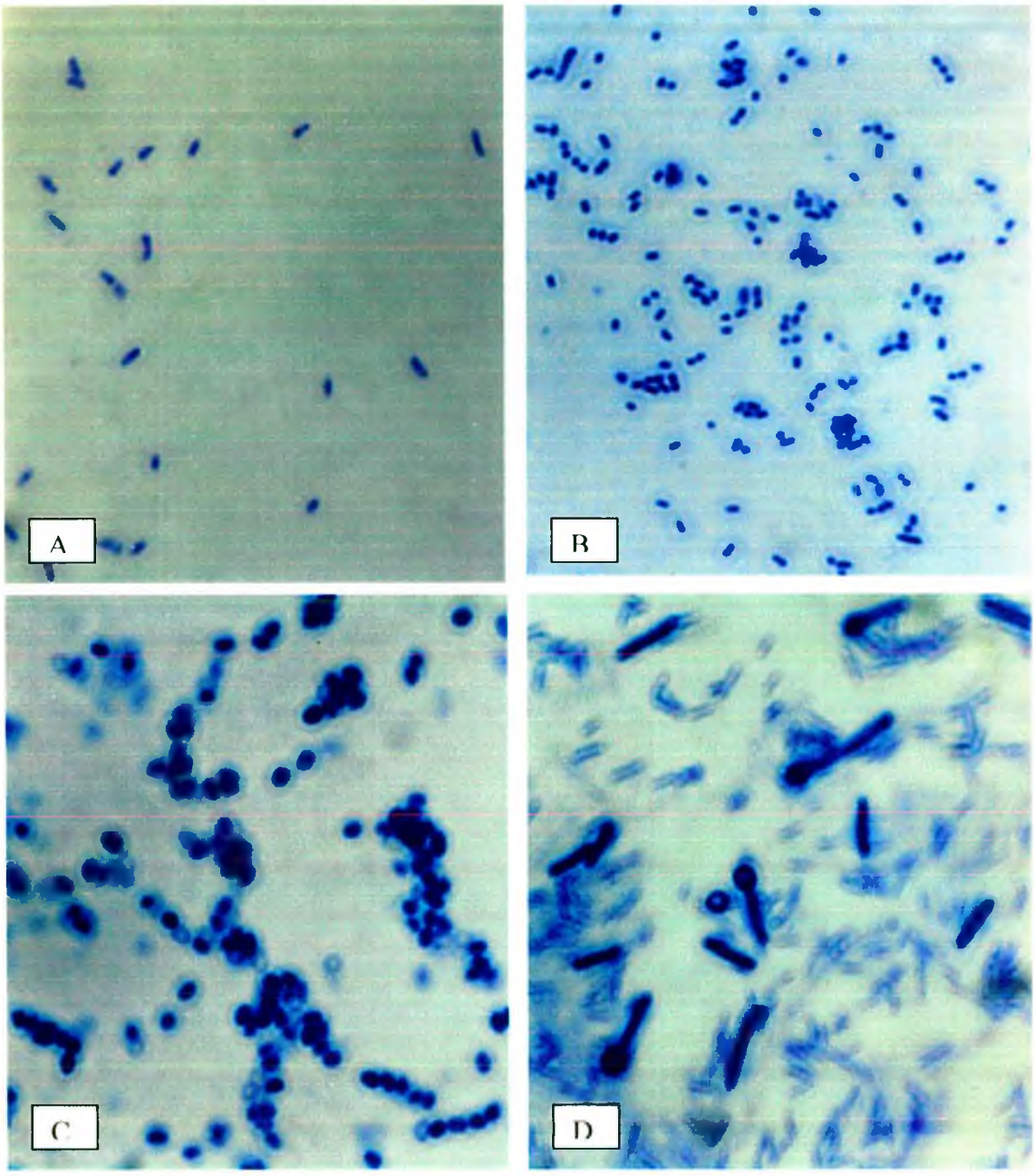


Fig. 18: (A-D) Bacterial cells stained with crystal violet (under compound Microscope, 1000X).
(A) Strain no- 13. (B) Strain no- 18.
(C) Strain no-16. (D) Strain no-29.
Bar = 10 μm

4.5.4.3 Hydrolysis of starch, gelatin and casein by the selected bacterial strains.

In this study it was observed that out of 31 bacterial strains 13 strains showed positive response in starch hydrolysis, 26 strains showed positive response in gelatin and 28 strains showed positive response in casein. It was noted that 13 strains were able to hydrolyze all the 3 ingredients while 2 strains were unable to hydrolyze any of 3 ingredients (Table.11 and Fig. 21).

4.5.4.4 Test for MR/VP, nitrate reduction and indole production.

Out of 31 bacterial strains 19 strains were positive in M.R. test, 15 strains were positive in Voges Proskaver tests and 17 strains were positive in nitrate reduction test. All the strains were negative in indole test (Table. 12 and Fig. 22).

4.5.4..5 Test for the utilization of citrate, deamination of phenaylalanine and degradation of tyrosine.

Out of 31 bacterial strains only six bacterial strains utilized citrate. All strains showed inability in the deamination of phynylalanine to phenylpyrovic acid. Nine strains degraded tyrosine (Table. 13).

4.5.4..6 Growth response at different concentration of NaCl

Growth response of the selected bacterial strains was tested at different concentrations of NaCl in nutrient broth medium. Different percentage of NaCl concentrations was 2, 5, 7 and 10. Most of the strains grew in all the concentrations of NaCl. Two strains (2 and 14) did not grow in 10% concentration of NaCl (Fig. 23 and Table. 14).

Table 9. Test for catalase production and motility

Strain no.	Catalase	Motility	Strain no.	Catalase	Motility
1	+	+	17	+	-
2	+	+	18	+	-
3	+	-	19	+	+
4	+	+	20	+	-
5	+	-	21	+	+
6	+	-	22	+	+
7	+	-	23	+	+
8	+	-	24	+	+
9	+	+	25	+	+
10	+	+	26	+	-
11	+	+	27	+	-
12	+	-	28	+	+
13	+	-	29	+	-
14	+	-	30	+	+
15	+	+	31	+	+
16	+	-	-	-	-

(+) Denotes the positive results; (-) denotes the negative results.

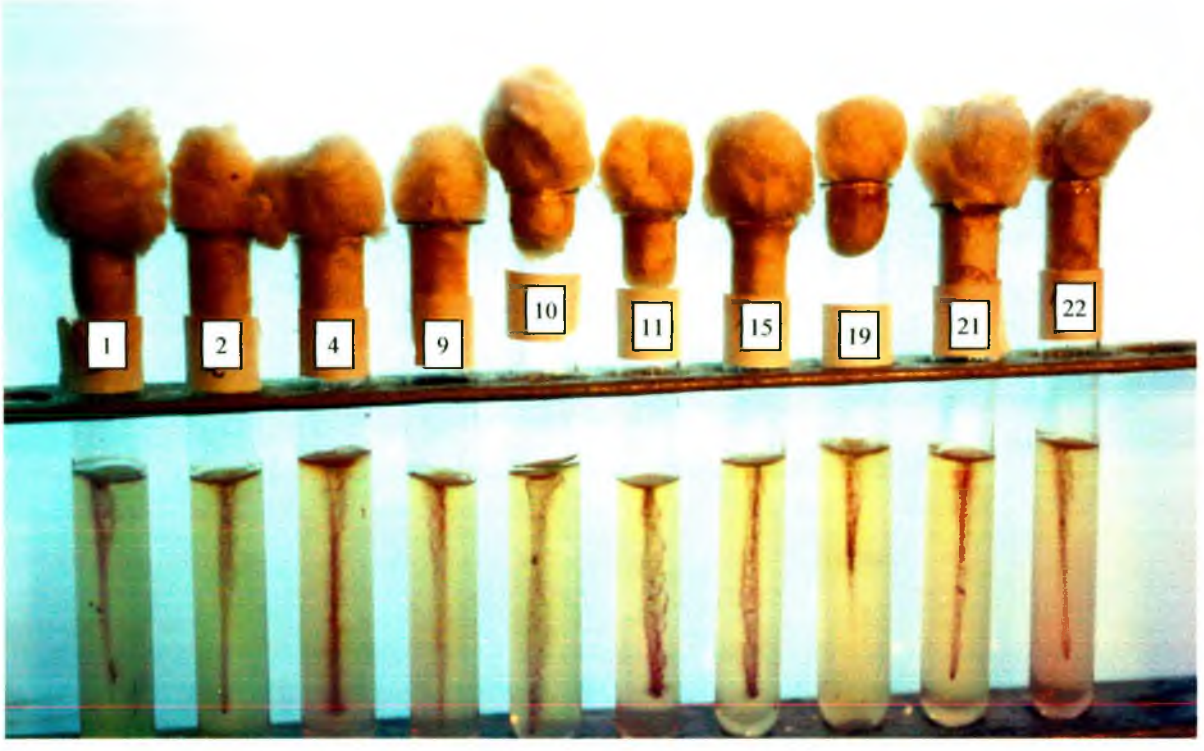


Fig. 19: Photograph showing the motility test of selected bacterial strains.

Table 10. **Fermentation tests of the selected strains.**

Strain no.	D-Glucose		L-Arabinose	D-Xylose	D-Mannitol
	Acid	Gas			
1	+	-	+	-	+
2	+	-	+	-	+
3	+	-	+	-	+
4	+	-	+	-	+
5	+	-	+	-	+
6	+	-	+	-	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	-	+	-	+
10	+	-	+	-	+
11	+	-	+	+	+
12	+	-	+	-	+
13	+	-	+	-	+
14	+	-	+	-	+
15	+	-	+	-	+
16	+	-	+	+	+

(+) Denotes the positive results; (-) denotes the negative results

(Continued)

Table 10. Fermentation tests of the selected strains.

Strain no.	D-Glucose		L-Arabinose	D-Xylose	D-Mannitol
	Acid	Gas			
17	+	-	+	-	+
18	+	-	+	+	-
19	+	-	+	-	+
20	+	-	+	+	+
21	+	-	+	-	+
22	+	-	-	-	+
23	+	-	+	-	-
24	+	-	-	-	-
25	+	-	+	+	+
26	+	-	+	-	+
27	-	-	+	-	+
28	+	-	+	-	+
29	-	-	+	-	+
30	-	-	+	-	+
31	+	-	-	-	-

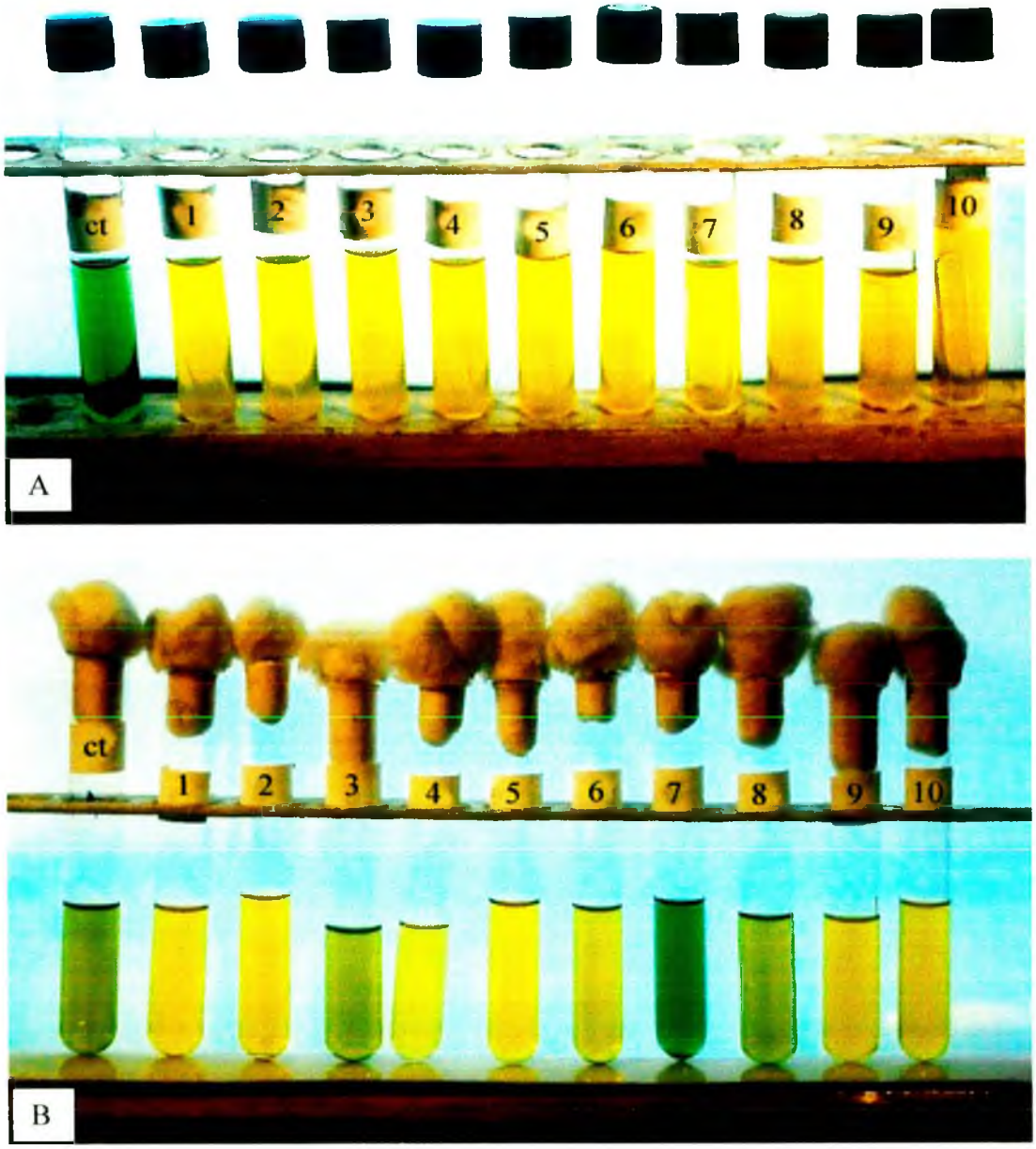


Fig. 20.a: (A-B) Fermentation test for selected bacterial strains, (A) Glucose (yellow- acid +ve), (B) Arabinose (yellow +ve)

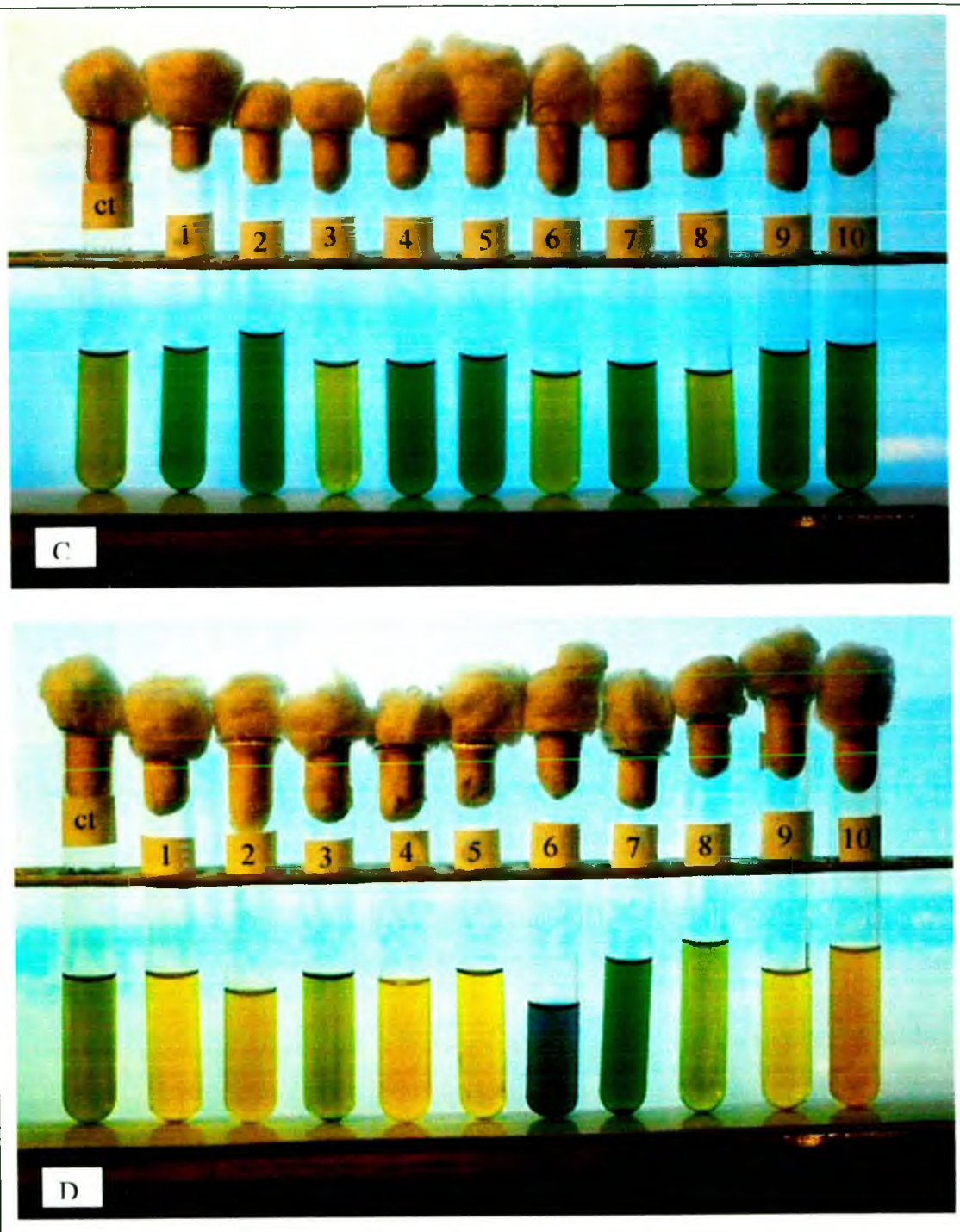


Fig. 20.b: (C-D) Fermentation test for selected bacterial strains.
(C) Xylose (yellow + ve), (D) Manitol (yellow + ve)

Table 11. Hydrolysis test of the selected strains.

Strain no.	Hydrolysis of		
	Starch	**Gelatin**	**Casein**
1	Not hydrolyzed	Hydrolyzed	Hydrolyzed
2	Not hydrolyzed	Hydrolyzed	Hydrolyzed
3	Hydrolyzed	Hydrolyzed	Hydrolyzed
4	Not hydrolyzed	Hydrolyzed	Hydrolyzed
5	Not hydrolyzed	Hydrolyzed	Hydrolyzed
6	Not hydrolyzed	Hydrolyzed	Hydrolyzed
7	Hydrolyzed	Hydrolyzed	Hydrolyzed
8	Hydrolyzed	Hydrolyzed	Hydrolyzed
9	Not hydrolyzed	Hydrolyzed	Hydrolyzed
10	Not hydrolyzed	Hydrolyzed	Hydrolyzed
11	Not hydrolyzed	Hydrolyzed	Hydrolyzed
12	Not hydrolyzed	Hydrolyzed	Hydrolyzed
13	Not hydrolyzed	Hydrolyzed	Hydrolyzed
14	Hydrolyzed	Hydrolyzed	Hydrolyzed
15	Not hydrolyzed	Hydrolyzed	Hydrolyzed
16	Hydrolyzed	Hydrolyzed	Hydrolyzed

(Continued)

Table 11. Hydrolysis tests of the selected strains.

Strain no.	Hydrolysis of		
	Starch	Gelatin	Casein
17	Hydrolyzed	Hydrolyzed	Hydrolyzed
18	Hydrolyzed	Hydrolyzed	Hydrolyzed
19	Not hydrolyzed	Not hydrolyzed	Not hydrolyzed
20	Hydrolyzed	Hydrolyzed	Hydrolyzed
21	Hydrolyzed	Hydrolyzed	Hydrolyzed
22	Not hydrolyzed	Not hydrolyzed	hydrolyzed
23	Hydrolyzed	Hydrolyzed	Hydrolyzed
24	Hydrolyzed	Not Hydrolyzed	Hydrolyzed
25	Hydrolyzed	Hydrolyzed	Hydrolyzed
26	Not hydrolyzed	Hydrolyzed	Hydrolyzed
27	Not hydrolyzed	Not hydrolyzed	Hydrolyzed
28	Not hydrolyzed	Not hydrolyzed	Not hydrolyzed
29	Not hydrolyzed	Hydrolyzed	Hydrolyzed
30	Not hydrolyzed	Hydrolyzed	Not hydrolyzed
31	Hydrolyzed	Hydrolyzed	Hydrolyzed

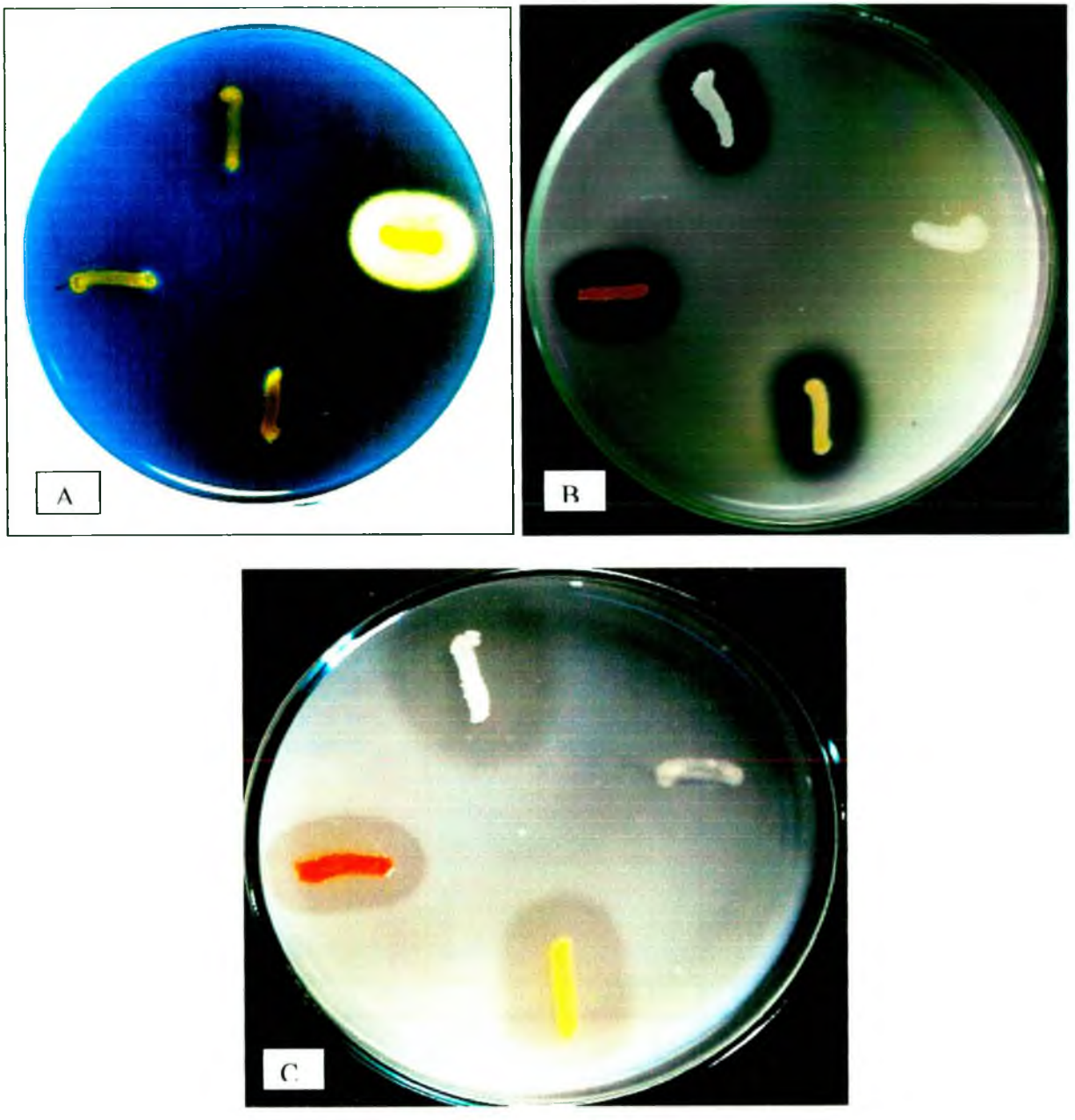


Fig. 21: (A-C) Photographs showing the Hydrolytic-activity of selected bacterial strains. (A) Starch, (B) Gelatin, (C) Casein.

Table 12. Tests for MR, VP, NO_3 reduction and Indole production by the selected strains.

Strain no.	MR	VP	Nitrate reduction	Indole
1	+	+	+	-
2	+	+	+	-
3	-	-	-	-
4	+	+	-	-
5	+	+	-	-
6	+	-	+	-
7	+	+	-	-
8	-	-	+	-
9	+	+	-	-
10	+	+	-	-
11	+	+	+	-
12	+	-	-	-
13	+	+	-	-
14	+	-	-	-
15	-	-	+	-
16	-	-	-	-

(+) Denotes the positive results; (-) denotes the negative results

(Continued)

Table 12. Tests for MR, VP, NO_3 reduction and Indole production by the selected strain.

Strain no.	MR	VP	Nitrate reduction	Indole
17	-	+	-	-
18	-	-	-	-
19	-	-	+	-
20	-	-	+	-
21	-	-	+	-
22	+	-	+	-
23	+	-	+	-
24	+	-	+	-
25	+	-	+	-
26	+	+	-	-
27	-	-	-	-
28	+	+	+	-
29	-	+	+	-
30	+	+	+	-
31	-	-	+	-

(+) Denotes the positive results;(-) denotes the negative results.

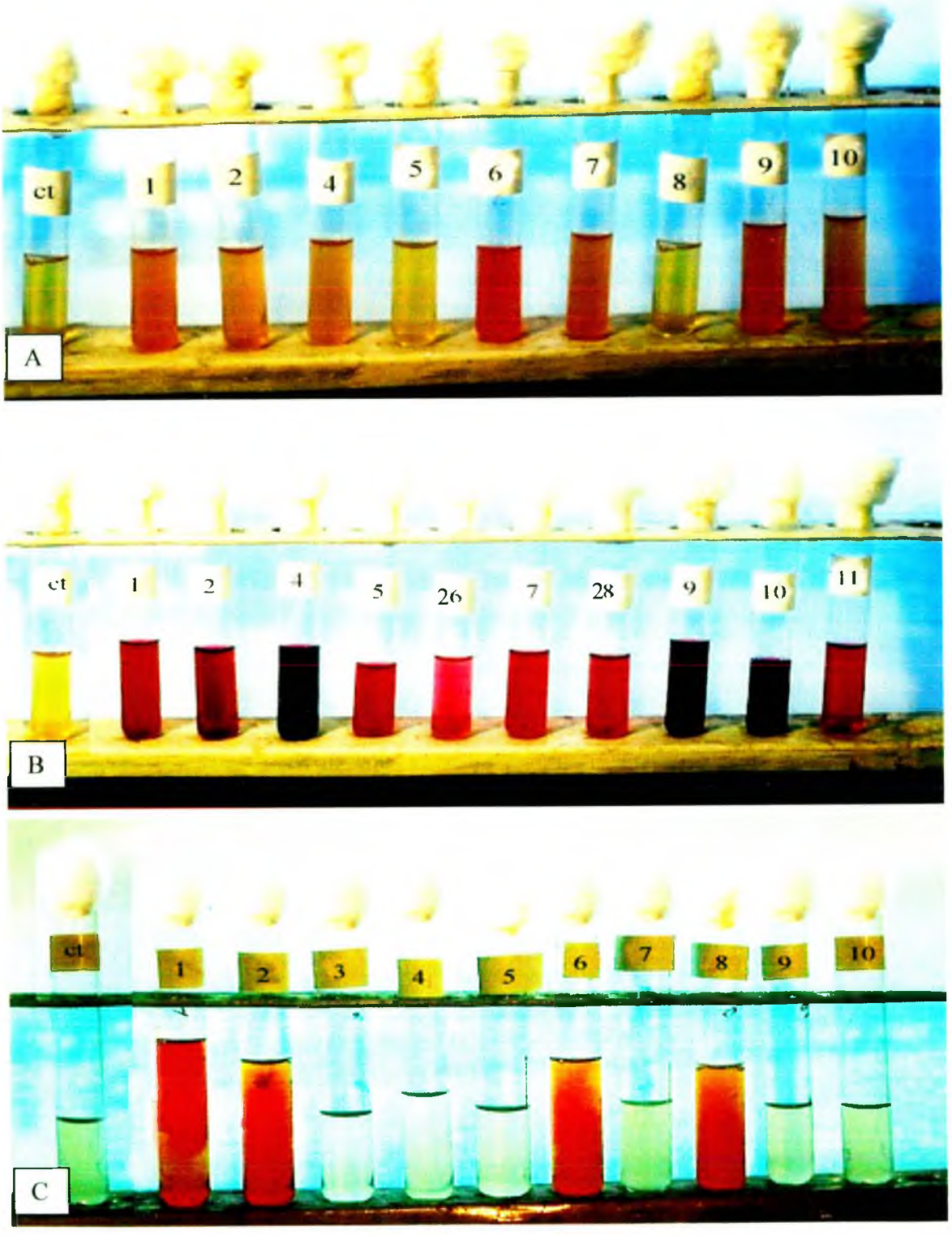


Fig. 22: (A-C) Photographs showing the test for (A) MR (Red + positive, Yellow – negative), (B) VP (Red + positive, Yellow – negative), (C) Nitrate reduction (Red + positive of nitrate, Yellow- absence of nitrate).

Table 13. Test for utilization of citrate and deamination of phenylalanine and degradation of tyrosine.

Strain no.	Utilization of Citrate	Deamination of phenylalanine	Degradation of tyrosine
1	-	-	-
2	-	-	-
3	-	-	-
4	+	-	-
5	-	-	-
6	-	-	+
7	-	-	-
8	-	-	-
9	-	-	-
10	+	-	-
11	-	-	-
12	-	-	-
13	+	-	-
14	-	-	-
15	-	-	+
16	-	-	-

(Continued)

Table 13. Test for utilization of citrate and deamination of phenylalanine and degradation of tyrosine.

Strain no.	Utilization of Citrate	Deamination of phenylalanine	Degradation of tyrosine
17	-	-	-
18	-	-	-
19	-	-	+
20	+	-	-
21	-	-	+
22	-	-	+
23	-	-	-
24	-	-	+
25	-	-	-
26	-	-	-
27	-	-	+
28	-	-	+
29	-	-	-
30	+	-	-
31	+	-	+

(+) Denotes the positive results; (-) denotes the negative results.

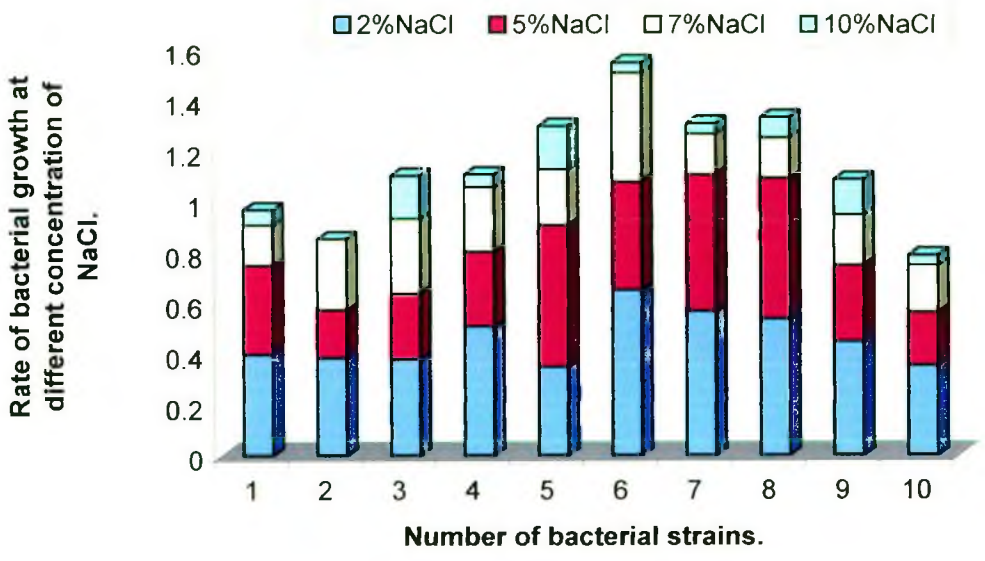


Fig. 23 Growth response of different bacterial strains in different concentration of NaCl.

4.5.4.7 Growth response of different bacterial strains at two selected pH.

All the selected strains grew well at pH 6.8. Except 3, 14, 17, 18, 21, 29 and 30, all other strains grew well at pH 5.7(Table. 14).

4.5.4.7.1 Effect of temperature on growth of the selected bacterial strains.

All the strains grew well at temperature 20°C, 30°C and 40°C.Only 20 no. strain was also able to grow at 50°C and strain no. 21 at 10°C (Table. 15).

4.6 Identification of the isolated bacteria.

It is difficult to identify bacterial strains in Bangladesh because all the required facilities are not available in the country. The isolated bacterial strains were therefore provisionally identified according to the Bergey's Manual (Sneath *et al.* 1986, Buchanan and Gibbons 1974). Out of the 31 bacterial strains, one belonged to the genus *Brochothrix*, five belonged to the genus *Listeria* and 25 bacterial strains were identified as 15 different species of *Bacillus* as follows, *B. alcalophilus, B. pumilus, B. firmus, B. badius, B. pantothenticus, B. alvei, B. polymyxa, B. lentus, B. licheniformis, B. megaterium, B. macerans, B. subtilis B. circulans, B. sphaericus* and *B. brevis*. Appen. 27 and 28 shows the standard chart for the identification of *Bacillus, Listeria and Brochothx*. spp. which have been used for comparing the selected strains.

Provisionally identified names of the selected strains are presented in the table. 16.

The strain nos. 1 and 2 were closely related to the standard strain *B. subtilis* differing only in three biochemical tests. Both the strains showed inability to produce acid from D-xylose. They also differed in their ability for the utilization of citrate. In case of hydrolysis tests the strains did not hydrolyse starch strain no. 3 resembled to *B. alcalophilus* but it differed in one biochemical test. The strain showed inability to produce acid from D-xylose.

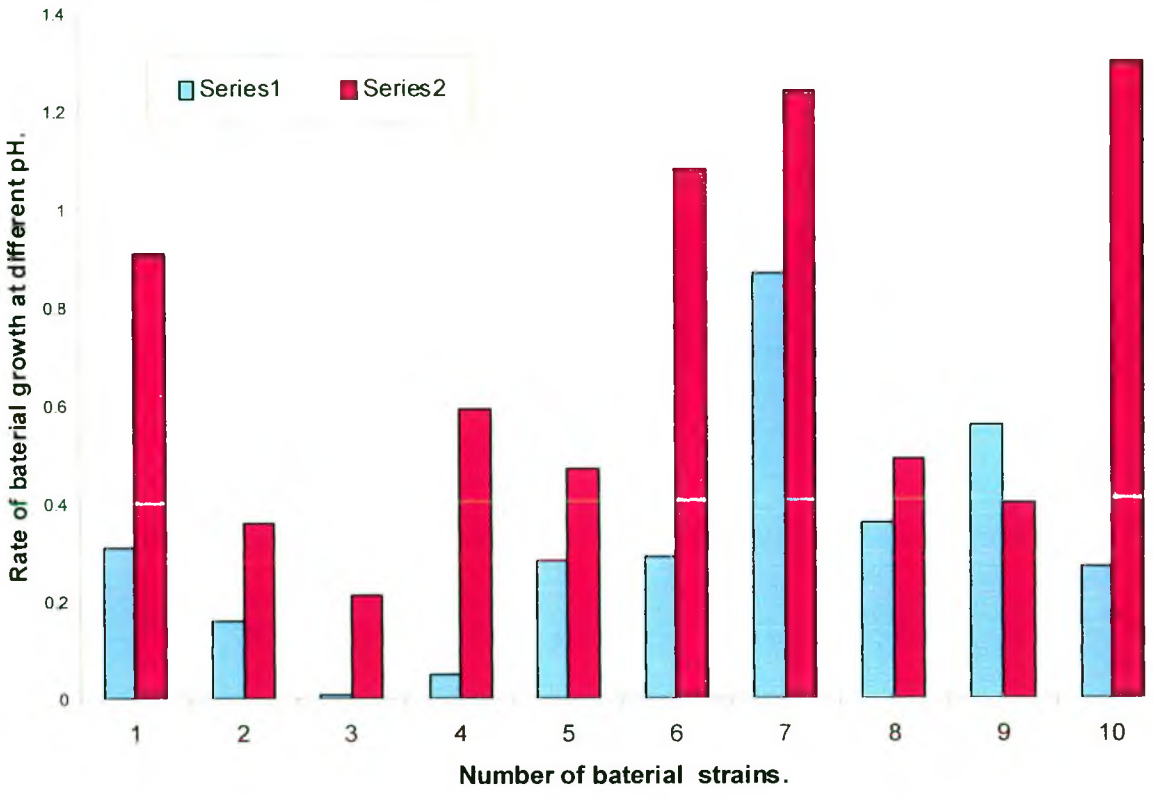


Fig. 24: Rate of bacterial growth at pH 5.7 and 6.8.

Table 14. Growth of bacterial strain in different pH and different percentage of NaCl.

Different bacterial strains	Growth of isolated bacterial strains in different pH		Growth of isolated bacterial strains in different % of NaCl			
	pH5.7	pH6.8	2 % NaCl	5% NaCl	7% NaCl	10% NaCl
1	0.36	1.06	0.4	0.35	0.16	0.06
2	0.16	0.36	0.385	0.19	0.28	0
3	0.01	0.21	0.38	0.26	0.295	0.17
4	0.05	0.59	0.51	0.295	0.255	0.05
5	0.28	0.47	0.35	0.56	0.22	0.17
6	0.29	1.08	0.655	0.425	0.43	0.04
7	0.87	1.24	0.57	0.54	0.16	0.04
8	0.37	0.49	0.54	0.555	0.16	0.08
9	0.56	0.7	0.45	0.3	0.2	0.14
10	0.27	1.3	0.355	0.21	0.185	0.04
11	0.42	0.85	0.65	0.405	0.15	0.2
12	0.15	0.37	0.44	0.345	0.28	0.2
13	0.25	0.58	0.4	0.41	0.25	0.14
14	0	0.29	0.57	0.47	0.05	0.007
15	0.24	0.5	0.45	0.62	0.35	0.12
16	0.23	0.93	0.38	0.365	0.2	0.03
17	0	0.17	0.645	0.57	0.21	0.01
18	0	0.12	0.43	0.415	0.24	0.17
19	0.14	0.43	1.39	0.93	1.3	0.52
20	0.12	0.34	0.15	0.25	0.32	0.07
21	0.12	0.17	0.66	0.255	0.07	0.02
22	0.04	0.3	1.32	0.81	0.69	0.35
23	0.05	0.33	0.545	0.76	0.25	0.26
24	0.01	0.19	0.38	0.21	0.14	0.03
25	0.41	1.12	0.87	0.79	0.53	0.11
26	0.4	0.86	0.41	0.64	0.32	0.18
27	0.03	0.07	0.55	0.145	0.04	0.04
28	0.06	0.12	0.465	0.065	0.03	0.04
29	0.01	0.21	0.701	0.3	0.04	0.06
30	0	0.36	0.41	0.48	0.37	0.06
31	0.17	0.53	0.854	0.655	0.51	0.07

Table 15. Growth response of the bacterial strains at different temperatures (°C)

Strain no	Growth of bacterial strains at different temperature (°C)				
	10°C	20°C	30°C	40°C	50°C
1	-	+++	++++	++++	-
2	-	++	++++	++++	-
3	-	+++	++++	++++	-
4	-	++	++++	++++	-
5	-	+++	++++	++++	-
6	-	+++	++++	++++	-
7	-	+++	++++	++++	-
8	-	++	++++	++++	-
9	-	++	++++	++++	-
1 0	-	+	++++	++++	-
11	-	+	++ ++	++++	-
12	-	+++	++++	++++	-
13	-	++	++++	++++	-
14	-	++	+++	++++	-
15	-	+++	++++	++++	-
16	-	++++	++++	++++	-

Table 15. Growth response of the bacterial strains at different temperatures (°C)

Strain no	Growth of bacterial strains at different temperature (°C)				
	10°C	20°C	30°C	40°C	50°C
17	-	+++	++++	++++	-
18	-	+++	++++	++++	-
19	-	+++	++++	++++	-
20	-	+	++++	++++	+++
21	+	+++	++++	++++	-
22	-	++	++++	++++	-
23	-	+++	++++	++++	-
24	+	++++	++++	++++	-
25	-	++++	++++	++++	-
26	-	++	++++	++++	-
27	+-	+++	++++	++	-
28	-	++	++++	++	-
29	-	+++	++++	++++	-
30	-	+	+	+	-
31	+-	+++	++++	++++	-

(+) Denotes the positive results; (-) denotes the negative results.

Strain nos. 4, 15, 22, 23 and 24 were closely related to *Listeria*. Strains, 4, 15 and 23 varied from the standard strains in their inability to hydrolyse casein and gelatin; strain nos. 22 and 24 were unable to hydrolyse casein.

The strain nos. 5, 10, 17 and 26 were provisionally identified as *B. pumilus*. But in certain cases they differed in their biochemical characters. Strain no. 5 was unable to produce acid from D-xylose and differed in citrate utilization test. Strain no. 10 differed from the type species only in it's inability to produce acid from D-xylose. Strain no. 17 was unable to hydrolyse starch and did not utilization of citrate. Strain no. 26 differed only in citrate utilization test. In this study citrate was not utilized by strain no. 26.

Strain no. 6 was closely related to the *B. firmus*, however, it varied from the type species in 5 biochemical tests. It was unable to produce acid from D-xylose and D- manitol to produced and acid from L- arabinose . It also showed inability to hydrolyse starch. The strain was also able to degrade tyrosine.

Strain nos. 7 and 27 resembled, the species *B. badius* , specially having vegetative cells with blunt ends . Strain no. 7 was able to hydrolyse starch, unlike the type species, incase of tyrosine test type species was able to degrade tyrosine while strain no. 7 failed to do so. Strain no. 27 was able to produce acid from L- arabinose and D- manitol unlike the type species.

Strain no. 8 resembled *B. pantothenticus*. It was not found to vary from the standard species in this study.

Stain no 9 was closely related to the standard stain of *B. alvei* but differed in 3 biochemical tests. Unlike standard strain, strain no. 9 was able to produce acid from L-arabinose and D-manitol, the strain 9 showed inability to hydrolyse starch, while the standard strain was able to hydrolyse it.

Strain nos. 11 and 28 were provisionally identified as *B. polymyxa*. Strain 11 differed from the standard species in 2 biochemical tests. It was unable to produce gas from D-glucose and could not hydrolyse starch. The strain no. 28 also differed in 2 biochemical tests from the standard strain. It

could not produce gas from D-glucose and was also unable to produce acid from D-xylose.

Strain no. 12 was found to be closely related to the standard strain of *B. lentus*. But it showed 3 differences. It could not produce acid from D-xylose, produced acid from D-mannitol and also showed negative reaction in starch hydrolysis test.

Strain no. 13 was provisionally identified as *B. licheniformis. This* strain differed in two biochemical tests from the standard strain. It was unable to produce acid from D-xylose and also showed negative reaction in starch hydrolysis test.

Strain no. 14 resembled the genus *Brochothrix* . However, it was able to grow at 37°C, while the type species grew at lower temperature (20°C and 22°C).

Strain no.16 was provisionally identified as *B megaterium*. It differed only in citrate test where the strain yielded negative result.

Strain no. 19 and 25 were provisionally identified as *B. macerans*. Strain no.19 was able to produce acid from L-arabinose and was unable to hydrolyse starch, strain no. 19 was also able to degrade tyrosine. Strain no. 25 was able to hydrolyse casein but standard strain was not. Strain no. 25 was unable to produce gas from glucose while the standard strain was able to produce gas from glucose.

Strain no. 20 was closely related to *B. circulans*. In this study, it did not show any variation from the standard species.

Strain no. 29 was provisionally identified as *B. sphaericus*. It differed in 3 biochemical tests from the standard strain, i.e., L-arabinose, D-manitol and V.P test. The strain no. 29 was able to produce acid from L-arabinose and D-manitol. In case of VP test, strain no. 29-yeided positive result but negative result was yielded by type species.

Strain no. 31 was found to be closely related to *B. brevis*. It did not show any variation in this study.

Venkataraman (1983) isolated bacteria from *Spirulina* culture as *Bacillus magaterium, B. subtilis B. licheniformis* , *B. firmus*, *B. polymyxa, B. alvei, B. circulans*, *B. firmus, B. macerans*. No pathogenic forms like *Salmonella* or *Staphylococcus aureus* were found either in the culture or the sun-dried *Spirulina* powder. The above report agrees with the present study, in that, no Salmonella or *Staphylococcus aureus* were observed.

Mahadevaswamy and Venkataraman (1987) mentioned that pathogenic forms were totally absent both in culture and dried powder. Where *E. coli, Staphylococcus aureus, Clostridium perfringens, Salmonella* did not survive if introduced in to the *Spirulina* culture, growing in CFTRI medium. The present study also revealed similar results and no pathogenic forms were found as mentioned before.

Table-16. Selected bacterial strains with their names (provisionally identified).

Strain no	Identified Name
1	*Bacillus subtilis* (Ehrenberg 1835) Cohn 1872
2	*Bacillus subtilis* (Ehrenberg 1835) Cohn 1872
3	*Bacillus alcalophilus* Vedder 1934
4	*Listeria spp* Pirie 1940
5	*Bacillus pumilus* Meyer and Gottheil, 1901.
6	*Bacillus firmus* Bredemann and Werner 1933
7	*Bacillus badius* Batchelor 1919
8	*Bacillus pantothenticus* Proom and Knight 1950
9	*Bacillus alvei* Cheshire and Cheyne 1885
10	*Bacillus pumilus* Meyer and Gotteil, 1901
11	*Bacillus polymyxa* (Prazmowski 1880) Mace 1889
12	*Bacillus lentus* Gibson 1935
13	*Bacillus licheniformis* (Weigmann 1898) Chester 1901
14	*Brochothris spp*
15	*Listeria spp* Pirie,1940
16	*Bacillus megaterium* de Bary 1884
17	*Bacillus pumilus* Meyer and Gottheil, 1901
18	*Bacillus lentus* Gibson 1935
19	*Bacillus macerans* Schardinger 1905
20	*Bacillus circulans* Jordan 1890
21	*Bacillus lentus* Gibson 1935
22	*Listeria spp* Pirie,1940
23	*Listeria spp* Pirie,1940
24	*Listeria spp* Pirie,1940
25	*Bacillus macerans* Schardinger 1905
26	*Bacillus pumilus* Meyer and Gottheil, 1901
27	*Bacillus pumilus* Meyer and Gottheil, 1901
28	*Bacillus polymyxa* (Prazmowski 1880) Mace 1889
29	*Bacillus sphaericus* Meyer and Neide 1904
30	*Bacillus polymyxa* (Prazmowski 1880) Mace 1889
31	*Bacillus brevis* Migula 1900

4.7. Effect of sun-dried *Spirulina platensis* extracts on the isolated bacteria.

Appen. 29. representing the effects of alga extracts with different solvents (water, methanol and ethanol), on the growth of 31 bacterial strains. No inhibition zone was observed around the paper discs, which was loaded with the algal extracts. No inhibition zone was observed in this study during experiments with (i) water extracts (ii) methanol extracts and (iii) ethanol extracts of *Spirulina platensis.*

According to Jordan (1978) extracts of *Spirulina* inhibited the growth of bacteria, yeast and fungi. According to Henrikson (1989) extracts of *Spirulina* inhibited the growth of yeast and fungi. Bacterial inhibition was not mentioned in his report.

4.8 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by different treatments.

4.8.1 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by different antibiotic treatments (Doxycyclin and Amoxicillin).

Extensive studies were conducted using different ppm of Doxycyclin and Amoxicillin in *Spirulina platensis* culture. The conditions under which lowest bacterial count could be obtained and at the same time condition of *Spirulina platensis* was also good, were designated as optimum treatment conditions (Table. 17).

From the table.17, it was observed that when *Spirulina platensis* culture was treated with 6gm/l Doxycyclin for 1hr (optimum treatment condition), it showed bacterial count 0.33 $x10^4$CFU/ml. While the control culture showed $5x10^5$/CFU/ml (Fig.25). Reduction of bacteria was 99.3%. And when *Spirulina platensis* culture was treated with 60gm/l. Amoxicillin for 24hrs (optimum treatment condition), it showed bacterial count $0.5x10^5$CFU/ml. While the control showed $2x10^5$/CFU/ml (Fig. 25). Condition of *Spirulina platensis* was found good in both the cases under microscope.

In this study Doxycyclin was found to be superior to Amoxicillin for controlling the reduction of bacteria in *Spirulina platensis* culture.

Table. 17 **Control of bacteria in *Spirulina platensis* culture using antibiotics (Doxycyclin and Amoxicillin)**

Treatments	Optimum gm/l	Optimum treatment periods	CFU/ml	Reduction of baeteria after treatment (%)	Condition of *Spirulina platensis* under microscope
Doxycyclin	6	One hour	$0.33x10^4$	99.3	As good as control
Amoxicillin	60	Twenty four hours	$0.5 x10^5$	89	As good as control
Control	0	0	$2 x10^5$	0	Good

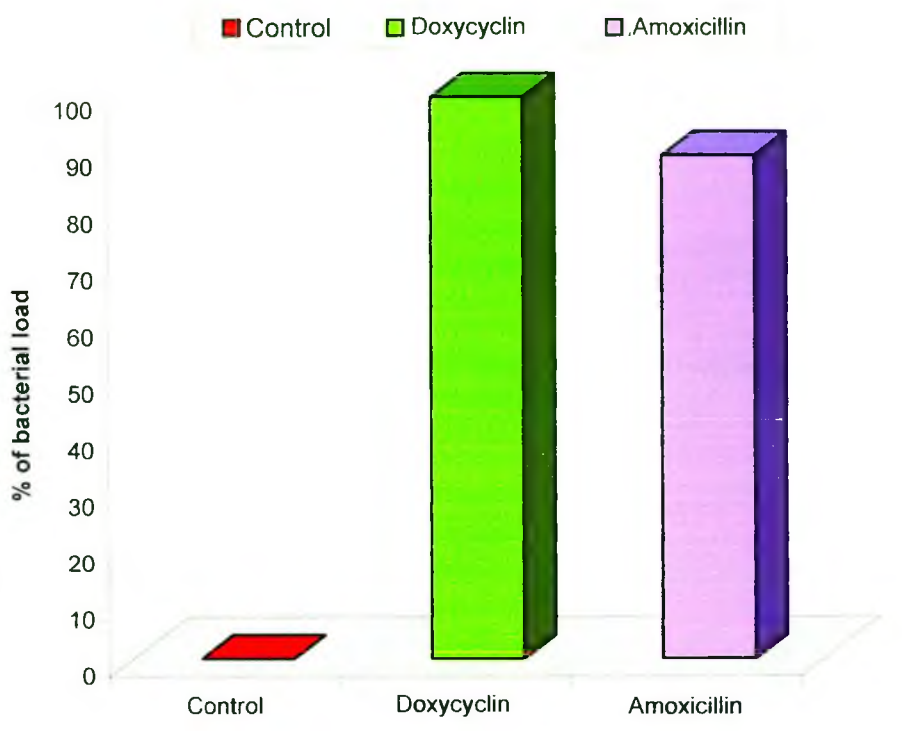


Fig. 25: Reduction of bacterial load in *Spirulina platensis* culture using antibiotics.

4.8.2 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by chemical treatments (Formaldehyde, Acetic acid and Alcohol).

Cultures treated with 50ppm of 37% formaldehyde for 10 minutes showed bacterial count $3x10^4$CFU/ml. That treated with 200ppm of 95% alcohol had bacterial count $5.3x10^4$ CFU/ml after 5 hours treatment while cultures treated with 50ppm of concentrated acetic acid showed bacterial count $3.3x10^4$ CFU/ml after 5 hours treatment. Results of the study are summarized in table. 18 and fig. 26.

Condition of *Spirulina platensis* was found good even after 30 days, when it was treated with formaldehyde. But the condition of *Spirulina platensis* deteriorated after 15 days, when the culture was individually treated with concentrated acetic acid and alcohol.

Reduction of bacteria was highest in formaldehyde (73%) followed by acetic acid (70%) and then alcohol (51.8%).

4.8.3 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture by chemical treatments (Sodium chloride and Sugar).

Cultures were treated with 10gm/l. of sterilized sugar for 3 hours showed bacterial count $30x10^4$CFU/ml that treated with 150gm/l of sterilized NaCl had bacterial count $1x10^4$ CFU/ml, after 1 hour treatment. Results of the study are summarized in table. 19 and presented in fig. 27.

Reduction of bacteria was better in NaCl than sugar treated *Spirulina* culture.

Table 18. **Control of bacteria in *Spirulina platensis* culture using Formaldehyde/Acetic acid/Alcohol under optimum conditions of treatment.**

Treatments	Optimum (ppm/l)	Optimum treatment periods	CFU/ml	Reduction of bacteria after treatment (%)	Condition of *Spirulina platensis*
37% Formaldehyde	50	10 minutes	$3x10^4$	73	As good as control, even after 30 days
Con. Acetic acid	50	5 hours	$3.3x10^4$	70	Condition deteriorated after 15 days
95% Alcohol	200	5 hours	$5.3x10^4$	51.8	Condition deteriorated after 15 days
Control	0	0	$11x10^4$	0	Good

Table. 19. **Control of bacteria in *Spirulina platensis* culture using Sodium Chloride /Sugar treatment.**

Treatments	Optimum gm/l	Optimum treatment periods	CFU/ml	Reduction of bacteria after treatment (%)	Condition of *Spirulina platensis*
Sodium chloride	150	One hour	$1x10^4$	99	Not good as control
Sugar	10	Three hours	$30x10^4$	95	As good as control (30 days)
Control	0	0	$7x10^4$	0	Good

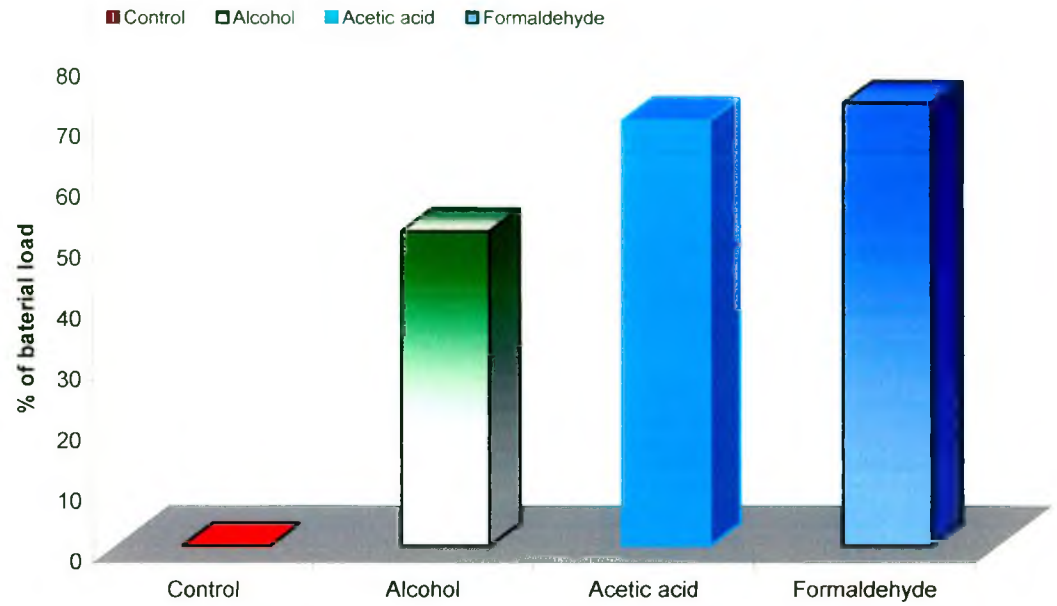


Fig. 26: Reduction of bacterial load in *Spirulina platensis* culture with different chemical treatments.

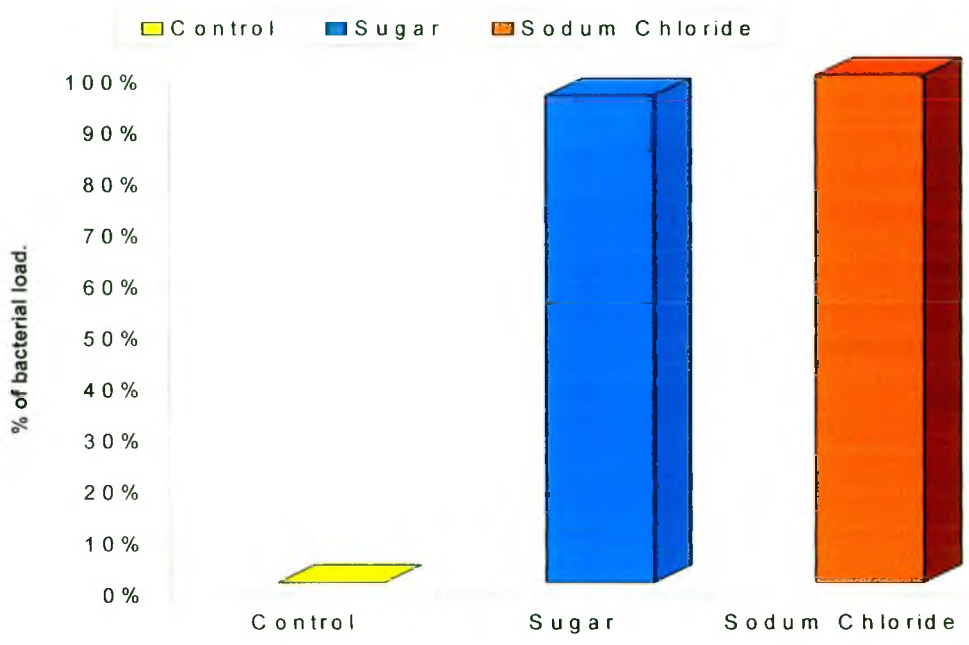


Fig. 27: Reduction of bacterial load in *Spirulina platensis* culture with sodium chloride and sugar treatment.

4.8.4 Reduction of bacterial load in laboratory scale open *Spirulina platensis* culture through washing

From the fig. 28 it was found that initial CFU/ml of the *Spirulina platensis* culture was $5x10.^{7}$ After five times washing with non- sterilized and sterilized tap water of BCSIR laboratories Dhaka, CFU/ml was reduced $2.2x10^{3}$ and $2x10^{3}$ respectively. Reduction of bacterial load was 99.9% when the water was non-sterilized and it was 99.95% when sterilized tap water was used.

Spirulina platensis has been cultured using tap water of BCSIR laboratories Dhaka, since 1989 with out any undesirable problems. However, nature of tap water may vary from place to place, creating problem in *Spirulina platensis* culture. It may be mentioned here that nature of tap water exerted adverse effect on *Spirulina platensis* culture elsewhere (personal communication, Majid). This indicates the good quality of BCSIR tap water.

Cifferri (1983) cited that if the trichom's are washed repeatedly with sterile physiological solution, the bacterial contribution to the total biomass becomes negligible.

According to Bonnin (1992) algal slurry has to be washed to eliminate undesirable chemicals, this operation eliminates also a large proportion of micro-organisms including bacteria, which have not been eliminated by filtration in the culture medium.

Venkataraman (1983) cited that the filtered slurry of *Spirulina* contains considerable amounts of unutilized nutrients and hence it needs to be washed to get a good quality product.

In the present study it was found that the bacterial load could be reduced by 99% by washing with water. The present study agrees with the findings of Venkataraman (1983) and Bonnin (1992).

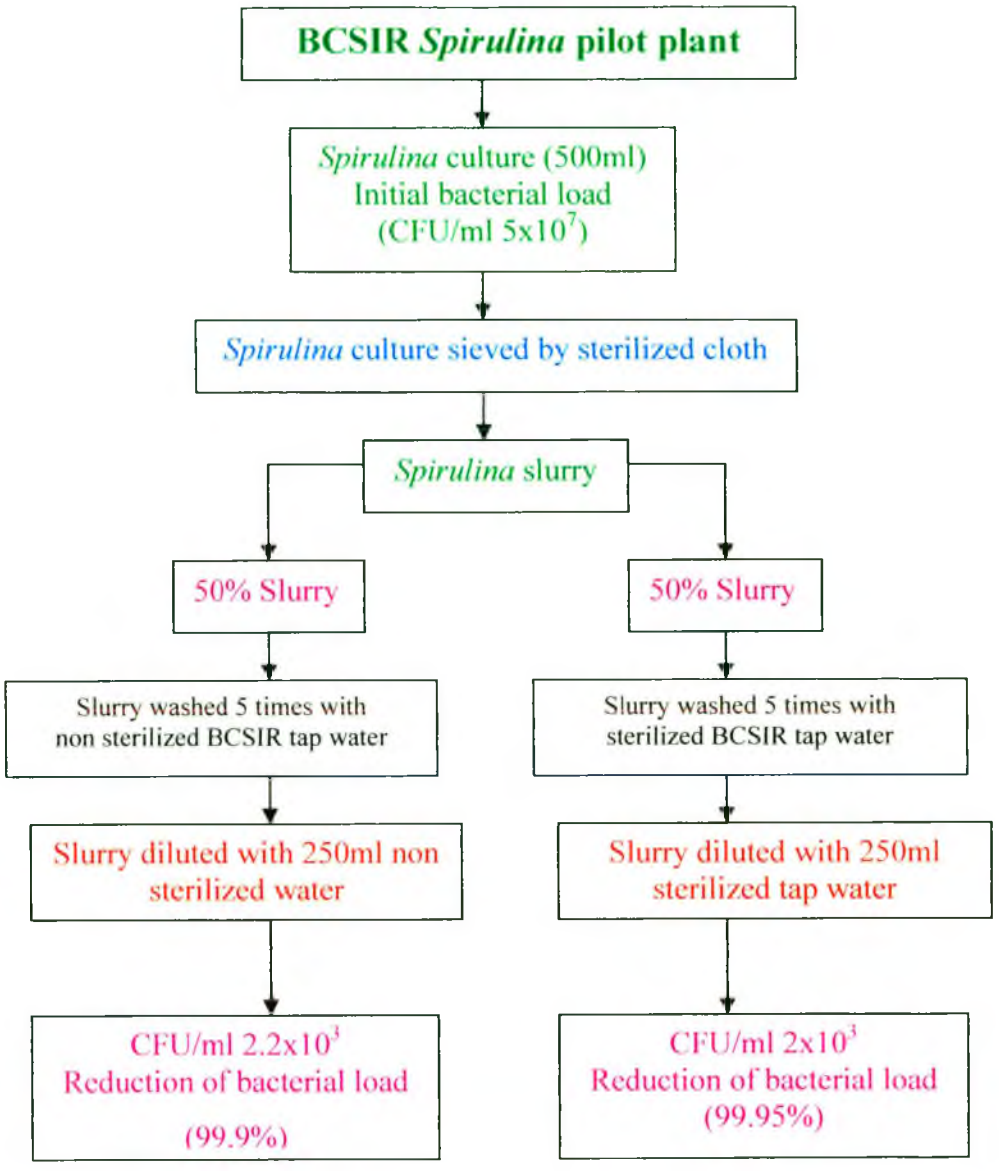


Fig. 28: Reduction of bacterial load in *Spirulina platensis* culture through washing.

4.9 Preservation of *Spirulina platensis* at room temperature.

4.9.1 Effect of storage period on protein content of sun-dried *Spirulina platensis* biomass.

The results of this study are presented in the appen. 30 and fig. 29. In appen. 30, initial protein content of sun-dried *Spirulina platensis* biomass was 65%, while it was 60% at the end of one-year storage period (61.68±1.6). It was observed that the protein content of sun-dried *Spirulina platensis* biomass was more or less same through out the one-year storage period. It was also observed that the protein content of stored *Spirulina platensis* biomass was not affected by humidity and temperature of the storage period (Fig. 29).

According to Venkataraman (1993) dried *Spirulina* flakes with moisture content of 8-10% can be packed and stored in polyethylene bags. This does not lower the nutrient quality of the product and also does not develop problem of rancidity for at least twelve months.

The present study agrees with the above findings of Venkataraman (1993).

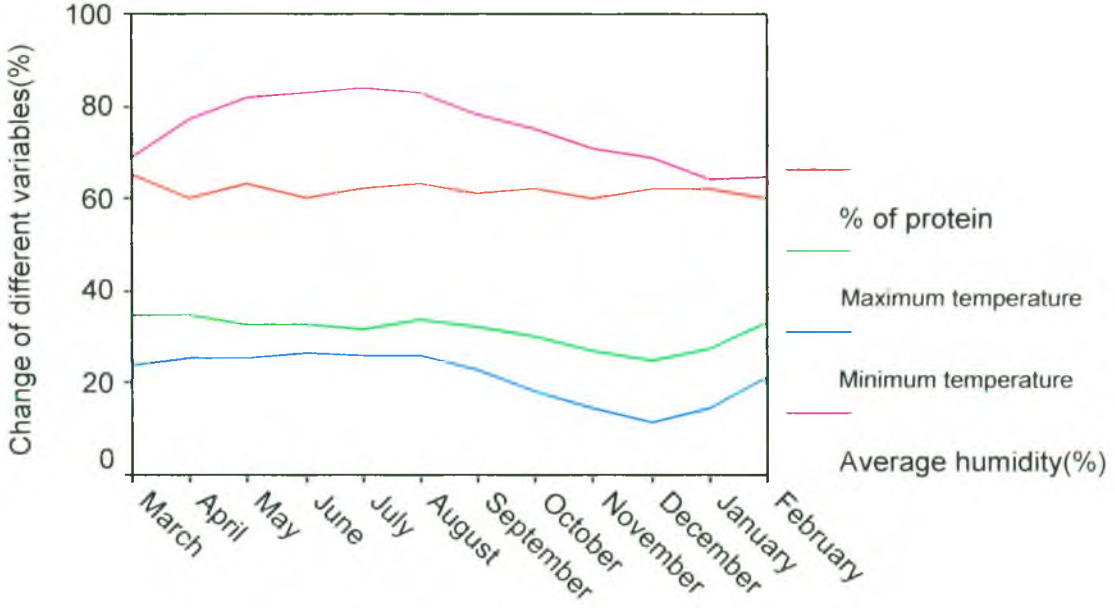


Fig. 29: Relation of protein damage percentage of Spirulina platensis with temparature and humidity

4.9.2 Effect of storage period on beta-carotene content of sun-dried *Spirulina platensis* biomass.

In this study it was observed that the percentage of beta-carotene content of sun-dried *Spirulina platensis* gradually decreased during the one-year storage period (Appen.31 and Fig. 30). In this study the initial percentage of beta-carotene was 0.2081177gm, and at the end of one-year storage period it was 0.004777gm (0.05±0.067), at room temperature (25.3°-33.3°C). It was obvious that percentage of beta-carotene content was adversely affected during the storage period at room temperature.

The percentage of beta-carotene content of sun-dried *Spirulina platensis* biomass was found to be maximum in March and minimum in February (Fig.30). There was no strong relation between percentage of beta-carotene of sun dried stored *Spirulina platensis* biomass with humidity (Fig. 32). It was observed that there was strong relation between beta-carotene of stored *Spirulina platensis* biomass with temperature (Fig. 31). It was noticed during in this study that, when temperature increased, the percentage of beta-carotene declined. It was also observed that there was no relation between beta-carotene content of stored *Spirulina platensis* biomass and humidity of the storage period (Fig. 32).

In this study strong relation between the percentages of beta-carotene of stored *Spirulina platensis* biomass with temperature of storage period was observed.

Annapurna *et al.* (1991) and Mathew *et al.* (1995) also reported loss of beta-carotene of *Spirulina*, when stored at room temperature. According to Seshadri *et al.* (1991), during storage in colored bottles, more than 50% beta-carotene was lost in less than 45 days. Similar result was also obtained in the present study, when *Spirulina* was stored in polyethylene bags.

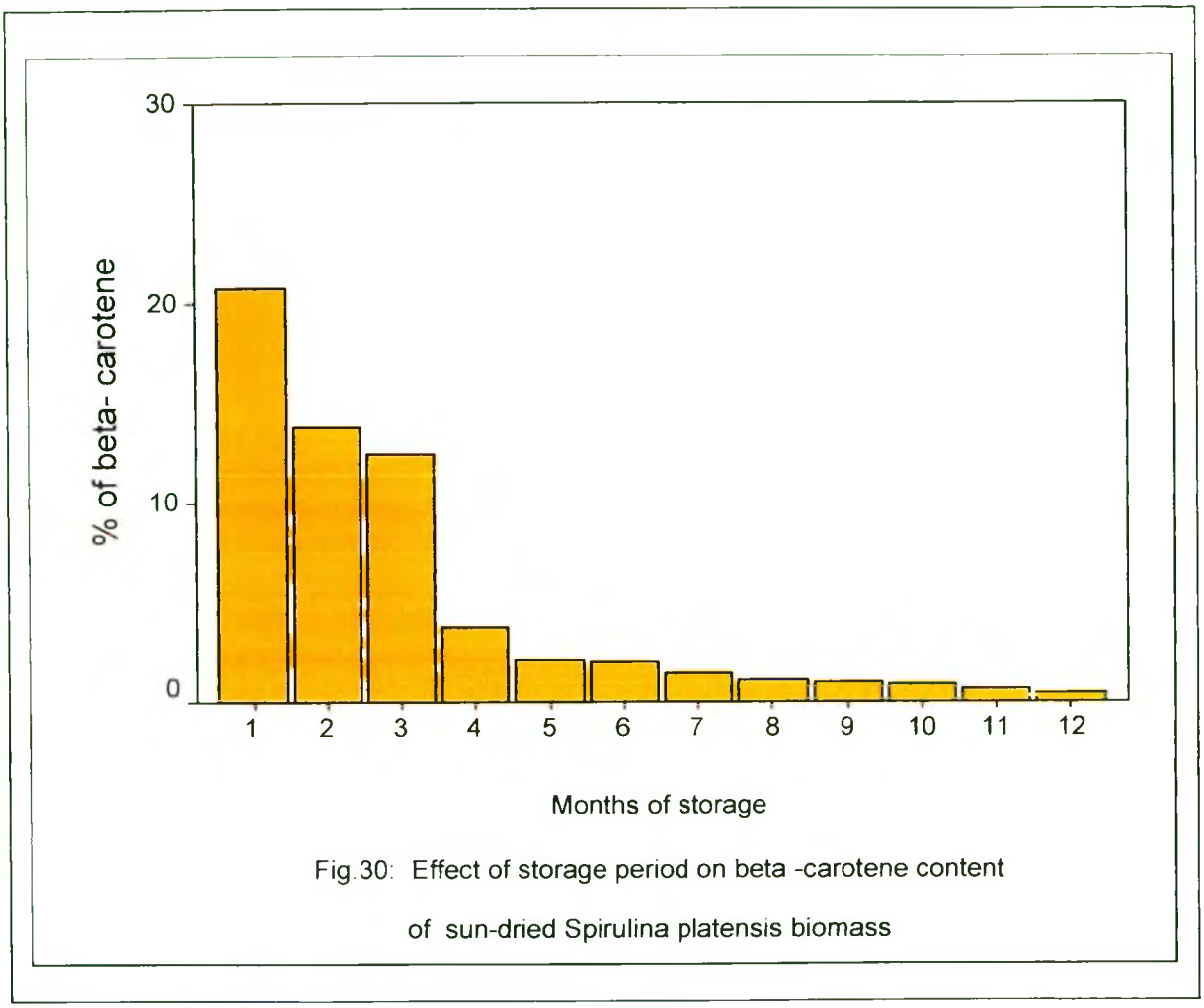


Fig.30: Effect of storage period on beta -carotene content of sun-dried Spirulina platensis biomass

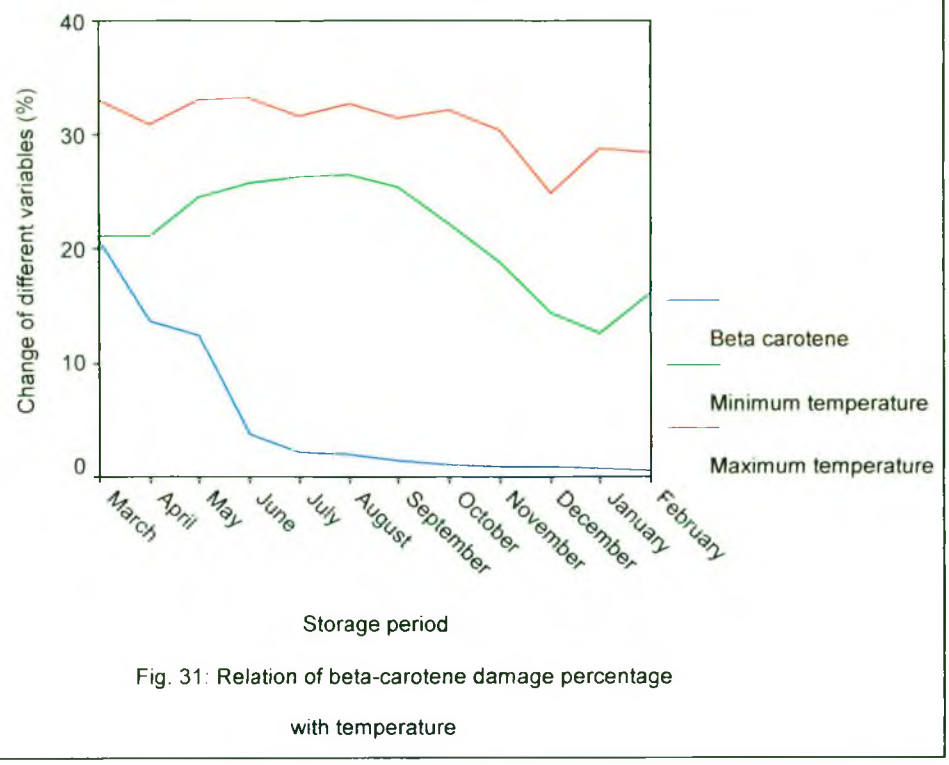


Fig. 31: Relation of beta-carotene damage percentage with temperature

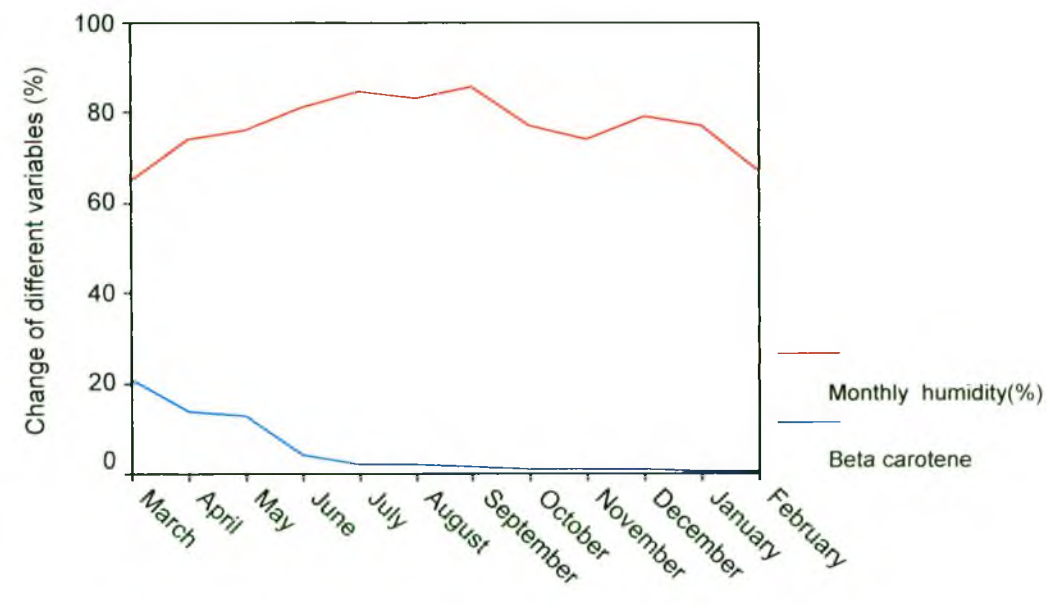


Fig. 32: Relation of beta-carotene damage percentage of Spirulina with humidity

4.9.3 Effect of storage period on bacterial load and moisture content of sun-dried *Spirulina platensis* biomass.

In the present study (Table. 20) it was observed that in the first observation the bacterial load of stored *Spirulina platensis* CFU/gm ranged from1000000-4000000 (2500000±1290994). The percentage of moisture content was 11.00-30.50 (19.63±8.23). At the second observation the CFU/gm was 2000000-5000000 (2750000±1500000). Percentage of moisture content was 10.99-26.70 (1815±6.59). At the third observation the CFU/gm was 4000000-5000000 (4250000±500000). The percentage of moisture content ranged 10.50-26.00 (17.48 ±6.52). And at fourth observation the CFU/gm was 5000000-8000000, (6500000±1290994). The percentage of moisture content was 13.00-31.50 (20.63±7.65).

It was observed that the CFU/gm of all samples gradually increased during the one-year storage period and it was found to fluctuate seasonally (Table. 21). It was also noticed that the percentage of moisture content of all samples decreased during the storage period up to third observation (8 months, pre-monsoon). But it increased at the fourth observation (12 months, monsoon period, Table. 21). Sample no. 2 having initial high moisture (30%), deteriorated after 8 months storage period. And at the end of the12 month's storage period, bad smell came from sample no.1 (initial moisture 20%).

Bacterial load, CFU/gm of different samples of sun-dried *Spirulina platensis* biomass was found to gradually increase during the storage period (Fig. 33). It was observed that, the maximum CFU/gm was recorded in monsoon followed by summer and then winter. Fig. 34 and 35 showed that the CFU/gm of *Spirulina platensis* biomass was related to humidity and temperature respectively.

Table 20. **Relation of climatic factors (humidity, temperature) and baeterial load (CFU/gm) on dried *Spirulina platensis* biomass**

Quarterly observation period	CFU/gm	Moisture (%)	Humidity (%)	Temperature (O°C)
	Mean / Range	**Mean / Range**	**Mean/ Range**	**Mean/ Range**
Initial observation: Monsoon (Oct'03)	2500000.0 ± 1290994.4 1 000000.0 – 4000000.0	19.63 ± 8.23 11.00 – 30.50	79.75 79.75 –79.75	26.80 26.80 – 26.80
2nd observation: Winter (Feb. '04)	2750000.0 ± 1500000 2000000.0- 5000000.0	18.15 ± 6.59 10.99 – 26.70	67.75 67.75 – 67.75	20.57 20.57 – 20.57
3rd observation: Summer (June '04)	4250000.0 ± 500000.0 4000000.0 – 5000000.0	71.48 ± 6.52 10.50 – 26.00	70.50 70.50 – 70.50	28.42 28.42 – 28.42
4th observation: Monsoon (Oct. '04)	6500000.0 ± 1290994.4 5000000.0 – 8000000.0	20.99 ± 7.65 13.70 – 31.25	79.50 79.50 –79.50	28.09 28.09 – 28.09

Table 21. Effects of storage conditions on bacterial load (CFU/gm) and moisture content of sun-dried *Spirulina platensis* biomass.

Number of sample	Initial observation Monsoon October, 2003				2rd observation Winter Februray, 2004				3th observation Summer June, 2004				4th observation Monsoon October, 2004			
	Moisture (%)	CFU/gm	Temperature °C	Humidity	Moisture (%)	CFU/gm	Temperature °C	Humidity	Moisture (%)	CFU/gm	Temperature °C	Humidity	Moisture (%)	CFU/gm	Temperature °C	Humidity
1	20.50	2000000	Maximum- 31.90, Minimum-25.50	79.75	19.00	2000000	Maximum-26.82, Minimum-14.32	67.75	18.30	4000000	Maximum-32.47, Mnimum-24.4	70.50	22.00	6000000	Maximum-31.0, Minimum-25.17	79.50
2	30.50	3000000			26.70	2000000			26.00	4000000			31.25	7000000		
3	11.00	4000000			10.99	5000000			10.50	5000000			13.70	8000000		
4	16.50	1000000			15.90	2000000			15.10	4000000			17.00	5000000		

Climatic factors are average of four months

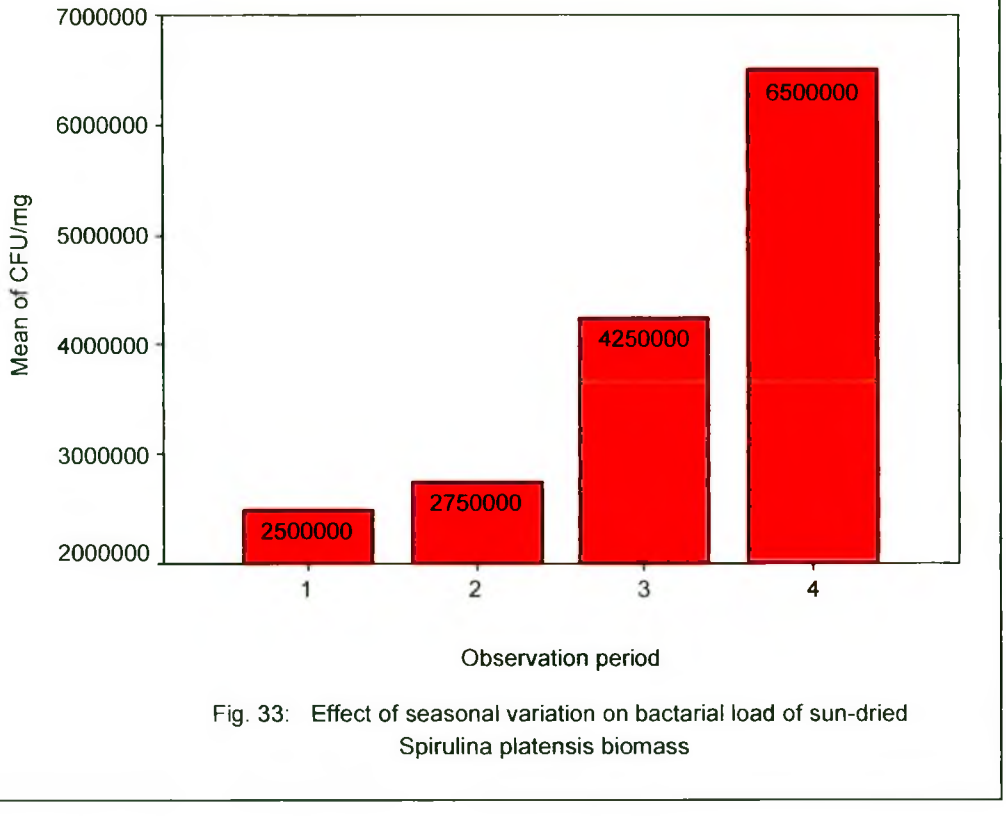


Fig. 33: Effect of seasonal variation on bactarial load of sun-dried Spirulina platensis biomass

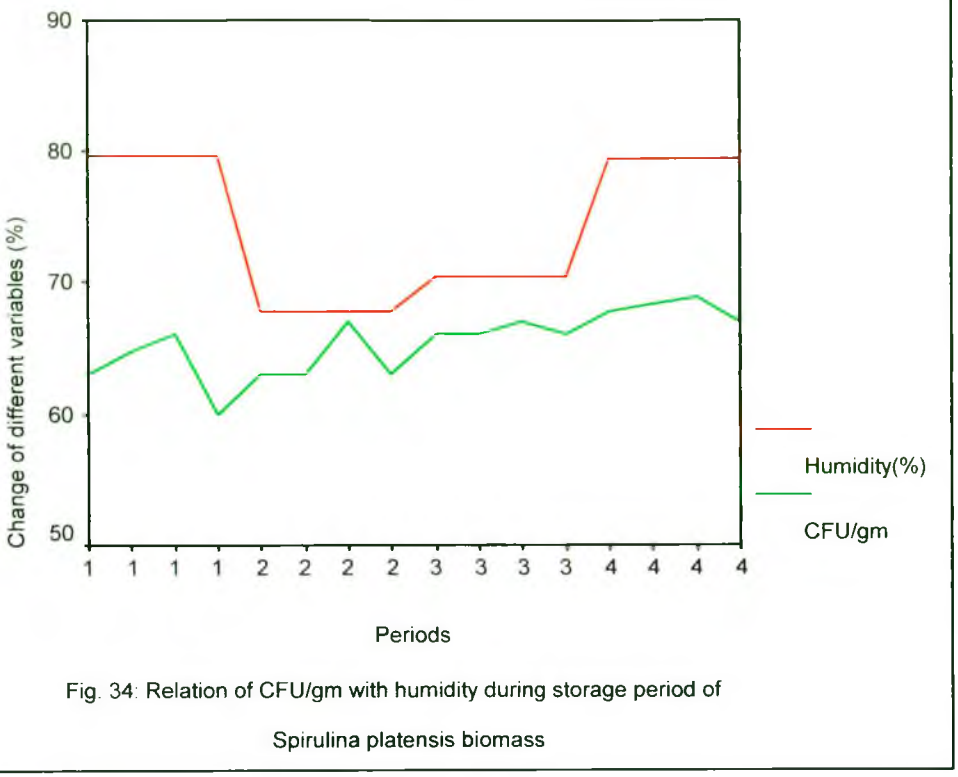


Fig. 34: Relation of CFU/gm with humidity during storage period of Spirulina platensis biomass

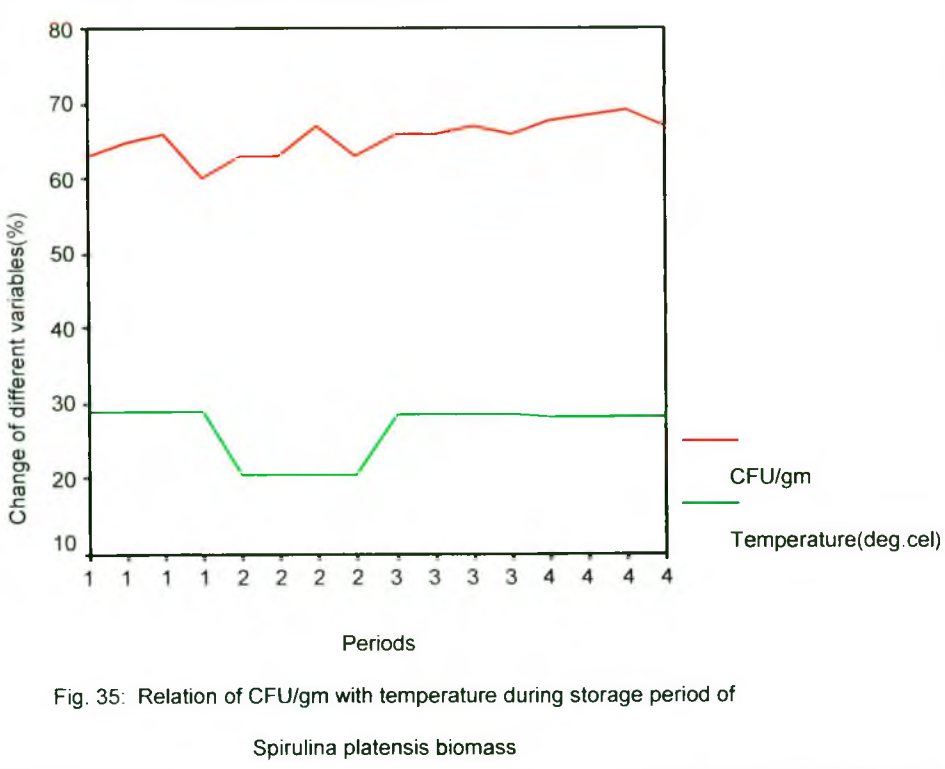


Fig. 35: Relation of CFU/gm with temperature during storage period of Spirulina platensis biomass

Mahadevwamy and Venkataraman (1982) cited that bacterial load of sun-dried *Spirulina* biomass was $40x10^4$ CFU/gm. Venkataraman and Becker (1982) reported bacterial load in sun-dried *Spirulina* biomass was $6x10^5$ CFU/gm. Venkateraman (1983) again reported that bacterial load of sun-dried *Spirulina* biomass was $6x10^5$ CFU/gm. In the present study it was found that, the range of bacterial load in sun-dried *Spirulina* biomass was $1x10^6$ to $4x10^6$CFU/gm.

Jacquet (1976) observed that long-term storage seemed to have positive influence in the decrease of the possible contaminating microbiological flora.

Venkateraman (1983) reported that dried *Spirulina* flakes with moisture content of about 8-10% could be packed and stored in polyethylene bags. Studies on bacterial contamination of the dried product have shown no increase of contamination levels with incremental storage time.

In the present study sun-dried *Spirulina platensis* was stored in polyethylene bags for a storage period of one year.

4.10. Regeneration of *Spirulina* from sun-dried *Spirulina platensis* biomass.

Different inoculum sizes of dry *Spirulina platensis* biomass (0.03%, 0.04%, 0.05% and 0.1% presented in the Table. 22) were used in the regeneration experiment. It was observed that maximum number of regenerated *Spirulina platensis*/ml was in 0.04% of dry *Spirulina platensis* biomass, in all batches (one week, one year and two years old). Hence 0.04% may be considered to be the optimum inoculum size for *Spirulina platensis* regeneration. Minimum of regenerated *Spirulina platensis* was found in 0.1% of dry biomass (Fig. 36).

Sun-dried *Spirulina platensis* biomass stored for one week, one year and two years, when put in culture medium and duly observed under the microscope (on first day, after 24 hrs, 48hrs, 10 days and 20days) showed the following results:

In the first day and after 24hrs microscopic observation long and short hyaline filaments were observed in the culture medium. After 48hrs very short broken filaments were found. After ten days, 2-3 coiled short filaments were found. After twenty days 4-5 coiled and straight *Spirulina platensis* filaments were found (Table. 22).

In the present study it was possible to regenerate *Spirulina platensis* from its sun-dried biomass after one week, one year and two years storage at room temperature. Maximum regeneration was recorded when inoculum size of sun-dried *Spirulina platensis* was 0.04% and minimum in 0.1% (Fig. 36).

Review of literature revealed no information in this line.

Table 22. **Regeneration of *Spirulina* filaments from sun-dried *Spirulina platensis* biomass**

Period of observation	Type of sample	Inoculum size of dry *Spirulina* biomass (%)	Moisture content (%)	Number of regenerated *Spirulina* filaments/ (ml)	Microscopic observation of regenerated period in different selected inoculums				
					1st obs. (First day)	2nd obs. (after 24 hours)	3rd obs. (after 48 hours)	4th obs. (after 10 days)	5th obs. (after 20 days)
1998	One week old	0.03	11.2	6,460	2-4 coiled hyaline filaments	2-3 coiled hyaline filaments	Small and broken fragments	2-3 coiled filaments and small fragments	2-5 coiled filaments and small fragments
		0.04		9,680					
		0.05		7,280					
		0.10		2,470					
	One year old	0.03	13.1	7,217					
		0.04		11,258					
		0.05		4,160					
		0.10		1,575					
	Two years old	0.03	14.0	2,080					
		0.04		13,275					
		0.05		10,920					
		0.10		1,070					

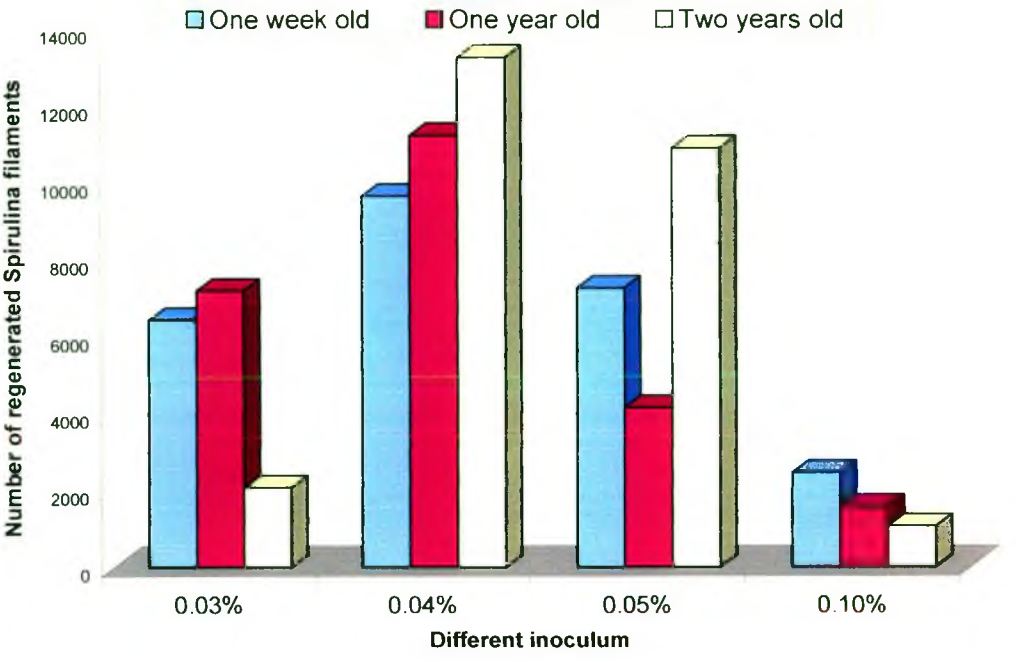


Fig. 36: Rate of *Spirulina* filaments regeneration from sun-dried *Spirulina platensis* biomass.

Chapter 5

Conclusion

Spirulina has already been claimed to be the miracle health food for the hungry people of the developing countries. In Bangladesh also it has been accepted as an item of health food in the form of capsules, tablets, powder, drinks etc. But the prevailing humid tropical climate has its inherent demerits. Here, most of the food, feed and medicaments under go very quick deterioration.

Besides physical (e.g. Temp, Light) and chemical (pH, Aw, Redox etc.) agents, microorganisms also play a significant role in the bio-degradation process.

From the facts and figures presented in this thesis, it can be logically concluded that

a. *Spirulina* when normally, grown in open culture system, is naturally associated with various microorganisms but, so far no pathogenic or toxigenic organisms have been reported

b. Under storage condition, the samples, having high moisture content, suffered quick deterioration. During preservation, the sample should be thoroughly dried, (preferably moisture should be below 11%) kept in air tight containers and stored in cool, dry, dark places. Cleaned sun-dried *Spirulina* has been successfully preserved for one year at room temperature in this study.

c. Well-planned study in future may yield data, which may be useful to further extend the shelf life of *Spirulina* biomass, at room temperature, or cold storage, with or without suitable desiccant like silica gel.

Chapter 6

References

Akhtar, N., P. Noor, M. A. A. Jahan and Md. M. Hossain. 1996. *Spirulina* culture in Bangladesh. V. Composition of rice husk ash and sodium bicarbonate as source of carbon feed back in *Spirulina* culture. Bang. J. Sci. Ind. Res. **31(1):** 142.

Annapurna, V. V., Y. G. Deosthale and M. S. Bamji. 1991. *Spirulina* as a source of vitamin A. Plant Foods for Human Nutrition. **41:** 125.

Annapurna,V. N. Shah, P. Bhaskaram, M. Bamji and V. Reddy, 1991.Bioavilability of *Spirulina* Carotenes in Pre-School Children, Jclin Biochem. Nutr. **10:**145-151.

Anusuya Davi, M., S. G. Mahdavi and L. V. Venkateraman.1981. Studies on the proteins of mass cultivated, blue- green alga (*Spirulina platensis*). J. Agric Food chem. **29:** 522-525.

Armida, H. -C., N-Irma, M-mariana, C- German, L-B Blanca. 2002. Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, IPN, Carpio y plan de Anala S/N Casco de Santo Tomas, Apartado Postal 102, **56(3):** 279-285.

Arvind, P. and P. Srivastava. 2001. Optimum photoperiod for the growth of *Spirulina platensis.* Journal o -Phytological-Research. **14(2):** 219-220.

AOAC (Association of Official Analytical Chemicals). 1984. Methods of analysis (S. William, Ed.). 14th ed. Washington DC. 834 pp.

Avery, S. V. and J. M. Tabin. 1992. Mechanism of strontium uptake by laboratory and brewing strains of *Saccharomyces cerevisiae.* Applied and Environmental Microbiology. **58:** 3883-3889.

Ayehunie, S., A. Belay, T. W Baba and R. R. Ruprecht. 1998. Inhibition of HIV-1 Replication by an Aqueous Extract of *Spirulina platensis*. Journal of Acquired immune Deficiency Syndromes and Human Retro virology. **18:** 7-12.

Azmat, F. 1991. Study of the Effects of *Spirulina* on blood glucose level of normal and experimentally induced diabetic rats (thesis MS Medical). Dept. of Pharmacology, IPGMR, Dhaka. 81 pp.

Balloni, W., L. Tomaselli, L. Giovannetti, and M. C. Margheri. 1980. Biologia fondamentale del genere *Spirulina* In Cantarelli, C. Ciferri, O., Florenzano, G., Kpsiotis, G., Materassi, R., Treccani, U., Eds Progetto finalizzato "Ricerca di nuove fonti proteiche e di nove formulazioni alimentari". Atti del Convegno:Prospettive della coltura di *Spirulina* in Italia. Consiglio Nazionale delle Richerche. Firenze-Academia dei Georgiofili, CNR, Tipografia Coppini, 49-82 pp.

Bauer, A. W., W. M. M. Kirby, J. C. Sherris and M. Turck. 1966. Antibiotic susceptibility testing by standardized single disc method. Am. J. Clin. Pathol. **44:** 493-496.

Becker, E. W. and L. V. Vankataraman. 1984. Production and utilization of the blue–green algae *Spirulina* in Indian Biomass. **4:** 105.

Becker, E. W., B. D. L. Jakober and R. M. Schmulling. 1986. Clinical and biochemical evaluations of alga *Spirulina* with regard to its application in the treatment obesity. A double blind crossover study. Nutr. Rep. Internal. **33(4):** 565-574.

Begum, H. A., SAR Choudury, Nargis Akhtar and M. Shahjan. (1993). Study of effects of *Spirulina* on serum lipid profile in normal and fat fed rats. *TAJ.* **6(1):** 52 pp.

Belay, A. 1997. Mass culture of *Spirulina* outdoors -the Earthrise Farms experience- in *Spirulina platensis* (Arthospora). Physiology, Cell-biology and Biotechnology. A. Vonsahak, Ed. Taylor and Francis. London. U.K. 131-158 pp.

Ben, A. 1987. Presentation Polysaccharides from microalgae work shop. Duke University.

Begum, S., P. Noor, N. Akhtar and F. Z. Majid. 1998. *Spirulina* culture in Bangladesh V1. Domestic Scale Production of *Spirulina*. Bangladesh J. Sci. Ind. Res. **33(3):** 474

Bonnin, G. 1992. *Spirulina* production Engineering Handbook. B.E.C. C.M.A. – 5 place Roger salengro 4400 Nantes France. 79 pp.

Brady, D. and J. R. Dimcam. 1994. Bioaccumul action of metal cation by *Saccharomyces cerevisiae*. Applied Micrebiologya and biotechnology. **41:** 149-154

Bryan, A. H. 1950. Manual of methods for pure culture study of Bacteria. **12:**1-10.

Bucaille, P. 1990. Importance and effectiveness of *Spirulina* in infant's nutrition having protein energetic malnutrition in tropical countries (Franch). Doctorate thesis. University Paul Sabatier toulouse. **3:** 45.

Buchanan, R. C. and N. E. Gibbons. 1974. Bergey's Manual of Dterminative Bacteriology (8th Ed.). The Williams and Wilkins Company Baltimore, USA. 1268 pp.

Camorro-G. 1996. Toxicologia Escuela Nacional de ciencias. Biologicas, Instituto Politecnico Nacional, Mexico D. F. Rev. Invest-clin. Sept-Oct. **48(5):** 389-99.

Castro, F., P. Viedman and D. Cotoras. 1989. Biomass de Rhizopus, oligosporus como adsorbent de iones metalicos Microbiologia SEM. **8:** 94-105.

Claus, G. W. 1995. Understanding Microbes. W. H. Freeman and company, NY, 547 pp.

Collins, C. H. and M. P. Lyne. 1984. Microbiological Methods (5th Ed). Butter worth C (publishers) Ltd. London. 448 pp.

Ciferri, O. 1983. *Spirulina,* the Edible Microorganism. Microbial *Rev.* **47:** 551-578.

Cocks, L. V. and C. V. Van Rede. 1966. Laboratory handbook for oil and - biology and Biotechnology fat analysis.

Cohen, Z. 1997. The chemicals of *Spirulina*. *In Spirulina platensis* (Arthrospira): Physiology, Cell-biology and Biotechnology. A. Vonshak, Ed.. Taylor and Francis Ltd., London, UK. 175-204 pp.

Compos-Takaki, G. M., d.e., Diu, M. B. S., M. L. Koening and E.C. Pereira. 1988. Screening of marine algae from Brazilian northeastern coast for antimicrobial activity. Bot. Mar. **31:** 375-377.

Crist, R. H., K. Oberholser, N. Shank and M. Nguyen. 1981. Nature of bonding between metallic ions and algal cell walls, Environ. Sci. Technol. **15(10):** 1212-1217.

Darnall, D. W., B. Greene and J. Gardea-Torresdey. 1988. Gold binding to algae, in P. R. Norris and D. P. Kelly (Eds.), Biohydrometallurgy, Science and Technology Letters, Kew, Surrey, U. K. 487-498 pp.

Dasgupta, T., S. Banerjee, P. K. Yadav and A.. R. Rao. 2001. Chemomodulation of carcinogen metabolising enzymes, antioxident profiles and skin and fore stomach papillomagenesis by *Spirulina platensis*. Molecular and Cellular Biochemistry. **226:** 27-38.

Dillon, J. C., A. P. Phuc and J. P. Dubacq. 1995. Nutritional value of Alga *Spirulina*, Simpoulos Ap (Ed): Plants in Human Nutrition. World Rev. Nutri Diet. Basel, Karger. **77:** 32-46.

Earthrise Farms. 1988. Five year testing heavy metals in *Spirulina* (1983-1987). 19. pp

Eklund, C. and C. E. Lankford. 1967. Laboratory Manual for General Microbiology. Prentice-Hall Inc. Englewood Cliff. New Jerse. 295 pp.

El-Fouly, M. M., A. M. Salen, F. K. El-Baz and W. A. El- Malky. 1998. Botany Department: National Research Centre, Cairo, Egypt Egyptian-Journal-of Botany. **38(1-2):** 63-85.

Eykelenburg, V. C. 1977. On the morphology and ultra structure of the wall of *Spirulina platensis.* Ant Van Leeuwenhook J. Microbial serol. **43:** 89-99.

Faggi, E. 1980. Caratteristiche microbiologiche delle biomass di *Spirulina In* : Prospettive della coltura die *Spirulina* in India. E. R. Materassi, Firenze-Accademia dei Georgofili. 127 pp.

Fariduddin, A. T. M. and M. Masbhuddin. 2004. Effect of *Spirulina* in the prevention of accumulation of arsenic in rats. Bangladesh Medical Journal. **33(3):** 93.

Fedkovic, Y., C. Astre, F. Pinguet, M. Gerber, M. Ychou and H. Poujol. 1993. Spiruline et cancer. I. Doumenge, F., Dunard- Chast, H., Toulemont, A., eds. Spiruline algue de vie. Musee Oceanographique. Bulletin de I 'Institut Oceanographique Monaco. Numero special. **12:** 117-120.

Ferguson, J. and B. Bubela. 1974. The concentration of Cu (11), Pb (11), and Zn (11) from aqueous solutions by particulate algal matter, Chem. Geol. **13:**163-186.

Fox, R. D. 1983. Algoculture Doctorate thesis Univ Lois Pasteur, France. 110 pp.

Fox, R. D. 1986. Algaculture: la *Spirulina,* un espoir pour le monde de la faim Edisud, France.

Gadd, G. M. and C. White. 1989. Heavy metal and radionuclide accumulation and toxicity in fungi and yeasts. In Metal – Microbe – Interactions (Ed. R. K. Poole and G. M. Gadd). oxford IRL press. 19-38 pp.

Gee, A. R. and A. W. L. Dudeney. 1988. Adsorption and crystallization of gold at biological surface, in P. R. Norris and D. P. Kelly (Eds.), Biohydrometallurgy, Science and Technology Letters, Kew, Surrey, U. K. 437-452 p

Greene, B., G. Torresdey, J. L. Hosea, J. M. McPherson, R. A. and Darnall, D. W. 1987. Algal ion exchangers for waste metal recalmation, Am. Chem. Soc Nat. Meeting preprints Extended Abstract, **272(2):** 800-804.

Greene, B. and D. W. Darnall. 1990. Microbial Mineral Recovery, McGraw Hill Publishing Co. Newyork, London, Paris. 283 pp.

Gugliemi, G. R. 1993. Rosmarie and Nicole Tandeaude Marsc. Sunday 3rd April.

Gustafson, K., J. Cardelina, R. Fullar, O. Weishow, R. Kiser, K. Sander, G. Patterson and M. Boyd. 1989. AIDS – Antiviral sulfolipids from cyanobacteria (blue-green algae). Journal of the National Cancer Institute, **16:** 1254.

Hayashi, O., T. Katoh and Y. Okuwaki. 1994. Enhancement of antibody production in mice by dietary *Spirulina platensis.* J. Nutr. Sci. Vitaminol **40:** 431-441.

Hayashi, K., T. Hayashi and I. Kojima. 1996. A Natural Sulfated Polysaccharide, Calcium Spirulan, Isolated from *Spirulina platensis. In Vitro* and *ex Vivo* Evaluation of Anti-Herpes Simplex Virus Activities AIDS RESEARCH AND HUMAN RETROVIRUSES. **12(15):** 1463.

Hayashi, T., K. Hayashi, M. Maeda and I. Kojoma. 1996. Calcium *Spirulina,* an inhibitor or enveloped virus replication from a blue green alga *Spirulina platensis.* J. Nat – Prod, USA. **59(1):** 83-87.

Henrikson, R. 1989. Earth food of *Spirulina,* Ronore Entireprises Inc. Laguna beach, California. 9-164 pp.

Holden, M. 1981. A Rapid and Simple Method for Determining Carotene in Plant Meterial. Bulletin No. 22. Xerophthalmia Club, U. K. 47-52pp.

Hove, H., H. Norgaard and P.B. Mortensen. 1999. Lactic acid bacteria and the human gastrointestinal tract. European Journal Clinical Nutrition. **53:** 339-350.

Jacquet. 1976. Micro flora. des preparations de *Spirulina* Ann. Nutr. et Alim; CNRS Ed. France.

Jahan, N. (1991). Study of effects of *Spirulina* on serum lipid profile in normal and fat fed rats and in normal human volunteers. Department of postgraduate Medicine and Research. Dhaka. Bangladesh. 78 pp.

Jassby, A. 1983. Nutritional and Therapeutic properties of *Spirulina*. Draft for proteus corporation.

Jahan, M. A. A., R. Khatun, P. Noor, M. Hossain, N. Akhtar and J. L .Munshi, 2002. Use of *Spirulina* as curative measure on Arsenic Affected People. The Study sponsored by Ministry of Science and Technology, Government of the People's Republic of Bangladesh. 6 pp.

Jahan, M. A. A., P. Noor and N. Akhtar. 1994. *Spirulina* culture in Bangladesh III. Development of a Medium, suitable for commercial cultivation of *Spirulina* in Bangladesh. Bangladesh J. Sci. Ind. Res. **29(4):** 149.

Jahan, M. A. A., R. Khatun and L. Nahar. 1999. *Spirulina* culture in Bangladesh IX. A comparative study on the Effect Three Different Ashes on Coil Filament Characteristics of *Spirulina.* Bangladesh J. Sci. Ind. Res. **34(2):** 261.

Jorjani, G. and P. Amirani. 1978. Antibacterial activities of *Spirulina platensis.* Maj.limy Puz. Danisk, jundi shap. **1:** 14-18.

Karambeer, C. 1987. Bigger profits through improved wastewater treatment. Finish. Manage. **32:** 36-37.

Khatun, R. and F. Z. Majid. 2004. A Report on *Spirulina* in Bangladesh Past, Present, and Future. 1 pp.

Kuyucak, N. and B. Vosesky. 1988. New algal biosorbent for a gold recovery process, in P. R. Norris and D. P. Kelly (eds.), Biohydrometallurgy, Science and Technology Letters, Kew, Surrey, U. K. 453-464 pp.

Lank Ford, C. E. and C. E. Eklund. 1965. Laboratory manual for general microbiology. Prentiee Hall, Inc. Englewood clifts, New Jersey.

Liu-Yufeng, xu- Lizhi, cheng-Ni, Lin- Lijun, Zhan- chengwu. (2000). Inhibitory effect of phycocyanin from *Sprulina platensis* of the growth of human Leukemia. K562. cell .Journal of Applied phycology. **12(2):**125-130.

Mahadevaswamy, M. and L. V. Venkataraman. 1987. Bacterial contaminants in blue green alga *Spirulina*. Produce for use as biomass protein. Arch. Hydrobiologie. **110(4):** 623.

Majid, F. Z, G. Rahman and L. Nahar. 1996. Best food *Spirulina*. 17pp.

Mathew, B., R. Sankaranaryanan, P. Padmanabhan, V. Cherian, B. T. Somanthan, P. Amma, N. S. Amma and M. K. Nair. (1995). Evaluation of chemoprevention of oral cancer with *Spirulina fusiformis*. Nutr. and cancer. **24:** 200.

Miao, Jan Ren. 1987. *Spirulina* in jiangxi, china Academy of agricultural Science, jiangxi province, China. Paper presented at SOC. App. Algology, Lille, France, September.

Miranda, MS., R.G Cintra, S. B. Barros, F. J. Mancini. 1998. Antioxidant activity of micro alga *Spirulina maxima*. Braz J Med. Bio Res. **31:**1075-1079.

Momtaz, S. 1991. Study of the effects *of Spirulina* on serum Gpt and GOT Levels of normal and paracetamol induced hepatotoxic Rats (Thesis M. S. Medical). Dept. of Pharmacology IPGMR, Dhaka. 72 pp.

Nader de Macias, M. E., N. C Romero, M. C. Apolla, S. N. Gonzalez and G. Oliver 1993. Prevention of infections produced by *Escherichia coli* and *Listeria* monocytogenes feeding milk fermented with *lactobacilli*. Journal of Food Protection. **56:** 401-405.

Nahar, L., S. Begum, K. Rahima and M. M. Hosain. 1994. *Spirulina* culture in Bangladesh. 1 A pilot plant scale study on the growth of *Spirulina platensis* and *Spirulina fusiformis*. Bangladesh J. Sci. Ind. Res. **29**(1): 85.

Nichols, B. and B. Wood. 1986. Theoccurance of biosynthesis of gama linolenic acid in a blue green algae, *Spirulina platensis*, lipids. **3:** 46-50.

Noor, P., M. A. A. Jahan, N. Akhtar and S. Begum. 1994. *Spirulina* culture in Bangladesh. II. A pilot plant scale study on the growth of *Spirulina platensis* and *fusiformis*. Bangladesh J. Sci. Ind. Res. **29(1):**109.

Noor, P., N. Akhtar and S. Begum. 1999. *Spirulina* culture in Bangladesh. X. Effects of different Lights and Depths of culture Medium on Domestic Scale of *Spirulina* culture in summer and winter. Bangladesh J. Sci. Ind. Res. **34(2):** 169.

Norris, P. R. and D. Kelly. 1979. Accumulation of metals by bacteria and yeasts. Developments in Industrial Microbiology. **20:** 299-308.

Parada, J. L., G. Zulpa de Caire, M. C. Zaccaro de Mulic and M. M. Storni de Cano. 1998. Lactic acid bacteria growth promoters from *Spirulina platensis*. International Journal of Food Microbiology. **45:** 222-228.

Paknikar, K .M. and A. D. Agate. 1995. Laboratory manual. International workshop on metal-microbe interactions and their applications. United nations environment programme. Pune Mircen Agharkar Research institute, G. G. Agarkar Road, Pune-411 004 India. 3-84 pp.

Perdigon, G., S. Alvarrcz and M. Mcdici. 1992. Systematic and local augmentation of the immune response in mice by feeding with milk fermented with Lactobacillus acidophilus and /or Lactobacillus casei. In food Nutrition and Immunity (Dynamic Nutrition Research), Eds. Paubert-Braquct, M., Dupont, Chand Paolctti, R. 66-76 pp.

Priyasethu, K. M., T. N. Prabha and L. V. Venkataraman. 1994. Preparation of protoplasts from the cyanobacterium *Spirulina platensis* and a novel viability assay. Letters in Applied Microbiology. **18:** 241-244.

Puyfoulhoux, G., J. M. Rouanet, P. Besancon, B. Baroux, J. C. Baccou and B. Caporiccio. 2001. Iron availability from iron –fortified *Spirulina* by an in vitro digestion/Caco-2 cell culture model. J. Agric Food Chem. **49:**1625-29.

Ramirez, D., R. Gonzalez, N. Merino, S. Rodriguez and O. Ancheta. 2002. Inhibitory effects of *Spirulina* in zymazan-induce arthritis in mice Mediators Inflamm. **11:** 75-9.

Richmond, A. 1984. Micro algae of economic potential *In*: Richmond, A., Ed. Handbook of micro algal mass culture. CRC press, Inc, Boca Raton. 199-243 pp.

Richmond, A. 1992. Mass culture of cyanobacteria. *In:* Mann, N., Carr, N., Eds. Photosynthetic prokariotes.2nd Ed Plenum Press, New York and London. 181-210 pp.

Saha, SK, M. Misbahuddin, R. Khatun and I. R. Mamun. 2005. Effect of Hexane Extract of *Spirulina* in the Removal of Arsenic from Isolated Liver Tissues of Rat. Mymenshingh Med J Jul. **14(2):** 194.

Seshadri, C.V. 1977. Murugappa Chettiar Research Centre (MCRC). Technical Notes Madras, India.

Seshadri, C.V., B. U. Umesh, R. Manoharan. 1991. Beta-carotene studies in *Spirulina.* Bioresource –Technology (United Kingdom) **38(2 and 3):** 111-113.

Shri, A. M. M., Murugappa Chettiar Research Centre Photosynthesis and Energy Division, MCRC II year. Thara Madras, India.

Sneath, P. H., A. N. S. Mair, M. E. Sharpe and J. G. Holt (Eds). 1986. Bergey's Manual of Systematic Bacteriology (9th Ed.). The Williams and Wilkins Compamy, Baltimore, USA. **2:** 1599.

Society of American Bacteriologist. 1957. Manual of Microbiological Methods. Mc Graw-Hill Book Company Inc. NY. 315 pp.

Society of American Bacteriologist. Pure culture of Bacteria Edition. 1948-49. M. J. Pelezar, Jr.

Strandberg, G. W., S. E. Shumate and J. R. Parrol. 1981. Microbial cells as biosorpents of heavy metals, accumulation of uranium by *Saccharomyces Creevisia* and *Pseudomonas acruginasa*. Applied and Environmental Microbiology. **41:** 237-245.

Switzer, L. 1982. *Spirulina,* The Whole Food Revolution, Bantam Books. London, Sydney. 49-126 pp.

Switzer, L. 1980. *Spirulina,* The whole food revolution. Proteous Corporation, USA. 1-69 pp.

Tabin, J. M., D. G. Cooper and R. J. Neufeld. 1984. Uptake of metal ions by Rhizopus arrhizus biomass. Applied and Environmental Microbiology. **47:** 821- 824.

Takaaki, M., J. Murata, M. Toyoshima, H. Fujii, M. Nakajima, T. Hyashi, T. Kato and 1. Shiki. 1998. Inhibition of tumor invasion and metastasis by calcium spirulan (Ca-SP), a novel sulfated polysaccharide derived from ablue-green alga, *Spirulina platensis.* Clinical-and Experimental-Metastasis. **16(60):** 541-550.

Takeuchi, T. 1978. Clinical experiences of administration of *Spirulina* to patients with hypochronic anemia Tokyo Medical and Dental University Japan.

Upasani, C. D., A. Khera and R. Balaraman. 2001. Effect of lead with vitamin E, C or *Spirulina* on malondialdehyde, conjugated dienes and hydroperoxide in rats. Indian J. Exp. Biol. **39:** 70-4.

Varga, L., J. Szigeti, R. Kovacs, T. Foldes and S. Buti. 2002. Influence of a *Spirulina platensis* Biomass on the Microflora of Fermented ABT Milks During Storage (RI). J. Dairy Sci. **85:** 1031-1038.

Venkataraman, L. V. 1983. Blue green alga *Spirulina*. Monograph on blue green alga *Spirulina platensis*. Biotechnology and application. CFTRI. Mysore. India. 18-73 pp.

Venkataraman, L. V. and E. W. Becker. 1982. Biotechnology and utilization of Algae. The Indian Experience, D.S.T.I. New Delhi and CFTRI. Mysore, India. 179-184 pp.

Venkataraman, L. V. and E. W. Becker. 1989. Biotechnology of utilization of algae. The Indian Experience. 1-228 pp.

Venkataraman, L. V. and M. Mahadevaswamy. 1992. Pond management in *Spirulina* production. CFTRI. Mysore. 115 pp.

Venkataraman L. V. and E. W. Becker. 1982. Biotechnology and utilization of algae. The Indian Experience. 184 pp.

Vela, G. R. and C. N. Guerra. 1966. On the nature of mixed culture of *Chlorella pyrenoidosa.* Tx. 71105 and various bacteria.- J. Gen. Microbiol. **42:** 123-131.

Vonshak, A.1997a. In *Spirulina platensis* (Arthrospira): Physiology, Cell-biology and Biotechnology. A. Vonshak, Ed. Taylor and Francis Ltd., London, UK. 213-226 pp.

Vonshak, A.1997b. Use of *Spirulina* biomass in *Spirulina platensis* (Arthrospira): Physiology, Cell-biology and Biotechnology. A. Vonshak, Ed. Taylor and Francis Ltd., London, UK. 205-212 pp.

Voslesky, B. 1987. Biosorbents for metal recovery, Trends Biotechnol. **6:** 96-101.

Wood, J. M. and H. K. Wang. 1983. Microbial resistance to heavy metal. Environ. Sci. Tcehnol. **17:** 582A-590A.

Yamane, Y. 1988. The effect of *Spirulina* on nephrotoxicity in rats. Present at Anual symposium of the pharmaceutical society of Japan. Apr. 15. pharmaceutical Dept. Chiba University Japan.

Yang, H. N., E.- H. Lee and H–M. Kim. (1997). *Spirulina platensis* inhibits anaphylatic Reaction. Life Sciences. **61(13):** 1237-1244.

Yoshida, R. 1977. *Spirulina* hydrolyzates for cosmetic packs. Japan. Kokai 7731, 838 (Inc. CI A6K7100). 10 Mar.

Zhan, H. Q., A. P. Lin, T. Sun and Y. M. Deng. 2001. Chemo and radio-protective effects of polysaccharide of *Spirulina platensis* on hemopoteotic system of mice and doses. Acta Pharmacol. Sin. **22:**1121- 1124.

Zulpa de Caire, G., J. L. Parada, M. C. Zaccaro and M. M. Storni de Cano. 2000. World Journal of Microbiology and Biochemistry. **16:** 563-565.

Chapter 7

Appendices

Appendix 1. BD_1-medium

$NaHCO_3$ 3.2 gm, $NaNO_3$ 0.5 gm, KCl 0.2gm, $MgSO_4$ 0.1gm, $CaCl_2$ 0.008gm, $FeSO_4$ $7H_2O$ 0.002gm, EDTA 0.008gm, Urea 0.05gm, T.S.P. 0.01gm, R.H.A. 0.4gm, H_2SO_4 (98%) 0.1ml, H_2PO_4 (85%) 0.04 ml, A8 0.02% B3 0.02%, water 1000 ml

Appendix 2. Nutrient agar medium (Bryan 1950)

Beef extract	3.0gm
Peptone	5.0gm
Agar	15.0gm
Distilled water	1000ml
pH	7.2

Appendix 3. Crystal violet solution (Claus 1995)

Solution A

Crystal violet (85% dye content)	2.0gm
Ethyl alcohol (95%)	20ml

Solution B

Ammonium oxalate	0.8gm
Distilled water	80ml

Solution A and Solution B were mixed

Appendix 4. Motility medium / Sloppy agar medium (Eklund and Lankford 1967)

Nutrient broth	100ml
Agar	0.5gm
Tetrazolium chloride	0.001gm
pH	7.2

Appendix 5. Fermentation medium (SAB 1957)

Beef extract	3.0gm
$NH_4H_2PO_4$	1.0gm
$MgSO_4$	0.2gm
KCl	0.2 gm
Carbohydrate	10.0gm
Distilled water	1000ml
pH	7.4

Appendix 6. Starch agar medium (Claus 1995).

Soluble starch	3.0gm
Beef extract soluble starch	10.0gm
Agar	12.0gm
Distilled water	1000ml
pH	7.5

Appendix 7. Gelatin agar medium (Collins and Lyne 1984).

Beef extract	3.0gm
Peptone	5.0gm
Gelatin agar (5%)	50.0gm
Agar	15.0gm
Distilled water	1000ml
pH	7.2

Appendix 8. Methyl red /Voges Proskauer Broth (Bryan 1950)

Peptone	5.0 gm
Glucose	5.0gm
$K_2H\ PO_4$	5.0 gm
Distilled water	1000 ml
pH	7.2

Appendix 8. Alpha- Napthol solution (Bryan 1950)

Alpha- Napthol	5.0 gm
Ethyl alcohol (95%)	100 ml

Appendix 8. KOH-creatine solution(Bryan 1950)

KOH	40.00gm
Creatine	0.3gm
Distilled water	100ml

Appendix 9. Nitrate Broth

Nutrient Broth	1000ml
KNO_3	1.0gm
Sol. A	
Sulfanic acid	8.0gm
5N acetic acid	1.0l
Sol. B	
Dimethyl α napthalamine	6.0ml
5N acetic acid	1.0liter

Nitrate Reduction (pure culture of bacteria)

Peptone	5.0gm
Beef extract	3.0gm
KNO_3	1.0gm
Distilled water	1000ml(Double distilled)
pH	7.0 ± 0.2 at 25°C

Appendix 10. Kovac's reagent (SAB 1957)

Para-dimethyl –amino-benzaldehyde	5.0gm
Butyl alcohol	75ml
HCl (Conc)	25ml
pH	7.0

Appendix 11. Simmon's citrate agar (Claus 1995)

$MgSO_4$	0.2gm
Mono-ammonium phosphate	1.0gm
Di-potassium phosphate	1.0gm
Na-citrate	2.0gm
NaCl	5.0gm
Bromo-thymol-blue	0.08gm
Agar	15.0gm
Distilled water	1000ml
pH	6.8

Appendix 12. Phenyalanine agar medium (Sneath *et al.* 1986)

Yeast extract	3.0gm
DL- Phenyalanine	2.0gm
Na_2HPO_4	1.0gm
NaCl	5.0gm
Agar	15.0gm
Distilled water	1000ml
pH	7.3

Appendix 13. Tyrosine agar medium (Sneath *et al.* 1986)

L-tyrosine	0.5gm
Distilled water	10ml
Nutrient agar	100ml

Tyrosine was sterilized separately and was added to the medium before inoculation

pH	7.0

Appendix 14. Nutrient broth medium (Bryan 1950)

Beef extract	3.0gm
Peptone	5.0gm
Distilled water	1000ml
pH	7.2

Appendix 15. IFP

	gm/l
$NaHCO_3$	16.800
$NaNO_3$	2.500
NaCl	1.000
K_2SO_4	1.000
K_2HPO_4	0.500
$MgSO_4$, H2O	0.200
Na_2 EDTA	0.080
$CaCl_2$	0.040
$FeSO_4$	0.010
A_5	1ml
B_6	1ml

Appendix 16. Chemical composition of sun-dried *Spirulina* biomass

Name of the sample	Moisture (%)	Protein (%)	Fat (%)	Fibre (%)	Ash (%)	Carbohydrate by difference (%)	Beta-carotene (%)
Spirulina	11.00	65.00	0.24	1.27	6.60	15.89	0.20

Appendix 17. Bangladesh Standard and Testing Institute (BSTI) Sun-dried *Spirulina platensis*.

Description of tests	Results
Moisture content % m/m	**12.47**
Ash content % m/m	**4.66**
Iron (As Fe) ppm	**577.0**
Phosphorus (as P) ppm	**1601.60**
Calcium (as Ca)ppm	**1876.36**
Magnesium (as Mg) ppm	**1967.03**
Chloride (as Cl) ppm	**1246.90**
Protein % m/m	**60.58**
Fat content m/m	**0.06/. 49**
Lead (pb) ppm	**No trace**

Appendix 18. Effect of pH (daily adjusted) on *Spirulina platensis* culture

Sample no.	Initial pH	Initial O.D.	pH after 3days	O.D after 3days	pH after 7days	O.D after 7days
1	6	0.04	8.6	.07	6.6	0.02
2	7		9.1	1.00	10.3	1.01
3	8		9.3	1.10	10.0	1.01
4	9		9.4	1.20	10.0	1.21
5	10		10.0	1.23	10.1	1.24
6	11		10.9	0.04	10.2	0.02

Appendix 19. Effect of pH (not adjusted) on *Spirulina platensis* culture

Sample no.	Initial pH	Initial O.D.	pH after 3days	O.D. after 3days	pH after 7days	O.D.after 7days
1	6	0.04	10.1	0.07	10.1	0.02
.2	7		10.3	1.00	10.2	1.00
3	8		10.2	1.20	10.4	1.00
4	9		10.4	1.50	10.6	1.52
5	10		10.5	1.6	10.5	1.53
6	11		10.3	0.04	10.1	0.02

Appendix 20. **Production of *Spirulina platensis* biomass in different seasons of Bangladesh.**

Observation of different seasons and Years	Growth rate gm/m^2	Monthly average temperature (0^0C)		Monthly average rain fall (mm)	Monthly average humidity (%)
		Maximum	Minimum		
Winter, 1995	11.3	25.3	11.3	8	66
Summer, 1997	13.3	33.0	21.1	82	65
Monsoon, 1997	7.8	33.3	24.5	249	76

Appendix 21. Rate of metal uptake by the *Spirulina platensis* biomass at different pH (50ppm concentration of lead nitrate solution).

Treatments	2pH	3pH	4pH	6pH
Control	0.480	0.483	0.465	0.450
Acid treated	0.459	0.350	0.325	0.294
Alcohol treated	0.417	0.225	0.094	0.074
Alkali treated	0.446	0.121	0.089	0.089

Appendix 22. Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH (50ppm concentration of lead nitrate solution).

Treatments	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	70 min.	80 min.	90 min.	100 min.
Control	0.415									
Acid treated	0.309	0.273	0.254	0.248	0.236	0.225	0.210	0.270	0.296	0.296
Alcohol treated	0.040	0.035	0.020	0.010	0.004	0.008	0.003	0.024	0.024	0.024
Alkali treated	0.078	0.056	0.046	0.032	0.032	0.031	0.031	0.066	0.088	0.088

Appendix 23. **Rate of metal uptake by the *Spirulina platensis* biomass in different concentration of lead nitrate solution at selected pH and selected incubation period.**

Treatment	50ppm	100ppm	150ppm	200ppm	250ppm	300ppm
Control	0.280	0.560	0.840	1.120	1.340	1.530
Acid treated	0.271	0.437	0.726	0.931	1.150	1.410
Alcohol treated	0.023	0.079	0.214	0.252	0.677	1.037
Alkali treated	0.040	0.174	0.410	0.523	0.890	1.144

Appendix 24. **Rate of metal uptake by the *Spirulina platensis* biomass at different pH (50ppm concentration of cadmium sulphate solution).**

Treatments	2pH	3pH	4pH	6pH
Control	1.236	1.228	1.220	1.212
Acid treated	1.238	1.242	1.330	1.185
Alcohol treated	1.196	0.850	0.806	0.798
Alkali treated	0.996	0.427	0.284	0.256

Appendix 25. Rate of metal uptake by the *Spirulina platensis* biomass after different incubation period at selected pH (50ppm concentration of cadmium sulphate solution).

Treatments	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	70 min.	80 min.	90 min.	100 min.
Control	1.212									
Acid treated	1.186	1.180	1.178	1.145	1.161	1.164	1.168	1.150	1.142	1.112
Alcohol treated	0.919	0.798	0.723	0.590	0.556	0.485	0.430	0.374	0.335	0.298
Alkali treated	0.368	0.271	0.240	0.210	0.190	0.190	0.170	0.164	0.164	0.164

Appendix 26. Rate of metal uptake by the *Spirulina platensis* biomass in different concentration of cadmium sulphate solution at selected pH and selected incubation period

Treatment	50ppm	100ppm	150ppm	200ppm	250ppm	300ppm
Control	1.317	1.353	1.438	1.540	1.590	1.6000
Acid treated	1.090	1.90	1.435	1.438	1.540	1.560
Alcohol treated	0.540	0.958	1.153	1.274	1.276	1.358
Alkali treated	0.306	1.048	0.962	1.190	1.283	1.384

Appendix. 27 Different characteristics of the species of the genus *Bacillus* (cell diameter < 1.0 μm)

Characteristics	1	3	4	6	7	8	10	11	13	14	15	16	17	18	19	20	21	22	23	26	27	28	29	30	31	32
Spores round	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	v	+	-	-	-	+	+
Sporangium swollen	-	+	+	+	-	+	+	v	-	+	-	+	+	+	-	-	+	+	-	+	+	+	+	-	+	+
Catalase	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	ND	+	-	+	+	+
VP test	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-
Acid from																										
D-Glucose	+	+	+	d	d	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	-	-
L-Arabinos	+	+	-	-	-	-	+	d	-	-	-	-	-	-	+	+	+	-	-	-	ND	+	-	+		-
D-Xylose	+	+	-	-	-	-	+	d	-	-	-	-	-	-	+	+	+	d	-	-	ND	+	-	+		-
D-Mannitol	+	+	-	-	-	d	+	d	+	-	-	d	+	+	-	+	+	+	-	-	ND	+	-	+		-
Gas from Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-		-
Hydrolysis of																										
Casein	+	+	+	+	+	+	d	d	+	d	-	+	+	-	d	+	-	-	d	d	d	+	-	+		d
Gelatin	+	+	+	ND	ND	+	d	-	+	+	-	+	d	-	d	+	+		+	+	+	+	-	+		d
Starch	+	+	+	-	-	+	+	+	+	d	-	-	-	-	+	+	+	+	-	+	-	+	-	-		-
Utilization of																										
Citrate	+	-	-	-	-	d	d	d	-	-	-	-	-	-	-	+	d	-	-	-	ND	-	-	+	-	d
Propionate	-	-	ND	ND	ND	ND	ND	-	-	ND	ND	ND	ND	ND	-	+	ND	ND	ND	ND	ND	ND	ND	-	-	ND
Tyrosine	-	-	d	+	+	+	-	-	d	-	-	-	+	-	-	-	-	-	ND	-	ND	-	-	-	-	-
Deam of PL	-	ND	-	-	-	-	-	-	d	+	d	-	-	-	d	-	-	-	ND	d	ND	-	-	-	ND	+
NO_3 to NO_2	+	-	-	-	-	d	d	d	d	d	-	d	+	-	d	+	+	-	d	d	+	+	-	-	+	-
Indole	-	-	+	-	-	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-	ND	-	-	-	-	-

(-) denotes 90% or more of strains are negative;(+) denotes 90% or more of strains are positive; (d) denotes 11-89%of strains are positive, v =strain instability;(ND) denotes no data available;(NG) denotes no growth.

Appendix 28 . Different characteristics of the species of the genus *Bacillus* (cell diameter > 1.0 μm)

Characteristics	5	7	9	12	15	23	24	25	34
Spores round	–	–	–	–	+	+	v	–	–
Sporangium swollen	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+
VP test	+	–	+	NG	–	–	–	+	d
Acid from									
D-Glucose	+	–	+	NG	–	+	+	+	+
L-Arabinos	–	–	–	NG	–	–	d	–	–
D-Xylose	–	–	–	NG	–	d	d	–	–
D-Mannitol	–	–	–	NG	–	–	d	–	–
Gas from Glucose	–	–	–	–	–	–	–	–	–
Hydrolysis of									
Casein	+	–	+	–	–	d	+	+	+
Gelatin	+	ND	+	–	–	+	+	+	+
Starch	+	–	+	–	–	–	+	+	+
Utilization of									
Citrate	d	–	+	–	–	–	+	d	+
Propionate	ND	ND	ND	–	ND	ND	ND	ND	ND
Tyrosine	d	+	+	–	–	ND	d	d	d
Deam of PL	–	–	–	–	d	ND	d	–	–
NO_3 to NO_2	+	–	+	–	–	d	d	+	+
Indole	ND	–	–	ND	–	–	–	–	–

(-) denotes 90% or more of strains are negative;(+) denotes 90% or more of strains are positive; (d) denotes 11-89%of strains are positive, v =strain instability;(ND) denotes no data available;(NG) denotes no growth.

Appendix 29. Effect of sun-dried *Spirulina platensis* extracts different solvents on the growth of isolated bacterial strains

Bacterial strain	Width of inhibition zone in mm.		
	water extract	methanol extract	ethanol extract
Bacillus subtilis	-	-	-
Bacillus subtilis	-	-	-
Bacillus alcalophilus	-	-	-
Listeria spp	-	-	-
Bacillus pumilus	-	-	-
Bacillus firmus	-	-	-
Bacillus badius	-	-	-
Bacillus pantothenticus	-	-	-
Bacillus alvei	-	-	-
Bacillus pumilus	-	-	-
Bacillus polymyxa	-	-	-
Bacillus lentus	-	-	-
Bacillus licheniformis		-	-
Brochothris spp	-	-	-
Listeria spp	-	-	-
Bacillus megaterium	-	-	-
Bacillus pumilus	-	-	-

Bacillus lentus	-	-	-
Bacillus macerans	-	-	-
Bacillus circulans	-	-	-
Bacillus lentus	-	-	-
Listeria spp	-	-	-
Listeris spp	-	-	-
Listeria spp	-	-	-
Bacillus macerans	-	-	-
Bacillus pumilus	-	-	-
Bacillus badius	-	-	-
Bacillus polymyxa	-	-	-
Bacillus sphaericus	-	-	-
Bacillus polymyxa	-	-	-
Bacillus brevis	-	-	-

Appendix 30. **Effect of storage period on protein of sun dried *Spirulina platensis* biomass.**

Year	Month	Protein content (%)	Monthly average temperature (O^0C)		Monthly average rainfall (mm)	Monthly average humidity (%)	Average protein content of one year (%)
			Minimum	Maximum			
1995	April	65.2	23.00	34.80	199	69	61.68 ± 1.6
	May	60.0	25.60	34.60	208	77	
	June	63.0	25.40	32.50	343	82	
	July	60.0	26.60	32.80	257	83	
	August	62.0	26.10	31.70	361	84	
	September	63.0	26.10	33.80	244	83	
	October	61.0	22.90	32.20	357	78	
	November	62.0	18.20	30.30	0	75	
	December	60.0	14.00	27.20	0	71	
1996	January	62.0	11.40	25.00	2	69	
	February	62.0	14.70	27.70	7	64	
	March	60.0	21.10	33.00	82	65	

Appendix 31. **Effect of storage period on Beta-carotene of sun-dried *Spirulina* biomass**

Year	Month	Percentage of Beta carotene (%)	Monthly average temperature (O^0C)		Monthly average humidity (%)	Average beta-carotene content of one year (%)
			Maximum	Minimum		
1997	March	0.208177	33	21.1	65	0.051 ± 0.0676
	April	0.137764	31	21.1	74	
	May	0.124889	33	24.5	76	
	June	0.038124	33.3	25.8	81	
	July	0.021302	31.6	26.3	85	
	August	0.019577	32.7	26.5	83	
	September	0.014868	31.5	25.4	86	
	October	0.011299	32.1	22.2	77	
	November	0.009769	30.4	18.8	74	
	December	0.009062	24.9	14.4	79	
1998	January	0.007087	28.8	12.6	77	
	February	0.004777	28.4	16.1	67	