# Chapter 11
# Engineering *Spirulina* for Enhanced Medicinal Application

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## 11.1 Introduction

Cyanobacteria are prokaryotes which can perform photosynthesis like higher plants. Their genomic organisation is very simple and thus is suitable for the study of detailed photosynthesis mechanism at a molecular level also for many other genomic manipulations relevant to benefit of living organisms. This unicellular alga, *Spirulina* has a thin thread like elongated structure and classified under *Cyanobacteriaceae* which is blue green in colour. Under microscope it looks like bunch of bright helical threads (Fig. 11.1).

This is also known as *Arthrospira platensis*. Their odour and taste is similar to seaweed. They grow well in fresh water like ponds, lakes and rivers. Pollution-free medium with abundant sunlight and moderate temperature is favourable for growth of *Spirulina*. However, they can also sustain and adapt harsher environmental condition. *Spirulina* can be referred as a complete nutritional food supplement, being a rich source of many important nutrients, including protein, carbohydrates, iron and vitamins like A, K and B complex. Most of the dry weight of this unicellular alga is protein which is very essential for growth and regeneration. It is a better substitute for meat and dairy products which are rich in fatty acid and cholesterol. A few grams of *Spirulina* intake can fulfill our everyday requirement of protein, iron and vitamins, especially vitamins A in form of beta carotene, B complex, D and K. *Spirulina* though contains no vitamin C, but it helps maintain potency of vitamin C. *Spirulina* has great antioxidant properties incurred by its yellow xanthophyll content. Thus, *Spirulina* can be a wise option to be used as a dietary supplement to maintain good health and resist several diseases.

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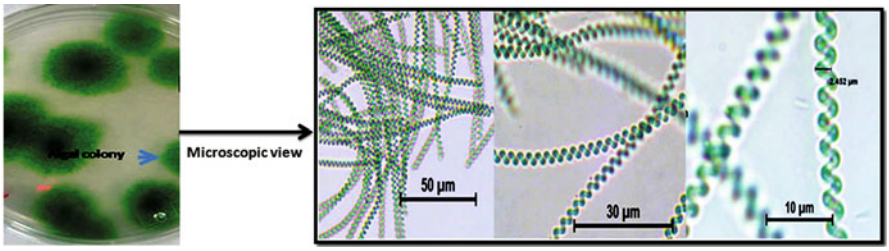


**Fig. 11.1** *Spirulina platensis* under light microscope at different magnification.

*Spirulina* can be genetically engineered for many practical purposes, but neither any stable gene transfer system nor any suitable expression vector has been established so far. Salvi et al. (1994) are the pioneers of gene transfer studies in *Spirulina* and concluded that identification of a suitable restriction modification system can accelerate the progress in this direction. Kaneko et al. (1996) sequenced the whole genome of *Synechocystis* sp. PCC 6803, a unicellular cyanobacterium which was followed by whole genome sequencing over 40 strains of cyanobacteria. Some nitrogen-fixing filamentous species, and some unicellular species, which are either nitrogen fixing or non-nitrogen fixing are to name among them. Only recently in 2010, Fujisawa et al. first sequenced the filamentous non-nitrogen-fixing species viz, *A. platensis*.

Sequencing of whole genome opened up the way to use genes from higher plants as probes (Amao and Nakamura, 2006) to identify and clone cyanobacterial genes involved in photosynthesis. Subsequently, many cyanobacterial photosynthetic mutants were possible to create by genetic manipulations conducted by Ruengjitchatchawalya et al. (2002) and Cao et al. (1999). They created some genetically engineered strains which can efficiently produce organic substances. One advantage of cyanobacteria is that they use solar energy, $CO_2$, $H_2O$ and inorganic substances as efficiently as higher plants. This feature can be exploited for production of useful organic products with minimum expenditure of resources and energy. A few decades ago Kuhlemeier et al. (1981) reported some expression vectors for *Anacystis nidulans* R2. Later, Kurdrid et al. (2011) developed cyanobacterial host vector systems to analyze the expression and structure function relation of the genes. One major advantage of *Spirulina* is its simpler growth requirements than other bacteria and is nonpathogenic. Therefore, the mass culture of *Spirulina* sp. can be performed efficiently (Vonshak, 1997). Because of these characteristics, many attempts to express foreign genes in *Spirulina* sp. have been made. One enzyme called human carbonic anhydrase, responsible for inter conversion of carbon dioxide and bicarbonate to maintain acid-base balance in blood and other tissues and removing extra carbon dioxide out of tissues and another called Mn superoxide dismutase from *E. coli* were cloned and expressed by Cao et al. in cyanobacteria. In

this chapter an effort has been made to summarise ongoing global research on the most efficient conditions for expression of the valuable genes of *Spirulina* sp. in other organisms and also research on how genes from other organisms can be cloned and expressed in *Spirulina*. The problems in engineering *Spirulina* and its probable solution have also been discussed.

## 11.2 Amazing Potential of *Spirulina* sp.

Algae are important, innovative solution for many health problems. They are also known to promote regular functioning of major physiological systems, including the immune system, humeral system, cardiovascular system, respiratory system, and nervous system (Edwards, 2013). Algal anti-inflammatory properties have been explored to produce several natural products in indigenous cultures as a sun protectant, a moisturising agent and as a treatment for wounds, burns and bruises. *Spirulina* offers an endless number of possibilities for enhancing health and wellness. Recent research has even suggested this algae as a therapeutic solution for a number of serious conditions, including diabetes, arthritis, heart disease, autoimmune diseases, and cognitive decline, in the form of dementia and Alzheimer's. Some biomolecules of *Spirulina* have been shown to stall the spreading of the HIV and SARS virus (http://newhope360.com). It also enhances natural cleansing and detoxification, improves gastrointestinal and digestive health, reducing cancer risks with antioxidant protection. In conclusion, there are an endless number of medical and health applications for algae. The naturally derived substance has broad implications for the prevention and/or treatment of a wide range of diseases, conditions, and minor ailments. The other economic value of these algae includes use of technologies in the area of energy production and pollution abatement. Health Enhancement Products, Inc. (HEPI) recognizes the benefits of algae, and strives to optimise the value of this natural product by conducting research and development on its vast capabilities. The company promotes overall health and wellness by seeking effective solutions for the most pressing medical issues faced by people today.

### *11.2.1 Healthy Dieting with* Spirulina *sp.*

*Spirulina* contains a very valuable compound called γ-linolenic acid or GLA. This is also found in human breast milk that contributes to generate strong immune system in new born babies. This microalga can be digested easily and is a rich source of several vitamins and nutrients. This is a great option to resist malnutrition in children and women. It helps the body absorb essential nutrients when it has lost its ability to absorb common forms of food. *Spirulina* favours growth of beneficial

microorganisms like *Lactobacillus* and *Bifidobacteria* in human digestive tract to promote healthy digestion and proper bowel function while reducing harmful microorganisms like *E. coli*, *Candida* and yeast. Beneficial microorganisms increase absorption and assimilation of nutrients from the foods we eat, and helps to protect against infection. It also acts as a natural cleanser by removing mercury and other lethal toxins ingested by the body. *Spirulina* also increases stamina and immunity levels in athletes, and its high protein content helps to build muscle mass. *Spirulina* can effectively curb the appetite of athletes and soldiers during their vigorous training schedules helping them to maintain their right body weight and immunity.

### *11.2.2 Disease Prevention by* Spirulina

Other than iron and beta-carotene, *Spirulina* contains many other micro-nutrients like copper, chromium, zinc, selenium and manganese. Human body generates a lot of toxic free radicals which react with these metals and subsequently eliminate from the body during excretion. A unique combination of phytonutrients viz. chlorophyll, phycocyanin and polysaccharides exists in *Spirulina* which makes it naturally suitable to fight many diseases. Some molecules which may harm human system and are absorbed by the body through pollution, poor diet, injury, or stress may also be eliminated with the help of these micronutrients. By removing free radicals, the nutrients help the immune system fight cancer and cellular degeneration. In some experimental findings, *Spirulina* sp. was found effective in regression of oral cancer in laboratory animals, and may pave way in cancer treatment. *Spirulina* sp. also can reduce low density lipoproteins (LDL) which are associated with severe cardiovascular disorders like atherosclerosis and strokes. LDL sticks to inner wall of the arteries and get hardened thus blocking the normal blood flow. Spirulina can lower the LDL accumulation in the body and helps prevent the many cardiovascular problems. It also helps lower blood pressure. *Spirulina* sp. also acts as antihistaminic component.

### *11.2.3 Anti-Aging with* Spirulina *sp.*

*Spirulina* being rich in various antioxidants prevents aging. *Spirulina* contains concentrated form of beta-carotene which is essential for eyes. *Spirulina* is rich in iron, magnesium and trace minerals, and these minerals get easily absorbed by the body than common iron supplements found in the market. *Spirulina* is the highest source of vitamin $B_{12}$, which is essential for healthy nerves and tissue, especially for vegetarians. In Table 11.1, the nutritional values of *Spirulina* and its medicinal importance are summarised.

**Table 11.1** Potential of *Spirulina* at a glance

| | Spirulina a rich source of nutrition | | Top 10 causes to engineer Spirulina for enhanced medicinal application |
|---|---|---|---|
| 1 | Protein content 65–71 % by dry weight, and contains all the essential amino acids except histidine. | 1 | Strengthens the immune system |
| 2 | It contain more than 13 types of minerals It has relatively high concentrations of K, Ca, Zn, Mg, Mn, Se, Fe and P. | 2 | Supports cardiovascular health and lowers cholesterol. |
| 3 | Vitamins $B_1$, $B_2$, $B_6$, $B_{12}$, E, biotin, pantothenic acid, folic acid, inositol and niacin are present. It is the richest vegetative source of $B_{12}$ in the world. | 3 | Improves gastrointestinal and digestive health. |
| 4 | Carotenoids present in the form of alpha-carotene, beta-carotene, xanthophylis, cryptoxanthin, echinenone, zeaxanthin and lutein. | 4 | Enhances natural cleansing and detoxification. |
| 5 | Pigment such as chlorophyll is found in great abundance in *Spirulina* along with phycocyanin and porphyrin. | 5 | Reduces cancer risks with better antioxidant protection. |
| 6 | *Spirulina* contains very little carbo-hydrates, and about 3.9 Kcal $g^{-1}$. There is also very little Na, which is important for some people. | 6 | Provides complete daily nutrition need for the body. |
| | | 7 | Promotes body metabolism. |
| | | 8 | Neutralizes body acidity. |
| | | 9 | Inhabits growth of cancerous cells. |
| | | 10 | Reported to combat HIV attack. |

## 11.3 Structural Features of *Spirulina* sp. Genome

A single closed circular genome of about 7.0 Mbp length is found in this cyanobacteria. However, no plasmid DNA has been sequenced so far. In general, the bacterial chromosome containing circular DNA sometimes contains uneven distribution of G and C bases on both strands; this can be detected by G-C skewing which help to locate ORFs and termination of DNA replication. In case of *Spirulina*, any such apparent shift was detected by this analysis unlike other cyanobacteria. *Spirulina* genome comprises 6630 potential protein coding genes, 49 RNA genes which consist of two sets of rRNA genes, 40 tRNA genes which code for tRNA, tmRNA, β subunit of RNAse P and signal-recognition particle RNA. Among the protein coding genes $3/4^{th}$ are of known function but no functional characterisation was done for about 1500 genes. Most of these genes are responsible for general cyanobacterian metabolism but the most strikingly the biosynthesis genes for cyanotoxins such as alkaloid toxins (anatoxins and saxitoxins), non-ribosomal peptide toxins (microcystins), urea-derived toxins (cylindrospremopsin) and ribosomal depsipeptide toxins (microviridins) were absent in the genome, which logically explains the use of

*Spirulina* as a food since long. The genes for photosynthesis viz, psa X for photosystem I, cytochrome c550-like genes and genes for cytochrome b6, ATP synthase and NDH are detected as in other cyanobacteria. Although there are two copies of cytochrome c6 gene, any gene coding for electron carrier proteins in the thylakoid lumen such as plastocyanin and cytochrome Cm is not yet characterized.

### *11.3.1 Important Genes in* Spirulina *sp. Genome*

One very unique feature of *Spirulina* is its ability to adapt in new or unfavourable environmental condition as in case of high salt concentrations. They have well equipped regulatory mechanism consisting of cAMP signalling cascade which helps them adapt in response to changes in the external environment (Ohmori et al., 2009). *Spirulina* develops a number of diverse cAMP- dependent signal cascades to adapt to different severe environmental conditions (Kashahara et al., 2001; Ohmori and Ohmori, 2002) as was revealed by comparative analysis of genes coding for adenylate cyclase. Amao and Nakamura (2006) highlighted the potential of *Spirulina* for production of biofuels due to presence of hydrogenase enzyme.

### *11.3.2 Lipid and Carotenoid Producing Genes*

Undoubtedly *Spirulina* is the richest source of beta-carotene containing about ten different kinds of carotenoids. Most important of them are alpha, beta and gamma carotenes. They also contain diverse variety of yellow xanthophylls. They work together to protect our body from antioxidants and carcinogens. Synthetic beta-carotenes do not always show these benefits. The reason is that natural one is easily assimilated and contains cis isomer, which is lacking in synthetic beta-carotenes.

Kerfeld et al. (2003) and Wilson et al. (2006) did in-depth comparative analysis of all known cyanobacterial genes involved in carotenoid biosysnthesis and concluded that *Spirulina* contains all of them excluding genes for β-carotene ketolase. Interestingly they found a product of ketolase viz, 3-hydroxyechinenone, to bind to an orange carotenoid protein during energy dissipation from phycoliposome in photosystem II. Therefore, it is possible that an unknown type of ketolase may be present in the genome. All known cyanobacterial genes involved in biosysnthesis of fatty acids, vitamin E, lipoic acid, glycerolipids, lipo-polysaccharides and polyhydroxy alkanoates were detected by them. However, a gene for ω-3 fatty acid production desatyrase (desB) was consistently missing in *Spirulina* as evidenced by the biochemical analysis (Murata et al., 1992).

### *11.3.3 Prevention of Reactive Oxygen Species Formation by* Spirulina

*Spirulina* harbours several genes encoding enzymes against reactive oxygen species. Genes for peroxiredoxin, thioredoxin peroxidase, and other putative peroxidases include five genes for bacterioferritin co-migratory proteins. However there was no evidence of gene for enzyme catalase. In a laboratory experiment by Pak et al. in 2012 using animal models it was revealed that several symptoms like increase in production of reactive oxygen species from liver mitochondria, activation of NF-kappa β, and imbalance in lymphocyte surface antigen ratio was significantly abated by administration of *Spirulina* and phycocyanin. It confirmed the therapeutic property of *Spirulina* sp. or phycocyanin that can inhibit the inflammatory response through anti-oxidative and anti-inflammatory mechanisms, breaking the crosstalk between oxidative stress and inflammation. Phycocyanin and phycocyanobilin from *Spirulina platensis* also protect against diabetic nephropathy by inhibiting oxidative stress. Zheng et al. (2012) have reported that bilirubin and its precursor biliverdin may have beneficial effects on diabetic vascular complications, including nephropathy, via its antioxidant effects. They investigated the role of chromophores viz. phycocyanin and phycocyanobilin obtained from *Spirulina platensis* and its chromophore in oxidative stress and renal dysfunction in diabetic rodent model.

### *11.3.4 Gene Transfer into* Spirulina *sp.*

The biggest limitation in engineering any beneficial gene into *Spirulina* is lack of complete understanding of the restriction modification system of this cyanbacterium, high polysaccharide content of genomic DNA and excessive methylation. Genomic DNA of some blue green alga obtained through cesium chloride gradient purification and ultracentrifugation is poorly digested while using common restriction enzymes. But digestion with these enzymes along the whole length of genome at specific restriction sites as in other microbes cannot be assured due to interference of excessive DNA methylation and polysachharide content.

One alternative strategy was proposed successfully by Promega Inc. which involves no restriction digestion of the genomic DNA. DNA is first extracted by incubating the cyanobacterial cells at 37 °C for 1 h with 10 mg $mL^{-1}$ lysozyme (Sigma) in 50 mM EDTA followed by addition of nuclei lysis solution. Briefly the procedure for genomic DNA library preparation involves sonication of g-DNA, blunting of the overhangs with exonuclease digestion, adding adenine nucleotide adapter at 3′ends using DNA-Taq polymerase enzyme and finally ligation into the pGEM ®-T Vector System. This method is called “T-vector method” where genomic DNA from *Spirulina platensis*, a typical filamentous cyanobacterium that is well known for the resistance of its DNA to restriction enzyme digestion has been used.

### *11.3.5 Vectors Used for Cloning in Cyanobacteria*

One of the crucial criteria in identifying suitable vector construction is its stability of exogenous DNA expression in microalgal expression systems. With the rapid advances of genome sequencing technologies, gene sequences of various microalgae was published and large-scale approaches have been developed to utilize the sequence information into functional information. Generally, function of any gene can be derived using various approaches, such as analysis of gene expression pattern by promoter activity assay, gene silencing, ectopic expression, structure-function analysis, protein sub-cellular localization examination, and *in vitro* or *in vivo* biochemical assays (Kumar and Hirochika, 2001; Curtis and Grossniklaus, 2003). The conventional method for engineering an expression vector construct depends on the restriction digestion of a suitable DNA fragment followed by ligation. This method is time consuming and is dependent on availability of appropriate restriction sites. This is a significant technical barrier for large-scale functional gene analysis studies. Recently, the Gateway cloning system has been developed to facilitate large-scale production of gene constructs, and it is able to achieve rapid cloning of one or more genes into multiple destination vectors using site-specific recombination. The recombination cloning system is based on a two-step process (Earley et al., 2006). Firstly, the desired DNA fragment is cloned into a general donor vector by "GATEWAY" – BP reaction. Then, the DNA fragment flanked by two site-specific recombination sites (attB1 and attB2) in the donor plasmid can be transferred precisely into a variety of expression vectors by site-specific recombination reactions. Once the DNA product is targeted into a donor vector, the transfer of the DNA constructs into an expression destination vector which becomes simple and requires no traditional restriction enzyme/ligase cloning. This Gateway technology have been widely used in the research community, and many Gateway-compatible open reading frame clone collections and expression plasmids have been created for functional genomic analysis in many organisms.

Two plasmids were constructed by Kuhlemeier et al. (1981), recombining the *E. coli* vector pACYC184 and the cyanobacterial plasmid pUC1. These recombinants plasmids, designated as pUC104 and pUC105, could be transformed to *E. coli* K12 as well as to the cyanobacterium *Anacystis nidulans* R2 and in both hosts they expressed their antibiotic markers. pUC104 and pUC105 differ with respect to the location and the orientation of the pACYC184 segment in pUC1. pUC104 was tested to be more stable in different environmental conditions. Transformation of pUC105 to *Spirulina* was done successfully with antibiotic resistance marker for chloramphenicol.

Another shuttle vector was constructed by introducing multiple cloning site of plasmid pUC18 between EcoRI and HindIll sites of pBR322 to produce pBR322M. Then a 4.4-kb BamHI-XhoI fragment of pBA1 that originated from *A. nidulans* 6301 was ligated to the 2.5-kb Pvu II Eco47III fragment of plasmid pBR322M and the ends were filled with T4 DNA polymerase. An ampicillin resistant marker was also introduced to give rise pBAX18R.

### *11.3.6 Methods for Transformation into Microalgae*

So far, microalgae have been successfully transformed through various transformation methods. The popular transformation methods are biolistic transformation and electroporation. In some cases, transformation resulted in the successful and stable expression of transgenes from either the nucleus or the plastid, but in most cases, only transient expression was observed. So far lot of research has been performed to increase the lipid yield of microalgae through optimization of growth and induction conditions, such as temperature, light intensity and duration, salt concentration, and nutrient requirement, while genetic modifications of microalgae to alter quantity, quality or composition of lipid are reported less. The main reason is probably the lack of a generally applicable transformation protocol for microalgae. Since microalgae are such a diverse group of organisms, it cannot be guaranteed that a method that works for one species could be applied to another one. Some antibiotics routinely used in the transformation of plants, such as kanamycin and zeocin, have been successfully used as markers in cyanobacterial gene manipulation. Also, heterologous gene expression in microalgae demands for preferential codon usage and appropriate promoter sequences to modulate each expression. This can be achieved by getting information on completely annotated and sequenced microalgal genomes. Any protocol for the genetic transformation of a microalgal strain needs to be customized to meet its specific requirements and overcome corresponding limitations.

## 11.4 Studies on Cloning, Structure and Function of Extrachromosomal DNA in *Spirulina* sp.

Cao Xue Cheng et al. (2005) described an efficient protocol for the extraction and purification of extrachromosomal DNA (exDNA) from *Spirulina platensis* strains, such as Sp-HO1, Sp-D, Sp-S, Sp-T etc. This method was commonly described as CTAB-Proteinase K method. The homology of several exDNAs, as well as between exDNA and chromosomal DNA was analyzed by Southern blotting. Partial sequences of exDNA from Sp-S were cloned and the primary and secondary structures, open reading frames (ORFs) of the cloned fragments, and function of proteins encoded by ORFs were analyzed through bioinformatics analyses. Briefly the CTAB method includes traditional method of cell lysis while adding Proteinase K during crude DNA extraction and purification by phenol-chloroform-isoamyl alcohol. In this way proteinase K helps removing protein contaminants and improves DNA quality. This method was successfully used to extract extrachromosomal DNA from more than ten strains of *Spirulina*. The results suggested that different strains of *Spirulina* contains exDNA of different size, e.g. strain HO1 exDNA has a length of 0.75 kb whereas strain D, J and Z has that of approximately 1.1 kb. Sp-S has two exDNA: shorter one is 1.8 kb long and longer one is 3.6 kb long. Same is the case for Sp-T. Although the longer one is double the length of shorter exDNAs in above

two strains 2D gel electrophoresis revealed no new bands and confirmed that they were two independent strains. However, these DNAs were not abundant and difficult to isolate and purify from total DNA. The results revealed that three methods viz. freeze-thaw extraction, DNA gel extraction kit and alkaline method, can be used for exDNA purification. However, the freeze-thaw extraction method is the best one. It is with recovery efficiency over 90 %. Molecular characteristics of exDNA in *Spirulina* through studies on the genetic stability, probable molecular conformation and restriction enzyme patterns of the exDNA, it was found that these DNAs were stable.

### *11.4.1 Analysis of Restriction Endonuclease Digestion of Genomic DNA from* Spirulina *sp.*

The modification of the CTAB extraction procedures (Poberski et al., 1997) includes adding proteinase K to lyse cells, high NaCl concentrations in the buffer to remove polysaccharides and an additional proteinase K digestion of crude DNA extracts to remove any excess proteins. The CTAB-proteinase K method is efficient to prepare digestible genomic DNA from *S. platensis*. Subsequently it was established that the genomic DNA can be digested by more than 12 restriction enzymes. The proper reaction condition involves an enzyme concentration of 5 U $g^{-1}$ DNA, and a digestion time of 4 h.

### *11.4.2* Spirulina *Cloning and Expression Analysis of the Serine/Threonine Kinase Gene Family*

Serine/threonine kinases (STKs) have been found by Qin Song et al. (1993) in an increasing number of cyanobacteria, showing its important roles in signal transduction. Their work aims at molecular cloning and functional elucidation of a putative STK sequence in *Spirulina*. Ongoing *Spirulina platensis* genome project offers us a wealth of information concerning the sequences and organisation of STK gene family in *Spirulina platensis*. Thirty three putative STK homologues were identified in *Spirulina platensis* draft map. Motifs and invariant amino acids typical in eukaryotic STKs were conserved well in these proteins. These STK proteins were classified into three major families according to their domain structures. Their research provides a fundamental clue for further study of signal transduction system in *Spirulina platensis*.

## 11.5 Important *Spirulina* Genes Cloned in other Organisms

Besides this, several genes obtained from *Spirulina platensis* were cloned in other organisms by several researchers. Tiboni et al. (1984) cloned the genes for the large and small subunits of ribulose-1,5-bisphosphate carboxylase from *S. platensis* in *E. coli*. Sanangelantoni et al. (1990) also cloned the gene for ribosomal S2protein and a part of the gene responsible for the peptide elongation from *S. platensis* in *E. coli*. Riccardi et al. (1991) constructed the genomic library of *S. platensis* DNA using lambda EMBL3 vector. After that, they cloned the acetohydroxy acid synthase gene from this recombinant library in *E. coli*.

Bini et al. (1992) cloned the gene for β-isopropylmalate dehydrogenase of *S. platensis* (*leuB*) from a λEMBL3 genomic library by heterologous hybridization using the *Nostoc* UCD 7801 *leuB* gene as a probe. Salvi et al. (1994) identified, cloned, characterized and expressed the gene encoding serine esterase from *S. platensis* in *E. coli*. Kawata et al. (1998) used TA cloning vector for construction of a genomic DNA library. In their studies, the researchers cloned the gene coding phytoene synthase from *S. platensis* in *Synechococcus* and *Synechocystis*. Lui et al. (2005) cloned one operon named C-phycocyanin from *Spirulina platensis* into pMD18-T followed by its sequencing and genomic characterization. Zhang et al. (2005) cloned and characterized the partial *hoxH* genes encoding large subunit of nickel-iron hydrogenase of two cyanobacterial genera, including five strains of *Arthrospira* and two strains of *Spirulina* in *E. coli*.

Several investigations have been studied on the acyl-lipid desaturases genes. Meesapyodsuk et al. (2001) cloned the *desC* gene from *S. platensis* in baker's yeast and thus, the cyanobacterial gene product appeared to be functional in yeast. Apiradee et al. (2004) cloned and successfully expressed for the first time the genes encoding the acyl-lipid desaturases (desC, desA, desD) which are involved in γ-linolenic (GLA) synthesis in *E. coli*. Later, Kurdrid et al. (2005) cloned, expressed and characterized the *desD* gene in *Sachharomyces cerevisiae*.

Buttarelli et al. in 1989 sequenced the 5.3 kb DNA segment containing the *str* operon of *S. platensis*. Kasahara et al. (1997) sequenced the *cyaC* gene encoding an adenylate cyclase of *S. platensis*. Kawamura et al. (1986) have purified three restriction endonucleases from *S. platensis* subspecies *siamese* and named them SplI, SplII and SplIII respectively. Milano et al. (1992) demonstrated the two isoenzymatic forms of the enzyme acetohydroxy acid synthase (AHS), which catalyse the first common step in the biosynthesis of isoleucine, leucine and valine in *S. platensis* and they sequenced the genes *ilvX* and *ilvW* encoding these two enzymes. Tanioka et al. (2010) characterized cobalamin-dependent methionine synthase to study the physiological function of pseudovitamin 12 B or adeninyl-cobamide in *Spirulina platensis* NIES-39 and finally cloned the full-length *Spirulina* MS. Linjawi (2011) investigated the protective effect of *Spirulina* against mitomycin C (MMC)-induced genotoxic damage in male rats and suggested that *Spirulina* exerts its anti-mutagenic properties by inhibiting alterations in the gene expression.

### 11.5.1 *Cloning, Sequencing and Over-Expression of* Spirulina *Phycocyanin Gene*

Phycobiliprotein is an important light-harvesting pigment-protein available in cyanobacteria (*Cyanophyceae*), red algae (*Rhodophyceae*), the hidden of algae (*Cryptophyceae*) and a few dinoflagellates (*Pyrrophyceae*). According to the different composition and absorption spectra, the phycobiliproteins are generally divided into three categories: the phycocyanin (phycocyanin, referred to as the PC, the absorption spectrum in the 610–640 Nm), the phycoerythrin (phycoerythrin, referred to as PE, absorption spectra in the 500 to 570) and allophycocyanin (allophycocyanin, referred to as the APC, absorption spectra in the 650–671 Nm). These proteins can be used as industrial raw materials or food additives. Phycobiliprotein is widely used in the food industry, chemical industry, medical diagnosis, and clinical medicine. In addition, many kinds of phycobiliproteins, fluorescent, high quantum yield, easy combination of a number of proteins with biotin antibodies, phycobiliprotein acts as a fluorescent probe. Phycobiliprotein can be isolated and purified from *Spirulina. Spirulina* farming difficulties, its outdoor large-scale cultivation of the lack of systematic and comprehensive study, and high farming costs are expensive. So, phycobilisome protein from *Spirulina* sp. is not only the high cost but also complex process.

The genetic engineering methods for the production of phycobiliprotein has played an important role. Qin Song (2008) showed that the *Spirulina maxima* phycocyanin gene is 1119 bp having 99 % homology with *Spirulina platensis* phycocyanin gene. The β subunit gene sequences in the alpha subunit gene sequences upstream connection between 111 bp fragment of the gene, β subunit and α-subunit gene contained 519 bp and 489 bp, encoding 172 and 162 amino acid residues. Possible ribosome binding sites in the upstream of the gene sequence of the β subunit 8–11 bp exists at Gaga the binding site and a prokaryote ribosome binding sites usually ggaa similar, but not identical. *Spirulina maxima* phycocyanin three chromophore binding sites are the α subunit amino acids-Cys 84 and β subunits of amino acids-Cys82 and Cys153. The third chromophore binding site Cys153 located in the carboxy terminus of the β-subunit, has proved that this binding site is due to the subunit 12 amino acid residues (146–157). The phycocyanin contains many hydrophobic amino acid residues which play an extremely important role in phycocyanin gathering process.

### 11.5.2 *Cloning of RuBisCO*

Tiboni et al. (1984) cloned the genes for two subunits of ribulose-1,5-bisphosphate carboxylase from filamentous cyanobacterium, *S. platensis* into *E coli*. Those genes were found located very close having a total length of 4.6 kb. The amount of large subunit produced in the bacterial host represents at least 10 % of the total extracted protein.

### *11.5.3 Cloning and Characterization of* Spirulina *Esterase*

Sergio et al. (1994) identified a 23 kDA gene known to code for a serine esterase from the cyanobacterium *Spirulina platensis*. It was cloned and expressed in *Escherichia coli* before functional characterization. DNA sequencing studies revealed the primary structure of the esterase which had partial similarity with the carboxyl-esterase (esterase II) encoded by *estB* of *Pseudomonas fluorescens.* Notably, the highest degree of homology was found in a stretch of 11 identical or highly conserved amino acid residues corresponding to the GXSXG consensus motif found in the catalytic site of many other esterases, lipases and serine proteases.

### *11.5.4 Generation of Mutants in* Spirulina

Mingyue Fang et al. in 2013 tried to improve the carbohydrate yield of *Spirulina platensis* by generation of mutants with increased growth rate and carbohydrate content. They constructed a mutant library of *S. platensis* with diverse phenotypes using a new high throughput rapid mutagenesis technique at room temperature. The screening of the mutants was performed in the 96-well microplate and identified with microplate reader. The mutants which were stable even after several subcultures were considered ideal and selected. The mutants in mutant library showed diverse phenotypes in terms of cell growth rate, carbohydrate content and flocculation intensity. This mutagenesis method was established to be an effective tool to generate the mutant library for multicellular microalgae.

### *11.5.5 Use of Transposons for Cloning in* Spirulina *sp.*

The oxidative photosynthesis of microalga is well known for its ability to reduce carbon dioxide emissions. This feature is employed industrially for wastewater treatment. Mass cultivation and extraction of useful substances from the microalgae are also in practice. Kawata et al. in 2004 developed artificial transposon systems by extracting essential elements from natural transposons. They mutated transposase and transposon complex system which improved the transformation efficiency by electroporation. They used Tn5, a natural transposon, transposase and cation liposome complex by electroporation to improve transformation efficiency for *Spirulina platensis* -C1 (Arthrospira sp. PCC9438) and selected the cloned cells growing in 2.0 μg $mL^{-1}$ chloramphenicol containing medium. This genetically modified *Spirulina* have the potential for industrial wastewater treatment and production of useful chemical materials.

## 11.6 Challenges in Engineering *Spirulina* sp.

Because of its several benefits, studies on cyanobacteria have been increased all over the world. Especially during the past few decades, they have been used to understand photosynthesis and genetic expression controlled by photoregulation, cell differentiation, $N_2$ fixation, metabolism of carbon and hydrogen, resistance to environmental stress and molecular evolution (Koksharova and Wolk, 2002). Recently some cloning vectors and other genetic tools have been developed for cyanobacteria. Transformation, electroporation and conjugation techniques are being used for gene transfer studies. Mutant strains for specific genes have been developed by mutagenesis also. Complete genomic sequences of some strains have been obtained and some genomic sequence projects are under way. Condition of efficient electroporation into *Spirulina* was studied by Toyomizu et al. (2001b). The plasmid pHSG399 was transferred into *Spirulina* using electroporation technique and suggested the best electroporation condition is 5.0 ms pulse duration with an electric field of 4–8 kV per cm. On the other hand, Kawata et al. (2004) transferred Tn5 transposon, transposase and cation liposome complex into *Spirulina* and suggested 5.0 ms pulse duration with an electric field of 7.5 kV per cm.

## 11.7 Some Harmful Effect of *Spirulina* sp.

Although *Spirulina* is a suitable organism for producing recombinant proteins, there are not much researches going on gene transfer studies on *Spirulina platensis*. Despite of having very high nutritional value, this alga may also cause some side effects to some individuals including allergic reactions like rashes, hives, and difficulty in breathing. Some commercial *Spirulina* supplements may contain toxic substances as additives. It is, therefore, absolutely necessary to purchase *Spirulina* only from authentic sources. People with the serious metabolic condition phenylketonuria (PKU) cannot metabolize phenylalanine, and therefore, should not consume *Spirulina*. Similarly, this is not advisable for people with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. The *Spirulina* may also antagonise the effectiveness of certain medicines, such as prednisone, which are commonly used for treating asthma and other inflammatory diseases. Although *Spirulina* is apparently safe at larger doses, but doctors may advice the right dosage.

## 11.8 Conclusion

Use of seaweeds and other algae in oriental countries is an old practice as a remedy to cure or prevent various physical ailments. Several researchers have tried to establish a connection between these nutrient-rich sea plants and the body's immune system response. Intensive studies have started to identify potential sources of

pharmacologically active agents. The mechanisms through which these algae have a preventive role include reduction of plasma cholesterol, binding of bile steroids, lipids, antioxidant activity, prevention of carcinogenic effect, binding and complement the significant pollutant trace elements in the diet. Since this microalga are non-toxic and have immense nutritious value to human and live stocks, *Spirulina platensis* is considered one of the most commercially important species among microalgae. It contains protein, carbohydrates, minerals, chlorophyll *a*, phycocyanin, vitamin 12B, β-carotene and essential fatty acids like γ-linoleic acid for human and animal nutrition. So, its production and consumption are increasing every year. The production is over 2000 tons per year. Some countries such as China, South Africa, Japan, Mexico, Australia and Chile are leading countries on production of *Spirulina*.

The research on stem cells is in vogue because of its potential application in repair and regenerative medicine. Some researchers have investigated the effects of *Spirulina* on stem cell growth and proliferation and revealed that this cyanobacteria has the potential to stimulate endogenous stem cells leading to healing and regeneration. Sequencing studies on *Spirulina* genome have helped to decipher functional role of many genes which can be exploited to produce useful neutraceuticals. The lack of suitable restriction modification system and expression vectors limits gene transfer into *Spirulina*; however several alternate strategies are coming up and construction of complete genomic library will help to understand structure function relationship at genomic level leading to engineering *Spirulina* for medicinal purposes. Recently, Verseux et al. (2015) indicated the potential of *Spirulina* in International Space Research stations. *Spirulina* can adapt itself in unfavourable environmental conditions like high salt concentration, adverse temperature and pH of medium where not much bacterial species can survive. So, pure cultures of *Spirulina* can be maintained easily. Cultivation of *Spirulina* will also help to enable environmental purification during its photosynthetic process. Thus beneficial effect of this cyanobacterium can be utilized in space too as in earth.

*Spirulina* can be obtained from commercial sources for human consumption but most of them are cultivated in laboratory. Some unavoidable toxic contamination may happen during preparation of tablet or powder form of *Spirulina* and unfortunately that cannot be removed during downstream processing of large scale production. Genetically improved variety of this cyanobacterium may eventually overcome this challenge in future.

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