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Spirulina, The importance and Significance of Cloning

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MANY items have impacts on recombinant protein production in making it affordable, the most important of which is host with its characteristics such as compatibility, yields, expression system, costs, environmental effects, etc. Humans have been using spirulina (*Arthrospira sp*.) algae for centuries. It enjoys features which propose it as a golden host cell. Although several researches have been conducted to develop a stable and reliable transformation system, only a few projects meet the need. The aim of current paper was to focus on spirulina transformation researches. So reason to select the host and procedures to optimize a transformation system were given.

Keywords: *Spirulina*, Arthrospira, Algae, Cloning, Genetic engineering.

Introduction

The first documented use of algae by humans was by the Chinese, dating back to 2,000 years ago. They used algae to survive during famine. However, the biotechnology of algae has been developing since the middle of the last century [1]. The biotechnology of algae is in the embryonic stages of its scientific advancement, producing strains that grow faster and produce high levels of cell masses, as well as strains that require cheaper food sources and at the same time yield more crops serving as one of the major goals of micro algae genetic engineering[2,3]-. Many items affect costs and yields of the recombinant protein production. To achieve an optimal production system, some issues should be considered: (i) host organism (ii) expression vector (iii) characteristics of regulatory elements, such as promoters or terminators (iv) appropriate condition like growth and production medium for the host cell (v) compatibility of foreign protein (vi) purification strategy [4]. Table 1 lists the types of systems available to produce a biotechnology product and compares the factors affecting them

Considering the two manipulated micro algae, the Chlorella and Spirulina are good candidates for the expression and production of foreign proteins due to their high protein content.

Introducing Spirulina
Spirulina (Spirulina spp.)

Humans have been using spirulina algae for centuries. Spirulina has been commercially produced in the last 20 years and has been used as a supplement in the human diet and as dietary supplements in the aquaculture and

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TABLE 1. Comparison of different systems in gene transfer and production of biotechnological products [3-5].

Study factor	Transgenic microalgae	Transgenic plant	yeasts	Animal cells	Transgenic animals	Bacteria
Production time	Short	Long	Medium	Long	Long	Short
Cost of production	Very short	Short	Medium	High	High	Medium
High production cost	Very short	Short	High	High	High	High
Production cost	Chip	Chip	Chip	Expensive	Expensive	Chip
Storage temperature	Room temp.	Room temp.	-20°	-20°	Room temp.	-20°
Production level	Widespread	Widespread	Limited	Limited	Limited	Limited
Distribution	Simple	Simple	possible	difficult	difficult	possible
Gene size	Unlimited	Unlimited	Unknown	Limited	Limited	Unknown
Perform glycosylation	Full	Full	Incomplete	Full	Full	No
Protein value	Unknown	High	High	Medium	High	Medium
Risk of infection with viruses and endotoxins	Unknown	Unknown	Unknown	Yes	Yes	Yes
Moral ethnic	Medium	Medium	Medium	Medium	High	Low

poultry industries. Commercial algae are usually produced in large pools outdoors under controlled conditions, and some companies directly use sea and lake production [5]. Consistent with FAO reports, spirulina production worldwide is increasing today, for example, in China, the production increased from 19080 tons in 2003 to 41570 tons worth $ 16.6 million in 2004 [5]. Spirulina is an aqueous, photosynthetic, filamentous, spiral, multicellular algae (integrated single cells). Two of the most important species of Spirulina are *Spirulina platensis* and *Spirulina maxima*, the name of which is derived from its spiral and filamentous form [5]. Based on the phylogenetic tests and the rRNA 16S subunit (Ribosomal Ribonucleic Acid), they are classified as Prokaryotes under Phylum Cyanobacteria category [6].

The Spirulina species is known as Spirulina or Arthrospira, which generally equates the two species and uses the general term Spirulina for their biomass, but in fact assigning the term Spirulina to Arthrospira species, especially to the two commercial and scientific species of A. platesis and A.maxima, have been a mistake referring to the reprint of the Old Geitler book (1932-1932) where a taxonomic error occurred. Arthrospira, under the trade name of Spirulina, is the most important photosynthetic microorganism cultivated for its biomass nutritional value. But the problem is that some genuine of Spirulina species (Spirulina spp.) are mistakenly cultivated which lack nutritional value or have poor quality.

The differences that classify these two species are trichome diameter, pore pattern, gas vesicles, GC content, and 16S rRNA sequences, etc. But the functional difference between the two species is that Arthrospira morphologically has cell septa whereas Spirulina lacks cell septa. Considering the chemotaxonomy feature, the presence of ϒ-linolenic acid (GLA) in Arthrospira and its absence in Spirulina as well as the differences in 16S rRNA sequences have been proven [6-8].
Spirulina has been known for many years for its high protein, vitamins, essential amino acids and essential fatty acids [9]. 55-70% of its dry matter is composed of spirulina protein [5, 10-12] and it is a rich source of vitamins especially B12 (commonly found in animal tissues) and a precursor of vitamin A (beta-carotene) and minerals especially iron. It contains a small amount of ϒ-linolenic acid (GLA) and also contains other beneficial herbal chemicals that are valuable to health [5].

Genetic structure of Spirulina

The Spirulina has a single circular chromosome structure (Figure 1). Its size is estimated at 6.8Mb.

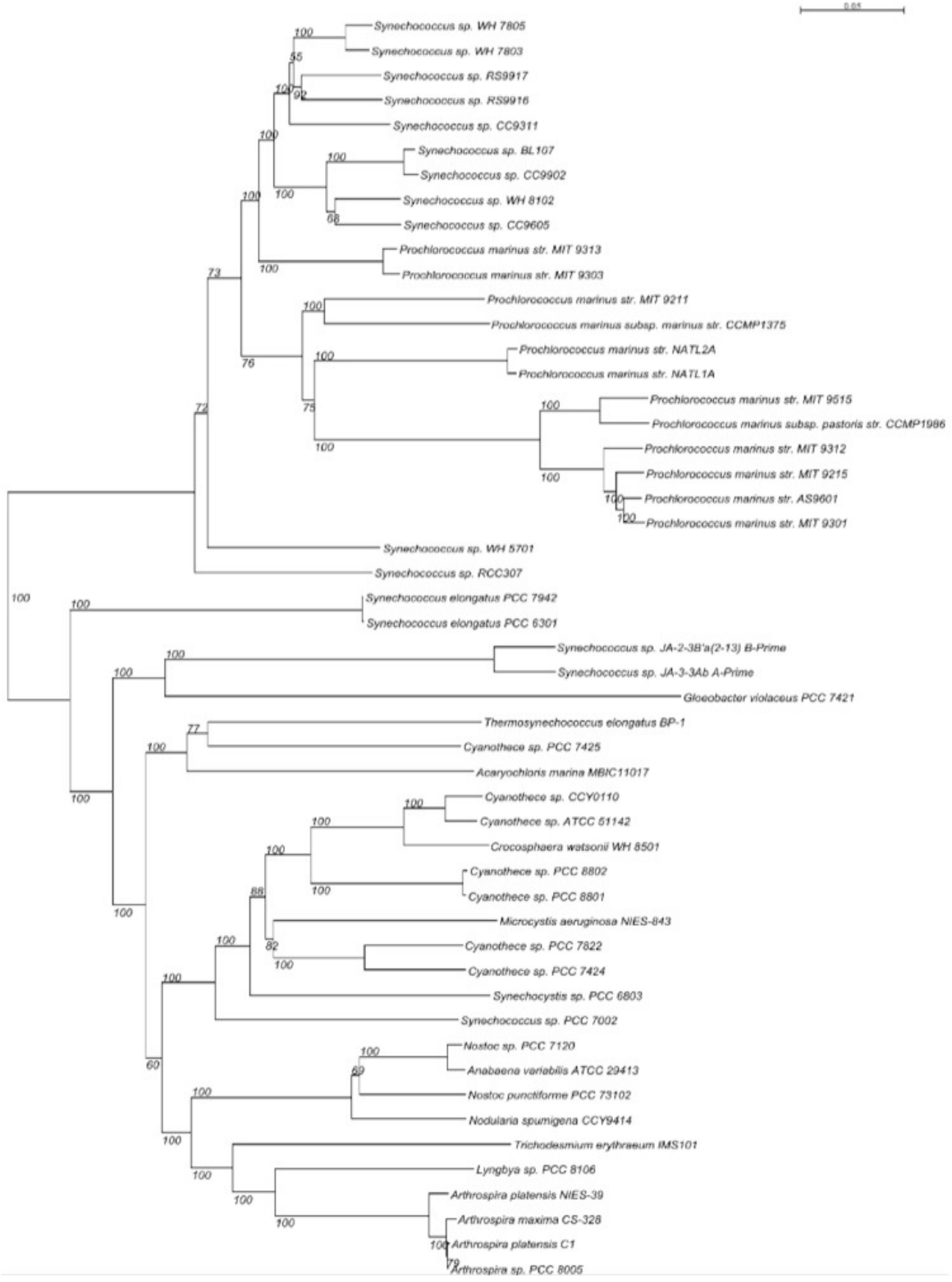

Fig.1. The phylogenetic tree of fifty-one cyanobacterial concatenated ribosomal proteins [13]. As it is clear *Arthrospira platensis* C1 was together with other strains in the order Oscillatoriales as well as was clearly separated from related species in the order Nostocales. This conserved, concatenated ribosomal protein phylogenetic tree proved the monophyly of Arthrospira genus [6].

The 6.7Mb probe of this sequence indicates the presence of 6630 protein-coding genes and two sets of rRNA genes and 40 tRNA genes. 78% of the spirulina encoding protein genes is similar to other organisms and the remaining 22% are currently unknown [14]. In the other hand, according to Cheevadhanarak *et al*. studies, the genome length was estimated to be 6.09Mb and the Spirulina encoding protein genes were 6108 and 45 RNA genes. This microorganism lacks an endogenous plasmid [6]. This genomic information is provided in NCBI ID 67617 database and with the access number of AFXD00000000). According to Cheevadhanarak *et al*. 2012 paper*:* projects of *Arthrospira* genome have been done in five research centers via using various *Arthrospira* strains (*A. platensis* PCC8005, *A. maxima* CS-328, *A. platensis* NIES- 39, *A. platensis* Paraca and *A. platensis* C1), such as: Genoscope, France; National Institute of Technology and Evaluation (NITE), Japan; DOE Joint Genome Institute, USA; King Mongkut's University of Technology Thonburi (KMUTT), Thailand and University of Applied Sciences, Switzerland.

The Importance of Spirulina Cloning

The reasons for the importance and necessity of *Spirulina* cloning include:

1. The absence of cellulose cell walls, which reduce the need for physical and chemical processes to be digested [16].
2. 55 to 70% of the spirulina weight goes to its protein content [5, 10-12].
3. As clear, promoters are one of the key regulatory elements which control the level of recombinant protein expression in the host [4]. The presence of phycocyanin specific promoter sequence, which is the strongest promoter in Spirulina, and 20% of the spirulina proteins weight is expressed by this promoter. [10-12].
4. Spirulina stimulates the immune system [17].
5. Spirulina has been used as a food source for centuries [1,2].
6. The US Food and Drug Administration (FDA) has classified Spirulina as Generally Recognized as Safe (GRAS) and allowed a range of 0.5 to 3 grams per serving [18].
7. It has no shared known disease with human
8. Spirulina is autotrophic which economically saves foreign protein production in this microorganism, in fact do as a live fermenter

But challenges in the spirulina cloning include:

1. No endogenous plasmid has been reported in Spirulina so far; and Spirulina is highly resistant to conjugal transgenic transfer.
2. The *Spirulina* genome is highly methylated.
3. Abundant endonuclease enzymes have been reported in Spirulina.
4. Given the limitations available, few studies have been performed on optimizing the Spirulina cloning pathway [19-21].

The Review of Literature on Spirulina Cloning

Considering the importance of acquiring the knowledge of gene transfer to Spirulina, the number of studies in this area is not extensively high and only a few relatively successful cases

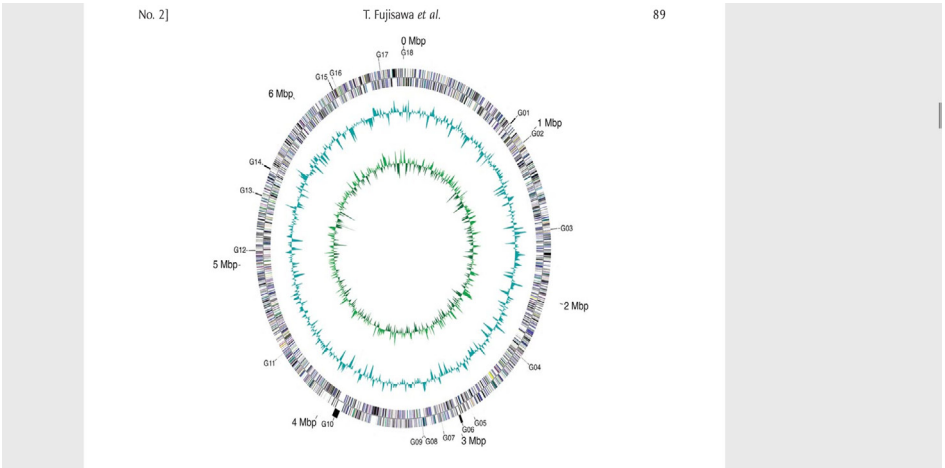

Fig. 2. The circular chromosome graphical map of Arthrospira platensis [14,15].

TABLE 2. Genome characteristic of *Arthrospira platensis* [6,14].

Strain	*Arthrospira platensis* C1 [6]	*Arthrospira platensis* (NIE-39) [14]
Genome	Single & circular Chromosome	Single & circular Chromosome
Plasmid	No	No
Genome size(~Mb)	6.08	6.8
Total genes	6153	-
Protein coding genes	6108	6630
G+C content	44.68%	44.3%
rRNA genes	6	2set
tRNA genes	39	40
Gene with protein function prediction	3757	2539

have been reported. This may be due to the unwillingness of researchers to investigate the outcome of the study.

The Vonshak et al. Research (1995-1999)

The project was an international project collaborated by France, China, Japan, Israel and Thailand and UNESCO's support for the development of a gene transfer system to Spirulina, but the main results of the project were not published, and only its final project documentation were made available.

The aims of this study were as follows:

1. Screening for plasmids in cyanobacterial Spirulina strains that can be used as a gene delivery tool.
2. Manufacturing the shuttle vectors that can perform the transfer to Spirulina with good performance and easy selection markers.
3. Developing protocols for molecular biology of working with Spirulina (electroporation, conjugation, and mutagenesis).
4. Observing the efficiency of transgenic spirulina in closed photo-bioreactors.

The team designed several recombinant plasmids (pCT1, pCT2, pCT3, pCT4) that carried a DNA fragment containing the phycocyanin promoter from Spirulina and the chloramphenicol resistance gene (Chloramphenicol). The initial results of transgenesis with pCT1 and pCT4 plasmids seemed promising. But these transgenesis only grew on plates containing the selected medium, but they all disappeared after sub-culturing. The research team suggested two possibilities for the destruction of transgenesis:

One possibility was that none of the inserted plasmids were able to permanently attach to the chromosome. The second possibility was that the free plasmids could not remain in the cells because of the endonuclease enzymes.

The researchers also attempted to design an improved version of the vector containing the pbsA gene isolated from Spirulina, and thus allowing it to be attached to the chromosome. To protect the new plasmids from the activity of restrictive enzymes, the researchers decided to incorporate a Methylase gene. Preliminary in vitro methylation studies using the initial constructed genes showed that the transgenics were able to survive for 25 days on a selective medium containing spectinomycin (0.5µg / ml), whereas those that had not methylated their plasmids survived 10 days less. Researchers at the project encountered problems that slowed the workflow. The last of these was the presence of several restrictive enzyme (NdeI) sites in the plasmid containing the methylase gene and a similar restrictive site in the psbA gene belonging to Spirulina. Researchers knocked out one of these sites. In general, despite the design of the new vector, these transgenics were also not stable.

Kawata et al., 's Research Collection (1998-2004)

Kawata *et al. (2004)* working on various projects on the spirulina transgenic, were not successful in early studies using the natural transposase Tn5. Subsequent studies by the same team using different approaches of natural transposase and Mini-Tn5 synthetic transposon along with liposome were successful in CAP gene expression and spirulina transgenics [22-24].

Invention of Integrative spirulina high-efficiency expression vector having edible safety and use thereof, (CN 103382482 A) belongs to inventors: Minggang et al. (2013)

The only invention related to Spirulina cloning is the "Integrative spirulina high-efficiency expression vector having edible safety and use" with the Patent of CN103382482A (2013). In this invention, the aim is to design structures for gene expression and transfer in spirulina. The designed vector contains a p21GZ backbone plasmid with suicide plasmid in host carrying the AP promoter (phycocyanin), the GFP marker gene and the non-operative insulin gene of (10LPG1) [25].

Jeamton et al.'s research (2017)

It seems this project is continuing of previous Research groups. Although Arthrospira sp. have strong restriction barriers, the group established a transformation system based on transposon and electroporation in the A. platensis C1 genome. They add type I restriction inhibitor to liposomes protocol to protect the foreign DNA from nuclease digestion. The highest transformation efficiency was 1.5 $\times 10^6$ cfu μg^{-1} DNA when both liposome (DOATP) and type I inhibitor used along with Transposome complex. The efficiency was 0, 3.4×10^5, 6.2×10^5 and1.2×10^6 cfu μg^{-1} DNA in control$^-$ group, Transposome complex group, Transposome complex with type I inhibitor group and Transposome complex with liposome (DOATP) group, respectively.

In the study, plasmid pAG44 carrying the transposon cassette including the GFP and spectinomycin resistance genes under the regulation of the PC promoter. Transformants were followed for more than eight passages in selective medium [26].

Conclusion

Since researchers became aware of the lack of any endogenous plasmid and the existence of restriction endonuclease, the research became more focused. So far, the best methods of gene transfer based on transposome, liposome and electroporation. Certainly, given the emergence of these successes, we will soon see the production of pharmaceutical and food products based on this cellular system.

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Conflict of interest

The authors declare no conflict of interest

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