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FINAL REPORT

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Development of a Gene Transfer System for the Cyanobacterium *Spirulina platensis*

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3. Executive Summary

The cyanobacterium (blue green algae) *Spirulina platensis* is now mass cultured mainly in the US, Thailand, Taiwan, China and India. The estimates are that by the end of the year 2000 the annual production will exceed 2000 metric tones with an estimate turnover of over $50x10^6$ US $. The purpose of this project was to develop a gene transfer system for this alga that will be used not only for strain improvement but also as a tool for increasing the know-how on the physiology, biochemistry and molecular biology of this new "agrobiotechnological" product.

The basic experimental approach was to design a transformable vector that will carry an easily selectable marker which can be used for detection of transformed cells. The goals were to achieve reliable and stable transformants either by inserting self replicating plasmids or by achieving integration of the inserted foreign DNA into the algal genome.

The main achievements of the project so far are:

i. Developing detailed experimental procedures that are specifically fitted to molecular biology work with *Spirulina.*

ii. Expending the knowledge and information available on different genes and their regulation in *Spirulina* such as pigment lipid and DNA metabolism.

iii. Demonstrating the ability to introduce foreign genes and express them in *Spirulina.*

iv. As an outcome of the work carried out in KMITT, an active research group dealing with all aspects of molecular biology in Spirulina was established. The group maintained an active research program, which is reflected in the number of graduate students currently employed in the lab. The collaborating PI has established international collaboration not only with Israel but also with many other research groups (US, Japan, France, and Hungary). There is no doubt that the group has managed to gain national and international recognition as a leading research center in molecular biology of *Spirulina.*

The main problem still to be solved is stabilization of the foreign transformed so that it can be replicated in cells grown at their optimal growth conditions. This goal has not been achieved yet, mainly due to a high level of nuclease activity in *Spirulina* cells. We hope that this goal will also be achieved with the new vector designed. The Thai collaborator has managed to receive extra funding from other resources, that will enable him to continue the work.

Strategy for Gene Transfer System Development for Spirulina platensis

Selectable marker Identification

↓

Antibiotic sensitivity test

↓

Restriction enzyme determination: HaeIII (major)

↓

Selectable Marker Identified

Vector construction

↓

- Chloramphenicol based
  - commercial
  - *Spirulina* promoter
- integrative spectinomycin based
  - *Synechocystis psba1* gene
  - *Spirulina psba1* gene

Gene Transfer system

↓

Growth determination

↓

conjugation
electroporation
- spheroplast
- sonicated cells
- whole trichome

4. Research Objectives

Micro-algal biotechnology as a tool for production of food, feed and high value products has been intensively reviewed and discussed. It carries a unique potential for many developing countries that are short of "sweet water" and fertile land. Those two resources represent the main limitation for further sustainable development of food and feed production in developing countries, many of which suffer from phenomena associated with desertification resulting from over use of land and water. Thus, the fact that many algal species can be grown on marginal water (brackish or saline) and land is just used as a supporting device, may turn algal biotechnology to be an appropriate answer or an alternative approach for future sustainable development.

Spirulina, a blue green filamentous alga has long been a traditional source of protein for people living along Lake Chad, in Africa. More recently it has been recognized for its value in the aquaculture feed formulation industry due to the relatively high content of some unsaturated fatty acids as well as high digestibility in post larval stages of fish and other marine animals development. It is a brackish-water alga, which grows profusely in alkaline-saline lakes in Central Africa and America. At present, *Spirulina* is mass cultivated under out-door conditions, the product intended mainly for the health food market and not as a food supplement. This is mainly due to the relatively high production cost. Any further development of this biotechnology will require a better understanding of the biology of this alga as well as improved strains that will fit the unique requirements of the production system.

The main goal of the proposed research was to establish a gene transfer system for *Spirulina* and to use it for studying the main limitation in *Spirulina* growth as well as obtaining strains of improved performance for mass cultivation in closed photobioreactors.

1. To screen for plasmids in strains of the cyanobacterium *Spirulina* to be used as vehicles for gene transfer.
2. To construct a shuttle vector that can be transformed to *Spirulina* with high efficiency and easily selected markers.
3. To develop protocols for molecular biology work in *Spirulina* (electroporation, conjugation, mutagenesis, and gene cloning and sequencing).
4. To evaluate the performance of the transformed *S. platensis* strain.

The know-how gained was aimed to provide the methodology for further studies in gene expression and regulation as well as construction of specific overproducing strains of a specific desirable chemical. The growing industry of *Spirulina* production should benefit from being able to genetically improve the strains that they are using. During the project, international collaboration with research groups in France, Japan and China were established. Additional funds for the work were received from UNESCO, The Israeli ministry of Science and the National Center for Biotechnology in Thailand.

5. Methods and Results

The experimental approach that we have adapted in the research work was that we would like to construct a shuttle vector that will be based and carry the following characteristics:

1. there will be no mutation occurring in the host strain, so that the prominent characteristics of the host strains are maintained.
2. facilitating an easy host-vector construction
3. facilitating gene knock-out to assist in gene structure and function analysis
4. facilitating the stability of the transformants

The research was divided into 3 phases:

Phase I: Developing the methodologies and screening procedures:

i. Screening for strains with fast growth rate and colony forming filaments.

ii. Evaluating procedures for preparation of cells to be transformed.

- whole trichome
- sonicated filament
- protoplast formation

iii. Screening for selection markers and transformation procedures:

- screening and evaluating antibiotic resistance
- chemical methods
- electroporation method

iv. Vector construction

- screening for plasmids in different *Spirulina* strains
- search for strong promoters

Phase II: optimization of the transformation system

- new versions of plasmids construction: search for more promoters
- optimization of transformation methodology
- how to identify the transformants
- characterization of transformants
- how to maintain the stability of the transformants

Phase III: how to construct stable transformants

- identify the factors promoting instability of transformants
- identify the presence of restriction enzymes in the trichome
- *in-vitro* DNA methylation of the DNA before transformation
- integrative plasmid construction:
- clone psba1 gene
- construct an integrative plasmid containing new selectable marker and parts of the psba1 gene
- search for new dominant selectable marker

Technical procedures and partial results were reported in the annual reports that are attached to this final report. The following is a short summary and update of the latest results.

Plasmid construction

Chromosomal DNA of S. platensis was isolated as described (see appendix). Partially digested Sau3A1 fragment of 2 to 4 kbp were isolated from agarose gel by electroelution and ligated to pKK232-8 (a promoter probe purchased from Pharmacia) at BamHI site. The recombinant plasmids were used to transform *E. coli* DH5a by $CaCl_2$ method. Transformants were selected on LB agar plate supplemented with Chloramphenicol at a concentration of 170 mg. ml^{-1}. Plasmids isolated from transformants resistant to Chloramphenicol were used in electrotransformation of *S. platensis*.

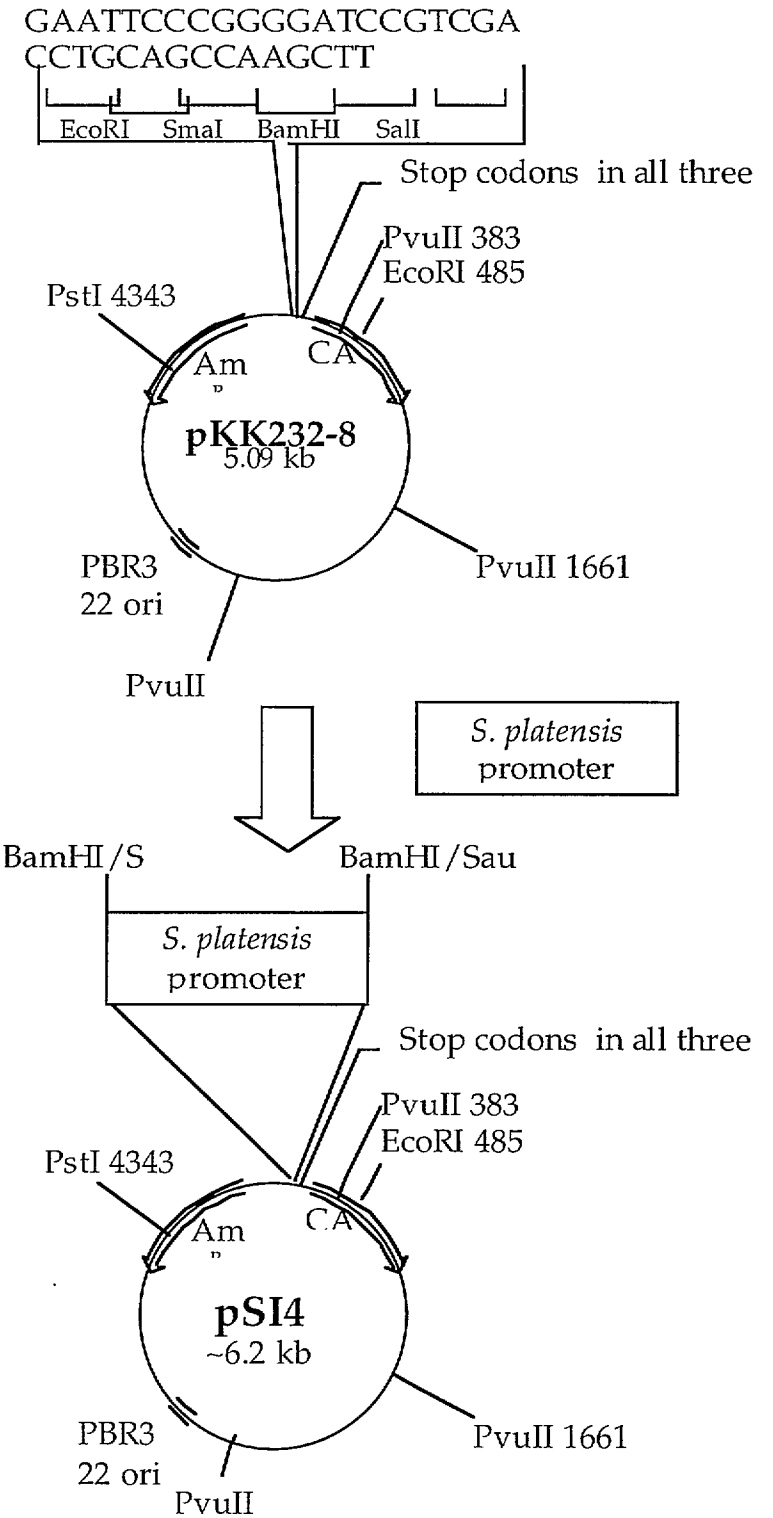


Fig. 1 Construction of plasmid pSI4. The 1.2 kb *Sau*3AI fragment of chromosomal DNA of *S. platensis*, which was expected as a promoter region, was subcloned into *Bam*HI site of plasmid pKK232-8, a Chloramphenicol acetyltransferase promoter probe vector (Pharmacia).

Main results

Recombinant plasmids harboring a DNA fragment containing a promoter of *S. platensis* in front of the CAT gene were constructed. The inserted fragments were about 1.2 kbp in size. Four recombinant plasmids designated pCT1, pCT2, pCT3, and pCT4 were used for transformation S. platensis to Chloramphenicol resistance as described above. The transformation frequencies were in the range of 56-237 transformants per microgram of the plasmid DNA as shown in Table 1 of which pCT4 gave the highest frequency while pCT2 gave the lowest. The green colonies of the transformants appeared after incubation for 30 days under low light intensity (10 $\mu E\ m^{-2}\ sec^{-1}$) against degraded filaments of the nontransformed cells.

Table 1: The frequency of the transformation of S. platensis using various recombinant plasmids

Plasmids	Transformation frequency (transformants /mg plasmid DNA)
no plasmid	0
pKK232-8	0
pCT1	281
pCT2	56
pCT3	204
pCT4	237

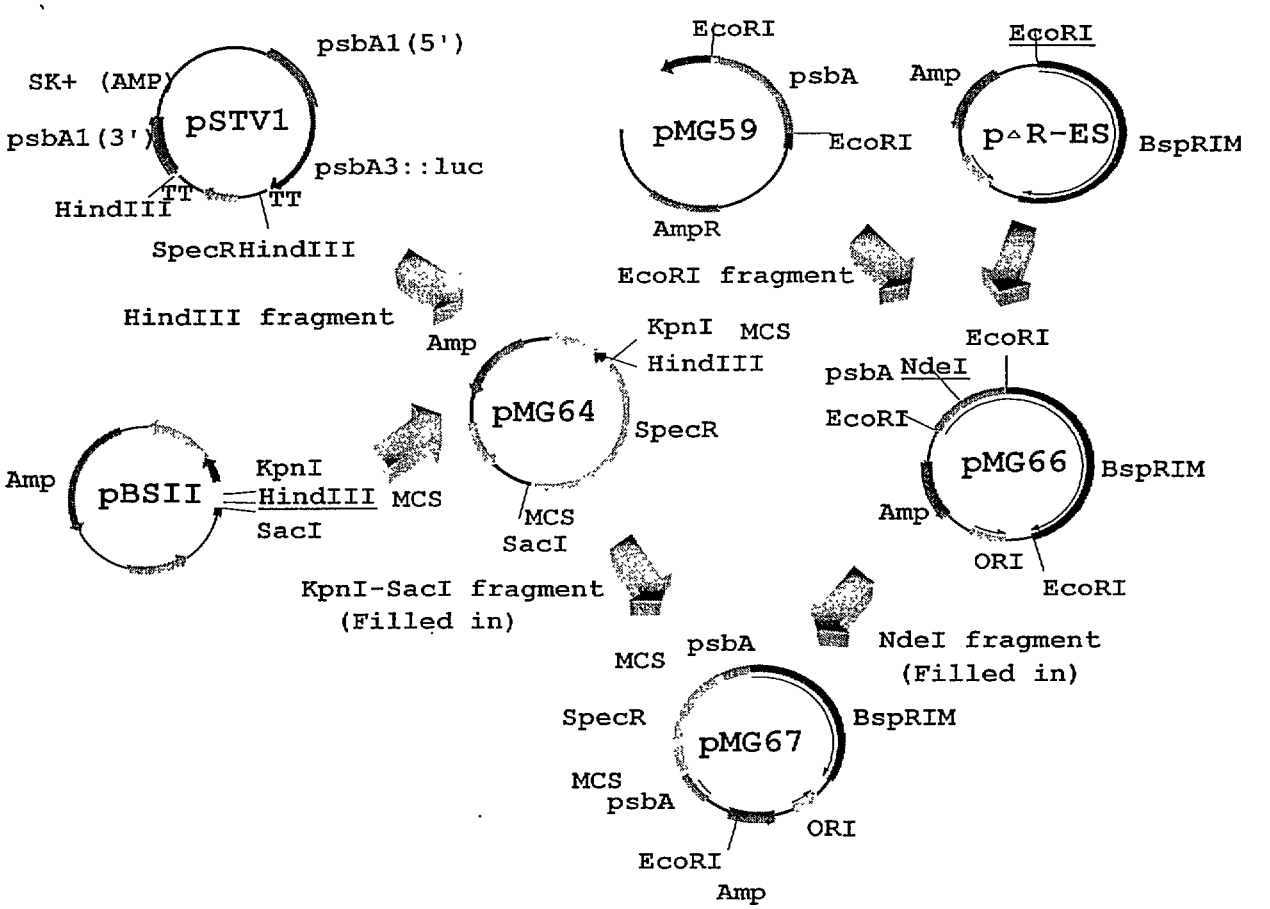
Transformants were stable for many generations upon subculturing onto medium containing Chloramphenicol at the concentration of 4 mg ml^{-1}. The results from amplification of the CAT gene by PCR technique and Southern hybridization's revealed that there was no free plasmid and the resistance of the transformants to Chloramphenicol was due to the CAT gene and the integration of the transformed plasmid into the chromosomes. Yet the main problem we faced was that all the transformants grow at a much slower growth rate and when transferred to liquid medium a loss in viability was observed. We have thus decided that further to our attempts to study the reasons for this instability, to redesign another shuttle vector with more defined inserts.

The following is a short summary of the steps taken in this approach

1. Cloning of DNA fragments from the cyanobacterium *Spirulina platensis*, three DNA fragments were cloned. One of them is an unsequencd segment, which has been inserted into a plasmid and sequenced.

2. Construction of vectors: In the phycocyanin operon, the promoter and the structural genes were separated and the promoterless CAT gene was inserted between them. So a vector which harbors homologous fragments from *Spirulina* and can thus result in a site-specific integration was constructed.
3. Effects of foreign inserts on gene expression: The vector was transformed into *E.coli* in order to check whether the CAT insert could be promoted by phycocyanin operon promoter and transcribe and translate independently. The results showed that every part in the vector could work well as expected.
4. We have studied the enzymatic restriction activity in the cells in an attempt to provide protection to the inserted DNA by pre-metylation.
5. Another approch was that we have tried to introduce another resistance marker in order to replace the CAP resistance that may have problems in the stability of the transformants.

The following is a schematic description of the steps undertaken in order to construct the improved vector that we believe may help in overcoming the problem we have encountered during the project.



1st step: Construction of the plasmid that contained SpecR and Multicloning sites (MCS)

The SpecR gene (with cyanobacterial promoter and terminator, ~1850 bp *Hin*dIII cassette) of the plasmid pSTV1 was inserted at the *Hin*dIII site in MCS of plasmid pBluescriptII SK+. The resulting plasmid, pMG64, was 4811 bp and contained SpecR in the MCS.

2nd step: Introduction of *Spirulina* sequence as a target site for homologous recombination.

A 919 bp *Eco*RI fragment of *psbA* gene of the plasmid pMG59 was inserted into *Eco*RI site of the plasmid pΔR-ES that contained the *Bsp*RI-type methylated gene.

The resulting plasmid was designated pMG66 and further used for blunt cloning, the SpecR cassette of pMG64, at the *Nde*I site.

3rd step: Introduction of Spectinomycin resistant gene (SpecR)

A 1952 bp *Sac*I (blunt)-*Kpn*I (blunt) fragment of *SpecR* cassette of pMG64 was inserted at the *Nde*I site (blunt) within the middle of *psbA* flanks (docking sites for integration) of pMG 66. The resulting plasmid, pMG67 (7471 bp), is

- efficiently protected from *Spirulina* restriction,
- capable of stable integration at the complemented *psbA* site,
- conferring Spec resistance gene to cyanobacterium,
- internal cloning sites to introduce the reporter constructs of interested gene.

Latest results and summary

The results of the transformation with the constructed plasmids pCT1-pCT4, seemed initially very promising. The transformants grew on the plates containing the selection media. However, after sub-culturing we could not maintain the transformants and eventually lost them all. One possible reason for this would be that none of the inserted vectors was able to integrate into the chromosome permanently and popped out of the chromosome. Alternatively it may as well be that the cells could not maintain the free plasmids due to restriction enzymes that chopped the inserted DNA. As such, we have tried to design an improved version of the vector that will include the psbA gene isolated from *Spirulina* and thus will allow its integration into the chromosome. In order to protect the new plasmids from the restriction enzyme activity, we decided to insert a mythylase gene as well. Specifically we aimed at the restriction site of Hae III that we detected as one of the main restriction enzymes active in our *Spirulina* strains. Preliminary studies performing in-vitro methylation using the initially constructed plasmids showed that the transformants could survive 25 days on selection plates containing Spectinomycin (0.5 microgram per ml.), 10 days longer than the transformants containing the non-methylated plasmid. However, the transformants were not stable, a problem that we hoped to solve by the integration of the psbA gene included in the newly designed vector.

We came up with some problems that slowed down the construction. The last one was multiple sites of a restriction enzyme (NdeI) in the plasmid that contains the methylase gene and the same restriction site in the psbA gene of *Spirulina*. We

knocked one of the sites and at this stage we are isolating the product of the last cloning step in order to evaluate its size and components before we start with the transformation experiments.

6. Impact, Relevance and Technology Transfer

The findings and results of the project helped in establishing the required know how for molecular biology studies in *Spirulina*. The Thai collaborator has established its reputation as one of the leading scientists not only in Thailand but also on the international level. From a relatively small group that was not familiar to the international scientific community working in *Spirulina* the group in KMUTT is now one of the leading groups in the world in this field, with established international collaboration with scientists as well as local industrial producers of *Spirulina*.

7. Project Activities / Outputs

Meetings Attended as Invited speaker

May 1997 Structure and Light regulation of genes encoding phycocyanin and allophycocyanin from *Spirulina platensis* C1" 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology, Phuket, Thailand.

November 11, 1997 Molecular genetic study on the production of γ-linolenic acid of *Mucor rouxii* ATCC 24905: Cloning and characterization of 9 and 12 desaturase genes, the genes involved in the production of γ-linolenic acid. The BIOTEC FORUM. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.

September 25, 1999 Deshnium, P., Paithoonrangsarid, K., Suphatrakul, A., Meesapyodsuk, D., Tanticharoen, M. and Cheevadharak, S. 1999 Temperature regulation of the expression of desaturase genes in *Spirulina platensis* C1. 2 nd European Phycological Congress, Montecatini Terme, Italy. September 20-26, 1999.

Workshop and Training Courses Organized

September 1996 "Molecular Taxonomy of Cyanobacteria : A Practical Course" Co-organized with National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency

Meetings attended as Oral presentations

1. **Cheevadhanarak, S.**, Meesupyodsuk, D., Jeamton, W., Nomsawai, P., Tandeau de Marsac, N.,Chaisawadi, S. and Tanticharoen, M. Structure and light regulation of genes encoding phycocyanin and allophycocyanin from *Spirulina platensis* C1. Oral presentation at the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology. May 7-10, 1997. Phuket, Thailand.
2. Deshnium, P., Paithoonrangsarid, K., Supatrakul, A., Meesapyosuk, D., Tanticharoen, M. and **Cheevadhanarak, S.** Temperature regulation of the expression of desaturase genes in *Spirulina platensis* C1. Oral presentation at the 2nd European Phycological Congress. September 20 -26, 1999. Montecatini Terme, Italy.

Meetings attended as Poster presentations

1. Jeamton, W., Nomsawai, P., Chaisawadi, S., Castets, A., Coursin, T., de Marsac, N.T., Tanticharoen, M. and **Cheevadhanarak, S**. Molecular cloning and characterization of the phycocyanin gene from a cyanobacterium *Spirulina platensis* C1. Poster presented at the 8th Annual Meeting of the Thai Society for Biotechnology and the 1996 Annual Meeting of the National Center for Genetic Engineering and Biotechnology on BIOTECHNOLOGY: PROSPECTS FOR THE FUTURE. November 14-15, 1996. The Malia Hua Hin Hotel, Prachuap Khiri Khan, Thailand.
2. Meesapyodsuk, D., Nomsawai, P., Anjard,C., Chaisawadi, S., Tanticharoen, M. and **Cheevadhanarak, S**. Molecular cloning and characterization of the allophycocyanin gene from *Spirulina platensis* C1. Poster presented at the 8th Annual Meeting of the Thai Society for Biotechnology and the 1996 Annual Meeting of the National Center for Genetic Engineering and Biotechnology on BIOTECHNOLOGY: PROSPECTS FOR THE FUTURE. November 14-15, 1996. The Malia Hua Hin Hotel, Prachuap Khiri Khan, Thailand.
3. Deshnium, P., Suphatrakul, A., Anjard, C., Chaisawadi, S., Tanticharoen, M. and **Cheevadhanarak, S**. Effect of low temperature on the expression of _ 12 desaturase (*desA*) gene in a filamentous cyanobacterium *Spirulina platensis* C1. Poster presented at the 8th

Annual Meeting of the Thai Society for Biotechnology and the 1996 Annual Meeting of the National Center for Genetic Engineering and Biotechnology on BIOTECHNOLOGY: PROSPECTS FOR THE FUTURE. November 14-15, 1996. The Malia Hua Hin Hotel, Prachuap Khiri Khan, Thailand.

4. Deshnium, P., Suphatrakul, A., Anjard, C., Chaisawadi, S., **Cheevadhanarak, S.**, Tanticharoen, M. Effect of low temperature on the expression of Δ12 desaturase gene in *Spirulina platensis* C1. Poster presented at the 1st European Phycological Congress. August 11-18, 1996. Koln, Germany.
5. Suphatrakul,A., Deshnium, P., Chaisawadi, S., Tanticharoen, M. and **Cheevadhanarak, S.** The effect of temperature on the expression of the D12 desaturase gene (*desA*) in *Spirulina platensis* C1. Poster presented at the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology. May 7-10, 1997. Phuket, Thailand.
6. Jeamton, W., Nomsawai, P., Chaisawadi, S., Castets, A., Coursin, T., de Marsac, N.T., Tanticharoen, M. and **Cheevadhanarak, S.** Transcriptional organization of the phycocyanin subunit genes of the cyanobacterium *Spirulina platensis* C_1. Poster presented at 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology. May 7-10, 1997. Phuket, Thailand.
7. Meesapyodsuk, D., Nomsawai, P., Anjard,C., Chaisawadi, S., Tanticharoen, M. and **Cheevadhanarak, S.** Transcriptional organization of the allophycocyanin subunit genes of the cyanobacterium *Spirulina platensis* C_1. Poster presented at the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology. May 7-10, 1997. Phuket, Thailand.
8. Nomsawai, P. Tandeau de Marsac, N. Tanticharoen, M., Thomas, J.C. and **Cheevadhanarak, S.** The effect of light intensive on phycobilisome and phycocyanin biosynthesis in *Spirulina platensis* C_1. Poster presented at the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology. May 7-10, 1997. Phuket, Thailand.
9. **Cheevadhanarak, S.**, Meesupyodsuk, D., Jeamton, W., Nomsawai, P., Tandeau de Marsac, N.,Chaisawadi, S. and Tanticharoen, M. Structure and light regulation of genes encoding phycocyanin and allophycocyanin from *Arthrospira platensis* PCC 9483. Poster presented at the 9th International Symposium on Phototrophic Prokaryotes. September 6-12, 1997. Vienna, Austria.
10. Deshnium, P., Suphatrakul, A., Meesapyodsuk, D., Tanticharoen, M. and **Cheevadhanarak, S.** A genetic study of temperature regulation of fatty acid desaturation in *Spirulina platensis* C_1. Poster presented at 9th International Symposium on Phototrophic Prokaryotes. September 6-12, 1997. Vienna, Austria.
11. Nomsawai, P. Tandeau de Marsac, N. Tancharoen, M., Thomas, J.C. and **Cheevadhanarak, S.** The effect of light intensive on phycobilisome and phycocyanin biosynthesis in *Arthrospira* sp. PCC 9438. Poster presented at 9th International Symposium on Phototrophic Prokaryotes. September 6-12, 1997. Vienna, Austria.
12. Meesapyodsuk, D., Deshnium, P., Tanticharoen, M., and **Cheevadhanarak, S.** Cloning and Expression of _ 9 acyl-lipid desaturase gene from *Spirulina platensis* C_1. Poster presented at 10th Annual Meeting of the Thai Society for Biotechnology and The 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology, November 25-27, 1998. Sol Twin Towers Hotel, Bangkok, Thailand.
13. Deshnium, P., Suphatrakul, A., Paithoonrangsarid, K., Meesapyodsuk, M., Tanticharoen, M., and **Cheevadhanarak, S.** Regulation of the *desA* gene transcription by temperature in *Spirulina platensis* strain C_1. Poster presented at 10th Annual Meeting of the Thai Society for Biotechnology and The 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology, November 25- 27, 1998. Sol Twin Towers Hotel, Bangkok, Thailand.

14. Jeamton, W., Meesapyodsuk, M., Nomsawai, P., Tandeau de Marsac, N., Tanticharoen, M., and **Cheevadhanarak, S**. Organization and expression of phycocyanin and allophycocyanin genes from *Spirulina platensis* C_1. Poster presented at 10th Annual Meeting of the Thai Society for Biotechnology and The 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology, November 25- 27, 1998. Sol Twin Towers Hotel, Bangkok, Thailand.
15. Paitoonrangsarid, K., Suphatrakul, A., Deshnium, P., **Cheevadhanarak, S.** and Tanticharoen, M. Study of the effect of high temperature on the desaturation of fatty acid in cyanobacterium: *Spirulina platensis* strain C1. Poster presented at the 10th Annual Meeting of the Thai Society for Biotechnology and the 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology, November 25- 27, 1998. Sol Twin Towers Hotel, Bangkok, Thailand.
16. Yompakdee, C., Malasit, P. and **Cheevadhanarak, S.** Inorganic phosphate controlled expression of dengue envelope protein by *Saccharomyces cerevisiae*. Poster presented at the 10th Annual Meeting of the Thai Society for Biotechnology and the 1998 Annual Meeting of the National Center for Genetic Engineering and Biotechnology, November 25-27, 1998. Sol Twin Towers Hotel, Bangkok, Thailand.

Publications

1. Nomsawai, P., Tandeau de Marsac, N., Claude Thomas, J., Tanticharoen, M. and **Cheevadhanarak, S.** Light regulation of phycobilisome structure and gene expression in *Spirulina platensis* C1 (*Arthrospira* sp. PCC 9438). Plant Cell Physiol. (*In press*)
2. Deshnium, P., Paitoonrangsarid, K., Suphatrakul, A., Meesapyodsuk, D., Tanticharoen, M., and **Cheevadhanarak, S.** Temperature-independent and -dependent expression of desaturase genes in filamentous cyanobacterium *Spirulina platensis* strain C1 (*Arthrospira* sp. PCC 9438). (In submission)
3. Vachhani, A.K. and Vonshak, A. Recombination protein A gene, *rec*A, from *Spirulina platensis* sp. IAM-M135.J. of Appl. Phycol., 8: 563-564, 1997.

8. Project Productivity

In terms of the expected impact of the AID-CDR program on developing countries there is no doubt that the project accomplished its goal. This is reflected in the establishment of a scientific active research group in KMUTT. The project has helped in building up the physical infrastructure as well as building the human capacity that will enable future work in molecular biology of cyanobacteria.

Although the methodology for introducing foreign DNA into *Spirulina* cells and getting it expressed was accomplished, the goal of obtaining a stable transformant has not been achieved. The reason is mainly due to the limited funding resources at the beginning of the project and the need to devote significant part of time and resources to develop methodologies that we originally assumed can be directly adapted from other algal or bacterial systems. We have no doubt that with the additional funding obtained and the newly constructed vectors, this goal will also be achieved in the near future.

9. Future work

With the methodology developed and the infrastructure established the work will continue along two main lines:

1- Further attempts with the improved constructed vectors to achieve a stable transformation system.
2- Use the molecular biology methodology developed for studying of gene regulation in *Spirulina*.
3- Attempt to construct an overproducing strain for high levels of fatty acids.

Appendices

ANNUAL REPORT

Covering Period: November 96 to November 97
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Development of a Gene Transfer System for the Cyanobacterium *Spirulina platensis*

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Project Duration: November 95 - November 98.

Executive Summary

The purpose of this project is to develop a gene transfer system for the cyanobacteria (blue green algae) *Spirulina* . The main problems in such a project are reflected in the fact that very little work has been done so far on the molecular biology of *Spirulina*. Thus as part of the project basic procedures and methodology has to be developed and modified to fit this organism. So far the work is progressing according to the main time table as provided in the original proposal.

The main achievements of the project so far are:

i. developing detailed procedures that are specifically fitted to the work with *Spirulina* .

ii. demonstrating the ability to introduce foreign genes and get them to be expressed in *Spirulina.*

The main problem still to be solved is to get the foreign gene that was transformed into the cells stable and capable of being maintained at the cells grown at their optimal growth rate.

The achievements and the collaborative work had put the laboratory of the Thai collaborator in the scientific front in the study of the molecular biology of cyanobacteria. His participation on international meetings and other exchange programs are the best proof that this project had helped in building his scientific capacity.

Section I

A) Research Objectives

The main goal of the proposed research is to establish a gene transfer system for *Spirulina* and use it to study the main limitation in *Spirulina* growth as well as obtaining strains of improved performance for mass cultivation in closed photobioreactors..

1. To screen for plasmids in strains of the cyanobacterium Spirulina to be used as vehicles for gene transfer.
2. To construct a shuttle vector that can be transformed to *Spirulina* with good efficiency and easily selected markers.
3. To develop protocols for molecular biology work in *Spirulina* (electroporation, conjugation, mutagenesis).
4. To observe the performance of the transformed *S. platensis* in closed photobioreactor.

B) Research - accomplishments

Optimization of elctroporation conditions: During this year we have further optimized the conditions for electroporation of *Spirulina* in order to increase the efficiency and viability of the treated cells.

The effect of field strength on the viability of the electroporated cells

The field strength ranging from 1 to 10 kv cm-1 were applied to the cells with the electroporation condition as described above. It was found that the field strength that gave the high viability to the electroporated cells was in the range of 1 to 5 kv cm-1 as shown in Fig. It was found that the greater field strength the more broken trichomes with the less viable filaments. It was observed that at the field strength more than 6 kv cm-1 cells were bleached with a lot of phycocyanin leaked out into the medium. As such the field strength of 1 to 5 kv cm-1 was chosen in the subsequent experiments.

The effect of the age of the cells on the viability of the electroporated cells.

Cells at various growth phase were used for electroporation experiments. They were cells at early log phase, mid log phase and late log phase. Cells were given a single pulse as described above with the field strength of 4 kv cm-1. It was found that cells in the early and mid log phase gave the highest percentage of viability of 60%, whereas cells at the late log phase gave very low viability

The effect of DNA concentration on the transformation frequency

The frequency of transformation was dependent on the concentration of the DNA. When electrotransformed S. platensis with the DNA at the concentration of 0 to 10 mg, it was found that high concentration of DNA gave large number of transformants. However, the frequency of transformation did not increased when the DNA more than 5 mg was used

Plasmid construction

Chromosomal DNA of S. platensis was isolated as described by Cai and Wolk (1990). Partially digested Sau3A1 fragment of 2 to 4 kbp were isolated from agarose gel by electroelution and ligated to pKK232-8 (a promoter probe purchased from Pharmacia) at BamHI site. The recombinant plasmids were used to transform E. coli DH5a by CaCl2 method. Transformants were selected on LB agar plate supplemented with

chloramphenicol at a concentration of 170 mg. ml-1. Plasmids isolated from transformants resisted to chloramphenicol were used in electrotransformation of S. platensis.

Main results

Recombinant plasmids harboring a DNA fragment containing a promoter of S. platensis in front of the CAT gene were constructed. The inserted fragments were about 1.2 kbp in size. Four recombinant plasmids designated pCT1, pCT2, pCT3, and pCT4 were used for transformation S. platensis to chloramphenicol resistance as described above. The transformation frequencies were in the range of 56-237 transformants per microgram of the plasmid DNA as shown in Table 1 of which pCT4 gave the highest frequency while pCT2 gave the lowest. The green colonies of the transformants appeared after incubation for 30 days under low light intensity (10 mE.m-2.sec-1) against degraded filaments of the nontransformed cells.

Table 1 The frequency of the transformation of S. platensis using various recombinant plasmids

Plasmids	Transformation frequency (transformants /mg plasmid DNA)
no plasmid	0
pKK232-8	0
pCT1	281
pCT2	56
pCT3	204
pCT4	237

Transformants were stable for many generations upon subculturing onto medium containing chloramphenicol at the concentration of 4 mg ml-1. The results from amplification of the CAT gene by PCR technique and Southern hybridizations revealed that there was no free plasmid and the resistance to chloramphenicol of the transformants was due to the CAT gene and the integration of the transformed plasmid into the chromosomes. Yet the main problem we faced was that all the transformants grow at a much slower growth rate and when transfers to liquid medium a loss in viability was observed. We have thus decided that further to out attempts to study the reasons for this instability to redesign another shuttle vector with more defined inserts.

The following is a short summary of the steps already taken in this approach

1. Cloning of DNA fragments from the *cyanobacterium Spirulina platensis,* three DNA fragments were cloned. One of them is an unsequencd segment which has been inserted into a plasmid and now being sequenced.
2. Construction of vector: In the phycocyanin operon, the promoter and the structural genes were separated and the promoterless CAT gene was inserted between them. So a vector which harbors homologous fragments from *Spirulina* and can thus result in a site-specific integration was constructed.
3. Effects of foreign inserts on gene expression: The vector was transformed into E.coli in order to check whether the CAT insert could be promoted by phycocyanin operon promoter and transcribes and translates independently as well as the expression of downstream would be effected by the insertion of CAT gene. SDS-PAGE technique was employed in this study. The result showed that every part in the vector could work well as expected.
4. Construction of genomic library: Preparatory work has been conducted.

C). Scientific Impact of Collaboration:

The collaborating scientists have contributed significantly to the project in developing the first vector to be used in the transformation. They have also performed all the electroporation optimization conditions and all the other tasks as specified in the original working program.

During the second year the investigators met once during a scientific meeting in Phuket May 97.

D) Description of Project Impact : After two years of the project even though that the main goal as not yet been achieved. There is no doubt that the capacity building purpose in terms of building a unique scientific capability in Thailand is getting closer. The international reputation of the Thai group has build up as was reflected during the meeting of the Asian Pacific Society for applied algology and the fact that the poster presented by the students from this group won the first prize. Most of the methodology developed is in use by graduate students in KMITT as part of their research projects.

Others. At this stage no major problems has been faced the required changes in working plans , budget or task allocations.

ANNUAL REPORT

Covering Period: November 95 to November 96
Submitted to the Office of the Science Advisor
U.S. Agency for International Development

Development of a Gene Transfer System for the Cyanobacterium *Spirulina platensis*

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US-Israel AID-CDR Program

Project Duration: November 95 - November 98.

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Executive Summary

The purpose of this project is to develop a gene transfer system for the cyanobacteria (blue green algae) *Spirulina* . The main problems in such a project are reflected in the fact that very little work has been done so far on the molecular biology of *Spirulina*. Thus as part of the project basic procedures and methodology has to be developed and modified to fit this organism. So far the work is progressing according to the main time table as provided in the original proposal. The main finding so far are that although much efforts have been put into developing a fast screening method for plasmids in different strains of *Spirulina* no such plasmids have been found. On the other tasks we were more successful.

i. - Few strains have been isolated capable in forming colonies rather then gliding on agar plates. This is an important step for further transformation and isolation procedures.

ii. - A procedure for isolation of protoplasts was developed capable of high efficiency of recovery. This step is of advantage in further transformation experiments.

iii. - Conditions for electroportation were developed for high efficiency of DNA delivery into the cells

iv. A low restriction activity *Spirulina* strain was isolated. This is important in the transformation experiment to increase stability of the newly introduced DNA.

v. - The *recA* gene from *Spirulina* was cloned isolated and sequenced. Its promoter activity and expression will be further studied.

Section I

Note on Publication: The following paper has been excepted for publication in "J of Applied Phycology"

Recombination protein A gene, *recA*, from *Spirulina platensis* sp. IAM-M135.

Gene bulletin: Database accession number: U33924

Ajay K. Vachhani and Avigad Vonshak

(reprints are not available yet.)

A) Research Objectives

The main goal of the proposed research is to establish a gene transfer system for *Spirulina* and use it to study the main limitation in *Spirulina* growth as well as obtaining strains of improved performance for mass cultivation in closed photobioreactors..

1. To screen for plasmids in strains of the cyanobacterium Spirulina as vehicles for gene transfer.
2. To construct a shuttle vector that can be transformed to *Spirulina* with good efficiency and easily selected markers.
3. To develop protocols for molecular biology work in *Spirulina* (electroporation, conjugation, mutagenesis).
4. To observe the performance of the transformed *S. platensis* in closed photobioreactor.

B) Research - accomplishments

B.1 Antibiotic sensitivity test

To obtain an appropriate antibiotic resistant gene marker, S. platensis was subjected to antibiotic sensitivity test. In the preliminary study, S. platensis was introduced to the antibiotic sensitivity test with 5 types of antibiotics, namely, ampicillin (50, 100, 200, 300-1000 (μg/ml), Chloramphenicol (1, 2, 3, 4, 5, 10, 15, 20 (g/ml), kanamycin (50, 100, 200, 300-1000 (μg/ml), rifampicin (50, 100, 200, 300-1000 (g/ml), and penicillin (20, 40, 60, 80, 100 (μg/ml). It was found that S. platensis was resist to ampicillin, kanamycin at the minimal inhibitory concentration of 1000 (μg/ml and to penicillin at 10 (μg/ml. However, it was sensitive to cholramphenicol and rifampicin at the minimal inhibitory concentration of 1 and 500 (μg/ml, respectively. Since S. platensis was most sensitive to chloramphenicol among all the antibiotics tested, chloramphenicol resistant gene was chosen as a dominant selectable marker in transforming S. platensis to chloramphenicol resistance.

B.2 Spheroplast formation and regeneration

Spheroplast of S. platensis was generated essentially by the method of Lanfaloni et al., (1989). Filaments of S. platensis at the various phase of growth were washed with TNE buffer (10mM Tris-HCl, 1 mM EDTA, 10 mM NaCl, pH 7.6) twice and resuspended in 7 ml of TNE buffer and 3 ml of 5M NaCl (1.5M final concentration), shaking gently for 5 min. Washed filaments were collected by low centrifugation at

6,000 rpm, 5 min. Cell wall of the filaments were partially removed by incubating the filament pellet in 5 ml of solution A (1mM Tricine-NaoH, 2mM MgCl2, 2mM CaCl2, 40mM NaCl, 10% PEG4000, pH 8) at room temperature by shaking slowly. Spheroplast formation was observed under microscope every 15 min. The spheroplast formation was terminated by collecting the spheroplast pellet at 1,000 rpm., room temperature for 5 min. Zarrouk medium was added to the pellet for 1 ml and the spheroplasts were regenerated on Zarrouk agar under low light intensity (30 (E.m-2.s-1), at room temperature. It was found that protplasts of S. platensis could be obtained within 2 hours and the filaments of S. platensis were regenerated from spheroplasts within 2 weeks. The efficiency of regeneration was 40-75% depending on the age of the cells. The suitable age of S. platensis for spheroplast formation was in the early log phase. At this stage of growth its cell wall could be removed more easily than the cells at later stage in lysing solution.

B. 3 Chemical transformation of S. platensis

The chemical transformation was performed essentially by the method of Yelton (1984). 50 (l ml of spheroplasts at the concentration of 108/ml in Zarrouk medium was mixed with 5 (l of plasmid DNA (50 (g). Then 25 (l of PEG solution at various concentration (either 20, or 40, or 60% polyethylene glycol 4000, 10 mM CaCl2, 10 mM Tris-HCl, pH 7.5) was added to the mixture. After incubation on ice for 20 min., the mixture was added with 500 (l of PEG solution and incubated at room temperature for either 5, or 10, or 30, or 60 min. Then, the cells were collected by centrifugation briefly at 1000 rpm. The pellet was resuspended in 1 ml. of Zarrouk medium and the cells were incubated at room temperature under low light intensity (35 (E.m-2.s-1) for 24 hours. Subsequently, the transformation mix was spread on Zarrouk agar medium containing chloramphenicol at the concentration of 10 (µg/ml).

The results demonstrated that after chemical transformation with PEG at various concentration, spheroplast regenerated well. It was found that there was no significance difference between the concentration of PEG use in the % regeneration of spheroplast. The % regeneration were 51, 42, and 46 when the concentration of PEG were 20, 40 and 60, respectively. However, % regeneration varied between the incubation time of the transformation mix in 40 % PEG solution after administration on ice for 20 min. It was found the % regeneration were reduced to 43, 33, and 27 after incubation for 10, 30, and 60 min., respectively.

B. 4 - Isolation of Chromosomal DNA

500 ml of 3-4 days old culture of *Spirulina platensis* (OD_{600}=1) will be used for DNA extraction. Filaments will be harvest by centrifugation, washed once with sterile distilled water and suspended in 2 ml lysis buffer (25% sucrose, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5). The suspension is then frozen at -70°C for 30 minutes and thawed at 37°C. Lysozyme is added to a final concentration of 1 mg/ml and the suspension is incubated at 37°C for 30 minutes. SDS (final concentration 10 mg/ml) and Proteinase K (final concentration 0.1 mg/ml) are added and the mix is incubated overnight in a water bath set at 55°C. An equal volume of phenol-chloroform-isoamylalcohol is added and mixed gently for 10-15 minutes. The aqueous phase is collected by centrifugation and RNAse treated (final concentration of DNAse free RNAse 50 mg/ml) 37°C for 1 hr. This is followed by extraction with phenol-chloroform-isomyl alcohol and chloroform-isoamyl alcohol. Sodium acetate is added to a final concentration of 0.3 M. The DNA is collected by ethanol precipitation with two

volumes of ice cold ethanol. The pellet is rinsed with ice-cold 70% and 100% ethanol, air-dried, dissolved in TE (10 mM Tris-HCl, pH 8., 1.0 mM EDTA) and stored at -20°C.

B. 5 - A rapid procedure for isolation of plasmids from filamentous cyanobacteria

Solutions:
1) Wash solution - 120 mM NaCl, 50 mM EDTA, pH 8.0
2) Lysis solution - 25% W/V sucrose, 100 mM EDTA, 50 mM Tris , pH 8.0, 10 mg/ml lysozyme
   (The lysozyme should be fresh each time)
3) Detergent solution - 1% brij 58. 0.4% sodium deoxcholate, 0.2 N NaOH
4) KHAc - 5 M KHAc, pH 4.8
5) T. E. buffer - 10 mM Tris, 1 mM EDTA, pH 8.0
6) T 1/10 E buffer - 10 mM Tris, 0.1 mM EDTA, pH 8.0
7) RNAse 1 mg/ml heat treated (90°C, 10 min.)

Procedure

1) 100 ml of culture in the late logarithmic phase of growth are harvested by centrifugation (Sorvall SS34 rotor, 6,500-10,000 rpm, 10 min.)
2) Pellet is resuspended in 'wash solution" and centrifuged again. Each tube should contain ~200 µl of packed pellet.
3) The pellet undergoes fast freezing in an ethanol dry ice bath and then thawed at 37°C for 5 min. 400 µl of "lysis solution" is added to each tube and mixed well. At this stage, the total volume is ~600 µl (referred further as the original volume). The tubes are incubated at 37°C for 40-60 min. (till the filaments are broken into separate cells).
4) At this point, 1200 µl (2 volumes of the volume already in the tube) of "detergent solution" are added, and the <u>two phases are mixed</u>.
5) The suspension is heat shocked by incubation for 5 min. at 0°C, then 2 min. at 42°C and back again for 5 min. at 0°C. After this treatment, the suspension should be clear.
6) 900 µl of precooled KHAc (i.e. 1.5 times the original volume in each tube) is added and mixed. The tubes are placed on ice for 5 min. Then centrifuged for 5 min. at 40°C. For Eppendorf tubes, a microfuge is used. For other tubes, the Sorvall centrifuge 15,000 rpm, 5 min., is used. Cells debris protein and chromosomal DNA are removed in the pellet.
7) The supernatant is transferred into a clean tube and 3 M Tris pH 8.0 is added to a final concentration of 0.5 M. Then, one volume of cold isopropanol is added to each tube, mixed, and immediately immersed in dry ice to cool the tube rapidly, <u>but not to freeze it</u>. At this point, the tubes can be put in the freezer overnight. The procedure can also be continued immediately after cooling the tubes.
8) The tubes are centrifuged for 5 min. Plasmid DNA is precipitated from the supernatant with the isopropanol and is pelleted.
9) The pellet is washed gently with 70% ethanol (the pellet should remain intact at the bottom of the tube) and then dried thoroughly by putting the tubes in a desicator under vacuum.
10) The pellet is resuspended in 50 µl of T.E. buffer. At this point, pellets are united into one tube (if there were more than one tube for each kind of alga,).
    3 M Tris is added to the resuspended pellet to a final concentration of 0.5 M and two volumes of isopropanol are added and mixed. This is immediately cooled down, centrifuged, and the pellet washed with ethanol 70% and dried as before.

11) The final suspension of the pellet is done in 50 µl of T 1/10E buffer + 2 µg/ml RNAse.
On 0.7% agarose gel, it is sufficient to load 30 µl of the sample.

Useful advice

It is convenient to use Eppendorf tubes for the whole procedure. If these 1.5-ml tubes are used, it is necessary to divide the material in them into a double number of tubes before step no. 4 and at step no. 7. The added volumes of Detergent solution and KHAc should be halved accordingly, so the ratio among algal pellet+lysis solution; detergent solution; KHAc stays 1: 2: 1.5

At step 10, the pellets are united into one or two Eppendorf tubes. The volume of the resuspended pellet in each tube should not exceed 400 µl. Everything else is exactly as written in the protocol.

B.6 - Electro-poration of *Spirulina*

Cells were grown for 5 days on shaker (100 ml Zarouk medium, 30oC). Reaching a concentration of 0.5 $O.D._{560nn}$ (no more).

Cells are harvested by centrifugation (10', 12000 RPM, 4oC.

Cells are re-suspended in 10 ml of HEPES 1mM, pH 7.2,

Centrifuge and washed again. (total washes 3 times).

Cells are re-suspended (last wash) in HEPES (5 ml) and counted. Cell concentration is adjusted to a final concentration of $7\ 10^{6}$ trichomes per ml.

Cells are kept on ice.

Plasmids for electroporation at a concentration of 10µg / 100µl dissolved in HEPES.

Electroportiom mix prepared in ependorf tubes

40µl of cells + 100µl of plasmid suspension, mixed and transferred to pre cooled electroporation cuvettes.

The mix is kept on ice for 10 min. Mix the solution before placing in electroporator.

Elctroporation conditions :

4 KV/ cm', 0.8KV, 200 ohm, 25µF

Cuvett used 0.2 cm electrode. gap . 50

Time constant is 4.5 - 5.

After electroporation add immediately 1ml of modified Zarouk medium (5 g/l $NaHCO_3$) mix gentle,

Transfer, using sterile Pasteur pipette, electroporated cells to 1.5 ml Ependorff tubes and incubate for 48 h, 30oC, low light. Mix once a day.

0.1 ml aliquots are plated on agar plates containing 1 or 2 µg/ml of CAP.

Preparation of agar plates

For platting small petri dishes are used containing 5 ml of agar with Zarouk medium and the indicated CAP concentration. plates are prepared 24-48 h before use

CAP is added from a stock solution 0.25 g / 5 ml of 100% Et-OH

20 µl diluted in 980 µl of DDW this 1 ml is added to 100 ml of agar & Zarouk medium mix for plates with final concentration of 1 µg CAP / ml. Or twice the amount for the 2µg/ml plates.

From each electroporation treatment 4 x 0.1 ml are plated on 1 µg/ml and 4 x 0.1 ml are plated on 2 µg/ml CAP containing agar plates. To estimate viability 0.1 ml is plated on agar without CAP.

Control treatments

1 - Electroporated cell without DNA

2 - Cells incubated as treated cells without DNA and without elctroporation.
Results can be monitored microscopically after 5 days of incubation.
After 10-12 days plates are over layered with 1ml of 0.5 μg/ml CAP.
Transfer to liquid
After 3-4 weeks on agar plates a singl trichome is transfered to liquid medium (1ml 0.5 μg/ml CAP). after growth can be observed the culture is transfered to 5, 50. ml medium containig 0.5 μg/ml CAP. After good groth is observed CAP concentration is increase gradualy to 1.2.3.4 μg/ml CAP.

B.7 Plasmid construction.

In order to construct a plasmid that will contain a promoter of a Spirulina gene and a good selection marker (chloramphenicol resistance) to following procedure was used. Chromosomal DNA of S. platensis was isolated a and partially digested by Sau3AI. Fragments in the size of 2-4 kbp were isolated from the agarose and ligated into a promoter less plasmid pKK232-8 at its BamH1 site. (see fig 1 for illustration)The recombinant plasmid was used to transform *E.coli* . Transformants were selected on LB plates supplemented with chloramphenicol. Plasmid were isolated to verify that they contain an insert of 2-4 kbp DNA. This plasmids will be used for electroporation.

B.8 Testing of *Spirulina* strain extracts for restriction endonuclease activity.

Spirulina platensis strains C1, Italy, Cambridge IAM-M135 were tested for the presence of restriction enzymes. Exponentially growing cultures of *Spirulina* were harvested by centrifugation, washed once with an equal volume of 0.1 M sodium phosphate buffer and resuspended in 1/10 of the original volume of the same buffer. The suspension was sonicated using the sonicator with a fine-tip probe. The sample was centrifuged and 2-5 ul aliquots of the clear cell free suspension were used to digest lambda DNA in typical restriction digests. Samples were run on agarose gels and checked for the restriction patterns of the lambda DNA. Extracts of most of the *Spirulina* strains caused extensive digestion of lambda DNA. Only the extract from *S. platensis* IAM-M135 (hereinafter referred to as 'K' strain) showed minimal digestion of lambda DNA and resulted in the least number of bands. The *S.platensis* K extract linearised plasmids pUC19, pBR322 and pKK232-8 but did not digest the plasmid pACYC184. (see fig 2,3,4,5) It seems that *S.platensis* K probably has only a single restriction endonuclease which has very few sites in lambda DNA and single sites in pUC19, pBR322 and pKK232-8. This finding is making the K strain a good candidate for further transformation studies.

C). Scientific Impact of Collaboration:

The collaborating scientists have contributed significantly to the project in developing the plasmid to be used in the transformation. They have also performed all the electroporation optimization conditions and all the other tasks as specified in the original working program.
During the first year the investigators met twice and the next meeting is set to May 97.

D) Description of Project Impact : It is to early to evaluate the impact. Most of the methodology developed is in use by graduate students in KMITT as part of their research projects.
Others. At this stage no major problems has been faced the required changes in working plans , budget or task allocations.

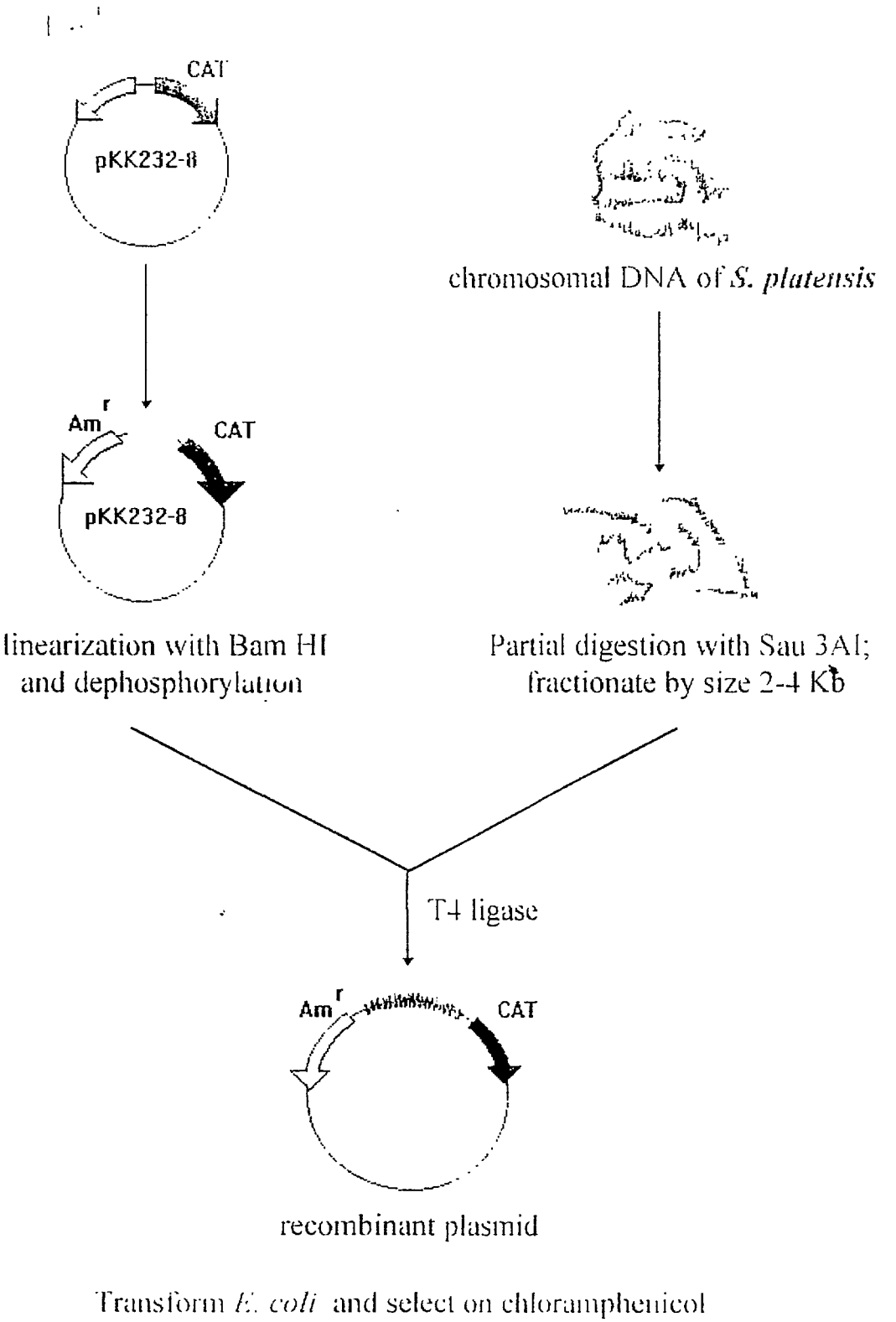

Figure 1 Cloning of promoter from *S. platensis* in *E. coli* with the aid of pKK232-8

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Fig. 2: Electrophoretogram showing the results obtained when cell-free extracts of *S. platensis* C1 were tested for endonuclease activity. Lane 1 contains the molecular weight markers: λ DNA cut by *Hind* III. Lanes 2, 3 and 5 contain the controls: λ DNA uncut, λ DNA uncut + high salt buffer (100 mM NaCl) and λ DNA uncut + medium salt buffer (50 mM NaCl) respectively. Lanes 4 and 6 contain the experimental samples: λ DNA uncut incubated with high salt (HS) buffer and 2 µl *S. platensis* C1 cell-free extract and λ DNA incubated with medium salt (MS) buffer + 2 µl *S. platensis* C1 cell-free extract.

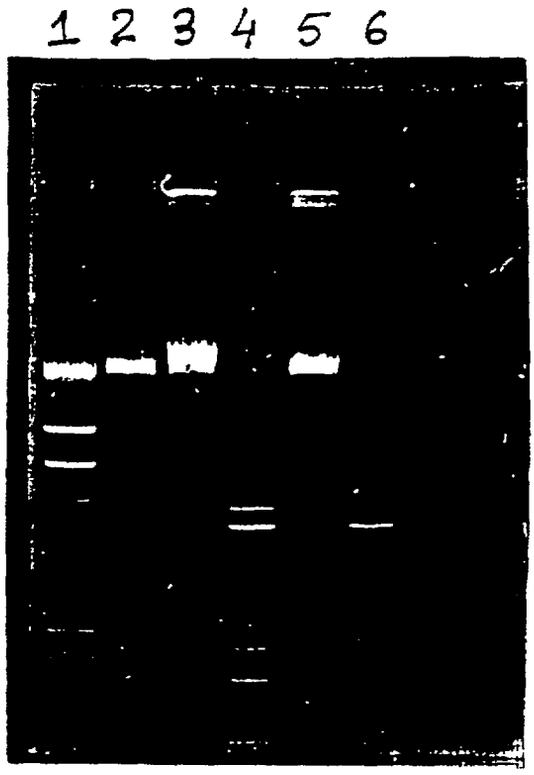

Fig. 3: Electrophoretogram showing the results obtained when cell-free extracts of *S. platensis* (Cambridge) were tested for endonuclease activity. Lane 1 contains the molecular weight markers: λ DNA cut by *Hind* III. Lane 2 contains the control: uncut λ DNA. Lanes 3 and 4 contain the experimental samples: λ DNA uncut incubated with high salt buffer (100 mM NaCl) and 2 µl *S. platensis* Cambridge cell-free extract and λ DNA uncut incubated with medium salt buffer (50 mM NaCl) and 2 µl *S. platensis* Cambridge cell-free extract.

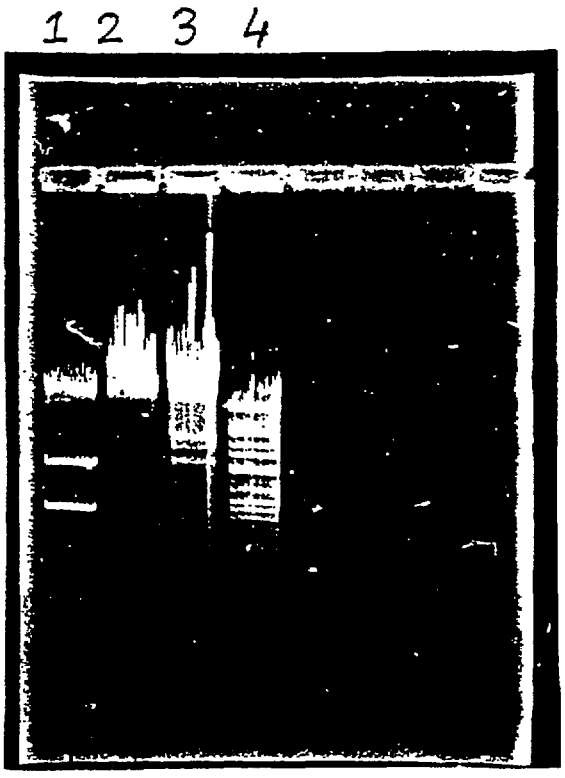

Fig. 4: Electrophoretogram showing the results obtained when cell-free extracts of *S. platensis* (Italy) were tested for endonuclease activity. Lane 1 contains the molecular weight markers: λ DNA cut by *Hind* III. Lane 2 contains the control: uncut λ DNA. Lanes 3 and 4 contain the experimental samples: λ DNA uncut incubated with high salt buffer (100 mM NaCl) and 2 µl *S. platensis* Italy cell free extract and λ DNA uncut incubated with medium salt buffer (50 mM NaCl) and 2 µl *S. platensis* Cambridge cell-free extract.

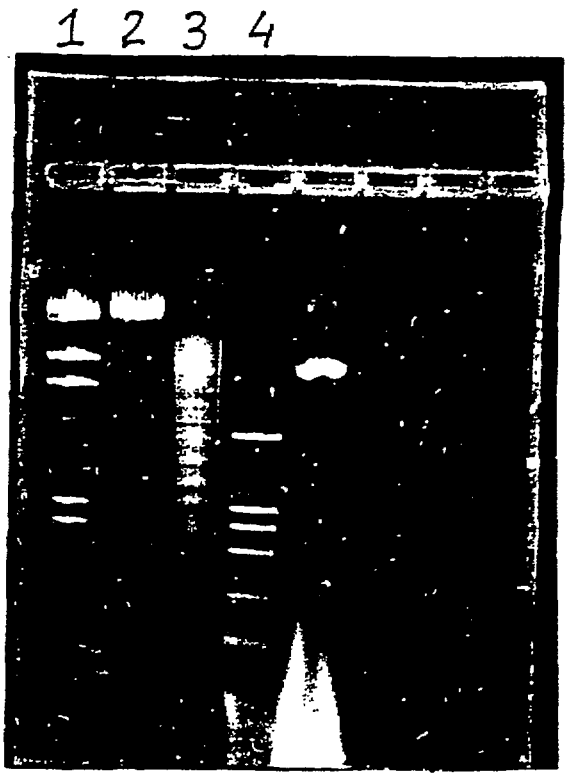
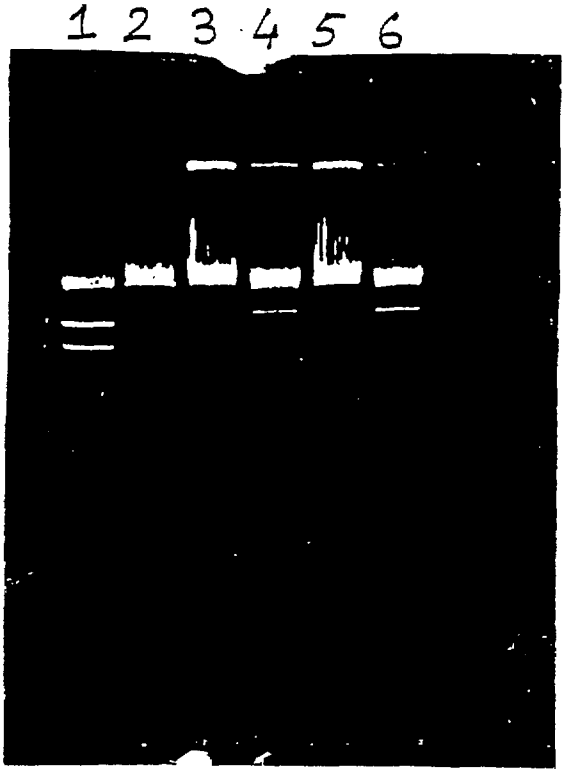
Fig. 5: Electrophoretogram showing the results obtained when cell-free extracts of *S. platensis* K were tested for endonuclease activity. Lane 1 contains the molecular weight markers: λ DNA cut by *Hind* III. Lanes 2, 3 and 5 contain the controls: λ DNA uncut, λ DNA uncut + high salt buffer (100 mM NaCl) and λ DNA uncut + medium salt buffer (50 mM NaCl) respectively. Lanes 4 and 6 contain the experimental samples: λ DNA uncut incubated with high salt (HS) buffer and 2 µl *S. platensis* K cell-free extract and λ DNA incubated with medium salt (MS) buffer + 2 µl *S. platensis* K cell-free extract.