## ГЕНОМИКА.
## ТРАНСКРИПТОМИКА

# EFFICIENCY OF LIGATION-MEDIATED PCR AND TAIL-PCR METHODS FOR ISOLATION OF *RbcS* PROMOTER SEQUENCES FROM GREEN MICROALGAE *Ankistrodesmus convolutus*

© 2012   Tran Thanh[a, b], Vu Thi Quynh Chi[a,b], Mohd Puad Abdullah[a],
Hishamuddin Omar[c], Suhaimi Napis[a,*]

[a]*Department of Cell & Molecular Biology, Faculty of Biotechnology & Biomolecular Sciences,
Putra-University Malaysia, 43400 UPM-Serdang, Selangor Darul Ehsan, Malaysia*
[b]*Rubber Research Institute of Vietnam, 236 Bis Nam Ky Khoi Nghia Street, District 3,
Ho Chi Minh City, Vietnam*
[c]*Department of Biology, Faculty of Science, University of Putra Malaysia,
43400 UPM-Serdang, Selangor Darul Ehsan, Malaysia*

**Isolation of promoter sequences from known gene sequences is a tedious task in genome-related research. An efficient method of obtaining the promoter sequences is necessary in order to successfully use targeted promoters for genetic manipulations. Here, efficiency and usefulness of two PCR-based methods, namely: ligation-mediated PCR and thermal asymmetric interlaced (TAIL) PCR, for isolation of promoter sequences of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*) gene from green microalgae *Ankistrodesmus convolutus (A. convolutus)* were evaluated. The results showed that the amplification efficiency of TAIL-PCR was higher than that of the ligation-mediated PCR method, i.e. the amplified promoter fragments of 1.2 and 0.8 kb in length or promoter sequences of 813 and 606 bp (after eliminating the unreadable sequences). The use of TAIL-PCR described here presents a low cost and efficient strategy for the isolation of promoter sequences of known genes, especially in GC-rich regions, and species with little or no available genome information such as *A. convolutus*.**

## INTRODUCTION

In organisms whose genome has not yet been sequenced or little or no genome information is available, isolation of genes of interest remains a tedious task even when these genes are tagged by insertion mutagenesis or express sequence tags [1]. Similarly, in these organisms, isolation of promoter sequences of known genes, especially genes in the GC-rich regions encounters the same challenge. Conventionally, a standard method for isolating the promoter sequences from known DNA sequences is through construction and screening of genomic libraries using cDNA probes. The conventional method is less desirable as it is laborious and time-consuming due to the meticulous experimental procedures. As a result, several PCR-based methods have been developed and applied in experiments to isolate gene promoter sequences. Two of the popular methods are ligation-mediated PCR [2, 3] and thermal asymmetric interlaced (TAIL) PCR [4, 5].

For the ligation-mediated PCR technique, a few commercial kits for genome walking have been devel-

oped including the GenomeWalker kit ("Clontech Laboratories Inc.", USA) and TaKaRa LA PCR *in vitro* Cloning Kit ("Takara Bio Inc.", Japan), which require restriction digestions of genomic DNA and ligation of a double-strand DNA cassette. Unfortunately, the GenomeWalker kit is ready to use only for human, mouse and rat genomes [6]. In the case of algae, preparation of a high-quality DNA extracts which would be completely digested by endonucleases was reported to be difficult [7, 8]. Michiels et al. [9] stated that the effectiveness of ligation-mediated PCR is diminished by undesirable amplification of PCR products that are flanked by adaptor sequences at both ends. Furthermore, commercial kits are also laborious, time-consuming and costly to use. Therefore, several scientific groups were engaged in development of simpler, more reliable and cost-effective methods for the isolation of promoter sequences. TAIL-PCR is currently one of the most efficient methods for the isolation of flanking regions from known DNA sequences. This method relies on PCR amplification with use of a short arbitrary degenerate primer coupled to nested known sequence-specific primers.

* E-mail: suhaimi@putra.upm.edu.my; suhaiminapis@gmail.com

**Table 1.** DNA sequences of primers used

| Primer | Sequence (5'−3') |
|--------|------------------|
| RSP1 | CGCGATCTGCTCGTCGTTCA |
| RSP2 | TTCACGGGCTGCCACACCAT |
| RSP3 | TGAGACGGAGGGCCATTGCT |
| C1 | GTACATATTGTCGTTAGAACGCGTAATACGACTCA |
| C2 | CGTTAGAACGCGTAATACGACTCACTATAGGGAGA |
| AD1 | (A/C/G/T)TCGA(C/G)T(A/T)T(C/G)G(A/T)GTT |
| AD2 | (A/C/G/T)GTCGA(C/G)(A/T)GA(A/C/G/T)A(A/T)GAA |
| AD3 | (A/T)GTG(A/C/G/T)AG(A/T)A(A/C/G/T)CA(A/C/G/T)AGA |
| AD4 | AG(A/T)G(A/C/G/T)AG(A/T)A(A/C/G/T)CA(A/T)AGG |
| AD5 | (A/T)GC(A/C/G/T)AGT(A/C/G/T)AG(A/T)A(A/C/G/T)AAG |
| AD6 | A(A/T)GCA(A/C/G/T)G(A/C/G/T)C(A/T)GA(A/C/G/T)ATA |
| AD7 | A(A/T)GCA(A/C/G/T)G(A/C/G/T)C(A/T)GA(A/C/G/T)ATA |

To our knowledge, although TAIL-PCR has been successfully applied to isolate promoter sequences of genes of interest from a number of organisms, only one report on usage of TAIL-PCR for isolation of promoter sequences from algae has been published [5]. At the time the present study was initiated, there was no report on comparative studies of the efficiency of these methods in isolating promoter sequences from algae and other organisms.

The present study has two objectives, which are: (i) to evaluate the efficiency of TAIL-PCR and ligation-mediated PCR methods, as well as to examine the versatility of TAIL-PCR, and (ii) to isolate a strong native promoter, which could be used to construct expression vectors with high efficiency for genetic engineering of green microalgae *A. convolutus* and other algae species.

## EXPERIMENTAL

**Algae culture and genomic DNA isolation.** Green microalga *Ankistrodesmus convolutus (A. convolutus)* was collected from axenic freshwater grown at 25°C and 150 rpm shaking in an incubator shaker under 12:12-h light-dark cycle, and maintained in Bold's basal medium [10]. *A. convolutus* genomic DNA (gDNA) was extracted as described in our previous report [11].

**Primer synthesis.** The nested specific primers, arbitrary degenerate (AD) primers and cassette primers used throughout the study are shown in table 1. Based on the known sequence of full-length *AcRbcS* cDNA (GenBank no. ACQ99367), which was reported in our previous study [11], three specific primers (RSP1, RSP2 and RSP3) in nested positions close to the 5'-end of the coding regions of *AcRbcS* were designed and synthesized. As calculated with the formula of Mazars et al. [12], melting points ($T_m$) for primers designed should be higher (60−62°C) than those (45−48°C) for AD primers.

**Isolation of *AcRbcS* promoter sequences using ligation-mediated PCR.** In order to isolate the promoter region of *AcRbcS* gene using the ligation-mediated PCR technique, a commercial genome walking kit, TaKaRa LA PCR *in vitro* Cloning Kit, was used. The method involves digestion of genomic DNA with suitable restriction enzymes, followed by ligation of the restriction fragments with adapters, which can be amplified by PCR. Using the first-specific primer that anneals to a complement sequence and a cassette primer C1 in this kit, which hybridizes to the adapter, an unknown flanking genomic sequence can be amplified. In order to improve the specificity, the second PCR was carried out using a sequence-specific primer (Cassette primer C2 in this kit), which anneals to the adapter, and the previous PCR product as a template. The manipulations were carried out according to the manufacturer's instructions, which are briefly described in fig. 1. Approximately 7.5 μg of genomic DNA was digested separately with 5 different restriction enzymes EcoRI, HindIII, PstI, SalI and XbaI. After purification, the digested genomic DNA was dissolved in 10 μL of sterilized distilled water (ddH₂O) and used for the cassette ligation reaction. The reaction was performed by combining 5 μL of digested products, 2.5 μL (20 ng/μL) of appropriate cassette, 15 μL of ligation solution I and 7.5 μL of ligation solution II. The mixture was then incubated at 16°C for 1 h and precipitated by 0.1 volume of 3 M sodium acetate and 2.5 volumes of cold absolute ethanol overnight. After centrifugation, the products were suspended in 5 μL of ddH₂O. In the first PCR reaction, approximately 100 ng of DNA-cassette ligation product was denatured at 94°C for 10 min before adding 1X LA PCR buffer II (Mg2⁺ plus), 400 μM dNTPs, 2.5 U LA *Taq* polymerase, 0.2 μM C1 as a forward primer, 0.2 μM first-specific primer RSP2 as a reverse primer, and ddH₂O to a final volume of 50 μL. Thirty cycles of 15 s denaturing at 98°C, 30 s annealing at 60°C and
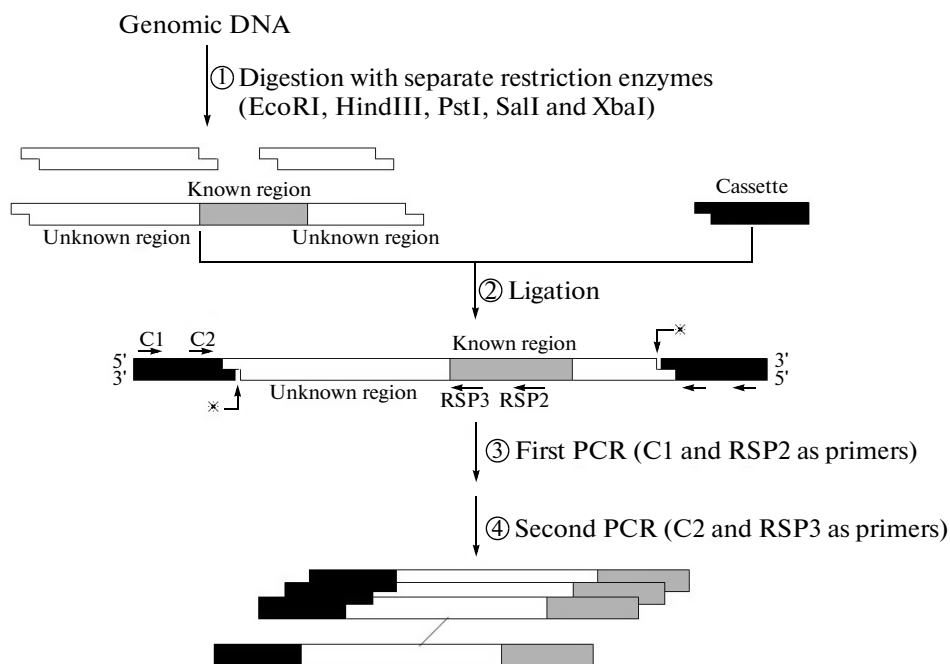
Genomic DNA

① Digestion with separate restriction enzymes
(EcoRI, HindIII, PstI, SalI and XbaI)

Known region

Cassette

Unknown region                      Unknown region

② Ligation

C1  C2                                    Known region                                    ✳

5'                                                                                                          3'
3'                                                                                                          5'

✳                    Unknown region          RSP3  RSP2

③ First PCR (C1 and RSP2 as primers)

④ Second PCR (C2 and RSP3 as primers)

**Fig. 1.** Schematic diagram of the ligation-mediated PCR method with use of the TaKaRa LA PCR *in vitro* Cloning Kit.

2 min extension at 72°C were completed, followed by a final extension at 72°C for 10 min. The second PCR was conducted using 50 ng of the first PCR product as a template, 0.2 μM second-specific primer RSP3 as a reverse primer, 0.2 μM C2 as a forward primer. The other reagents as well as the cycling conditions used for the second PCR were the same as the first PCR. All of the PCR reactions were carried out using a PTC-200 Peltier Thermal machine ("MJ Research", USA). After separating the products by 1.2% (w/v) agarose gel electrophoresis, target DNA bands were purified by a QiaquickGel Extraction Kit ("Qiagen", Germany), cloned into a pGEM-T Easy Vector System ("Promega", USA) and sequenced commercially.

**Isolation of *AcRbcS* promoter sequences using TAIL-PCR.** In order to allow chromosome walking beyond the known gene sequences into the unknown 5'-flanking regions, an alternative method called thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR), originally described by Liu and Whittier [13], was employed to isolate the promoter region of known DNA sequences. By using a set of nested known sequence-specific primers with a $T_m$ of more than 65°C in consecutive reactions together with a shorter (15−16 nucleotides) AD primer with a lower $T_m$ (about 45°C) and 64−256 folds of degeneracy, the relative amplification efficiencies of specific and non-specific products can be thermally controlled [13, 14].

Basically, one low-stringency PCR cycle was carried out to create an annealing site adapted for the arbitrary primer within the unknown target sequence flanking to the known segment. This sequence was then amplified preferentially over nonspecific products by interspersion of high-stringency PCR cycles with reduced-stringency PCR cycles. In this study, TAIL-PCR amplification was carried out using the nested sequence-specific primers RSP1, RSP2 and RSP3 as reverse primers pairing with AD primers as forward ones (fig. 2). The primary PCR reaction mixture consisted of *Taq* polymerase buffer supplied with the enzyme, 200 μM dNTPs, 0.2 μM RSP1 primer, 5 μM of any one of the AD primers, 1 U of HS *Taq* polymerase ("Takara Bio Inc.", Japan), and 50 ng of genomic DNA. The secondary PCR reaction consisted of *Taq* polymerase buffer supplied with the enzyme, 200 μM dNTPs, 0.8 U of HS *Taq* polymerase, 0.2 μM RSP2 primer, 4 μM of the AD primer used in the primary reaction, and 50-fold dilution of the primary PCR product. The tertiary PCR mixture consisted of *Taq* polymerase buffer supplied with the enzyme, 200 μM dNTPs, 0.5 U of HS *Taq* polymerase, 3 μM of the AD primer used in the previous reactions, 0.3 μM RSP3 primer, and 10-fold dilution of the secondary PCR product. The TAIL-PCR protocol was performed according to the method described by Liu and Whittier [13] with some minor modifications. The thermal cycling conditions are summarized in table 2. Finally, the products of the three PCRs were separated by 1.2% (w/v) agarose gel electrophoresis, and the target fragments were purified by a QiaquickGel Extraction Kit ("Qiagen", Germany) before cloning into the pGEM-T Easy Vector System ("Promega", USA) and then sequenced commercially to get the promoter sequence of *AcRbcS*.
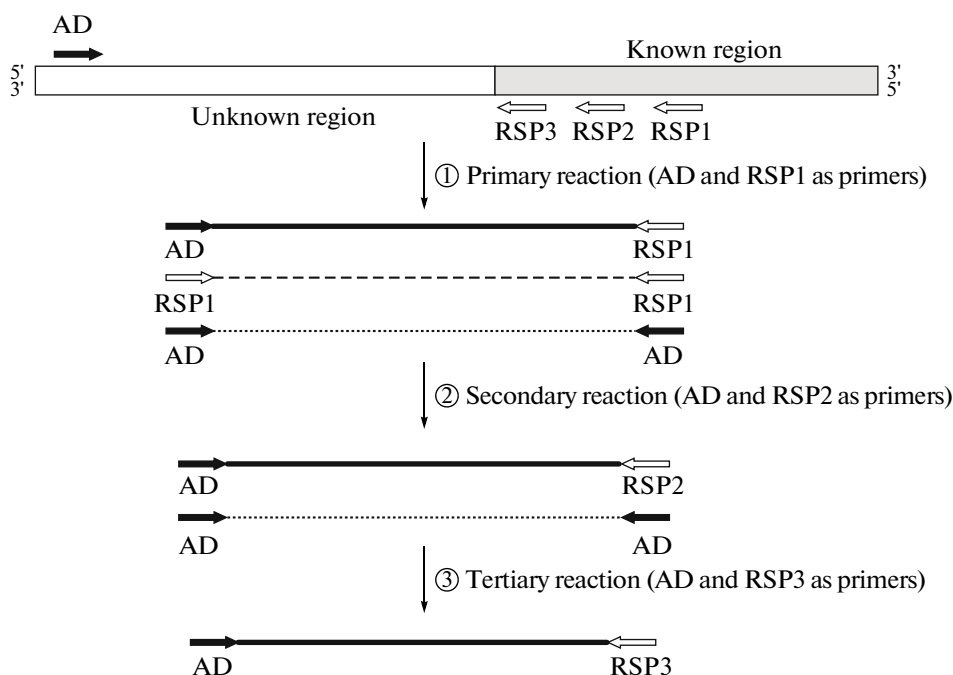
**Fig. 2.** Schematic diagram of TAIL-PCR showing the amplification of the target (full line) with non-targeted (dashed line) products.

## RESULTS AND DISCUSSION

Isolation of high molecular weight DNA from algae that is suitable for the isolation of promoters, construction of a genomic library, as well as digestion with restriction endonucleases, cloning, hybridization and PCR amplification is considered as an important issue [8, 15]. The diversity of cell wall, storage and secondary compounds further contribute to the difficulties and complications of isolation of high quality genomic DNA, especially in high quantities. Therefore, a method that works with one algae species will often fail with others. In this study, the genomic DNA extract of sufficiently high quality, with no sign of degradation (data not shown), in good supply has been obtained. The isolated DNA sample was perfectly suitable for further manipulations on the isolation of *A. convolutus RbcS* promoter sequences by using PCR-based methods.

In order to isolate the promoter sequences of the *A. convolutus RbcS* (*AcRbcS*) gene using the ligation-mediated PCR method, primers RSP2, RSP3 and five different templates (resulting from digestion of ge-

**Table 2.** Cycle settings used for TAIL-PCR

| Reaction (Primer combination) | Number of cycles | Thermal cycling conditions |
|---|---|---|
| Primary (ADs/RSP1) | 1 | 95°C, 1 min; 95°C, 1 min |
| | 5 | 97°C, 1 min; 62°C, 1 min; 72°C, 2.5 min |
| | 1 | 97°C, 1 min; 25°C, 3 min; ramping to 72°C, over 3 min; 72°C, 2.5 min |
| | 15* | 97°C, 30 s; 68°C, 1 min; 72°C, 2.5 min |
| | | 97°C, 30 s; 68°C, 1 min; 72°C, 2.5 min |
| | | 97°C, 30 s; 44°C, 1 min; 72°C, 2.5 min |
| | 1 | 72°C, 7 min |
| Secondary (ADs/RSP2) | 12* | 97°C, 30 s; 64°C, 1 min; 72°C, 2.5 min |
| | | 97°C, 30 s; 64°C, 1 min; 72°C, 2.5 min |
| | | 97°C, 30 s; 44°C, 1 min; 72°C, 2.5 min |
| | 1 | 72°C, 7 min |
| Tertiary (ADs/RSP3) | 20 | 97°C, 1 min; 44°C, 1 min; 72°C, 2.5 min |
| | 1 | 72°C, 7 min |

* These are nine-thermal-segment supercycles, each consisting of two high-stringency and one reduced-stringency cycle.
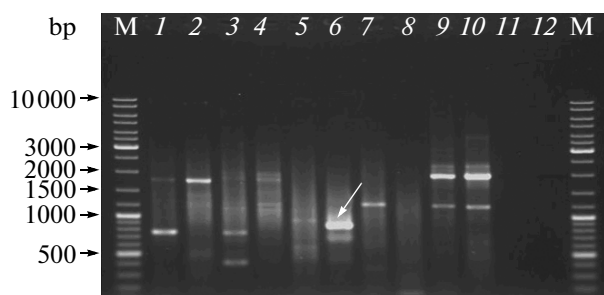
**Fig. 3.** Electrophoresis of products of ligation-mediated PCR carried out with use of the TaKaRa LA PCR *in vitro* Cloning Kit. The recovered fragment is marked with a white arrow. Line M − 1 kb DNA ladder; *1* and *2* − products of the first and second PCR using EcoRI-restricted gDNA as a template; *3* and *4* − products of the first and second PCR using HindIII-restricted gDNA as a template; *5* and *6* − products of the first and second PCR using PstI-restricted gDNA as a template; *7* and *8* − products of the first and second PCR using SalI-restricted gDNA as a template; *9* and *10* − products of the first and second PCR using XbaI-restricted gDNA as a template; *11* and *12* − negative controls of the first and second PCR.
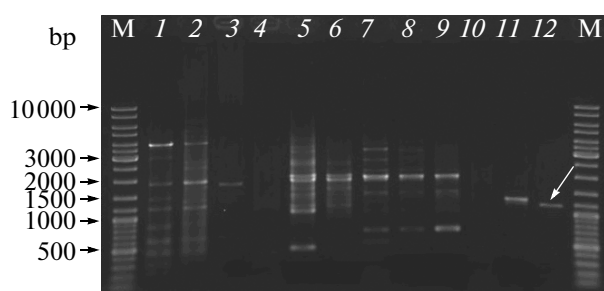


**Fig. 4.** Triple electrophoresis of the TAIL-PCR products. The recovered fragment is marked with a white arrow. Lane M − 1-kb DNA ladder; *1, 2* and *3* − products of the primary, secondary and tertiary TAIL-PCR using the AD1 primer; *4, 5* and *6* − products of the primary, secondary and tertiary TAIL-PCR using the AD3 primer; *7, 8* and *9* − products of the primary, secondary and tertiary TAIL-PCR using the AD4 primer; *10, 11* and *12* − products of the primary, secondary and tertiary TAIL-PCR using the AD7 primer.

nomic DNA with EcoRI, HindIII, PstI, SalI and XbaI) were used. Among the amplification products, the DNA fragment of approximately 800 bp in length (fig. 3, *lane 6*), obtained from the second PCR product using PstI-restricted gDNA as a template, was recovered, cloned into the pGEM-T Easy Vector System and sequenced. Unreadable segments of the obtained DNA sequence were removed, and the residual 606 bp fragment had a 44 bp sequence at the 3'-end, which perfectly overlapped with the sequence at the 5'-end of *AcRbcS* cDNA. This indicated that the fragment obtained by ligation-mediated PCR method was undoubtedly a fragment of the *AcRbcS* gene promoter.

Using this method allowed us to obtain good amplification only in one of the five templates derived from restriction digestion of genomic DNA with different enzymes. As may be supposed, too many or too few restriction sites for the selected enzymes in the genome of *A. convolutus* are presented, so the restricted fragments were too small to give a specific amplification or the effective size of the digested DNA fragment was too long to be amplified. In short, finding suitable restriction sites and subsequently ligating them is often a trick, when using the ligation-mediated PCR method is unfeasible because the information on restriction sites of the fragment is usually not available in advance. In addition, there is no guarantee that the digestion with a particular restriction enzyme will be successful, thus making it necessary to try different restriction enzymes. The success of the ligation-mediated PCR to amplify target products greatly depends on the quality of the genomic DNA and location of the restriction sites in the genome.

Meanwhile, for the second experiment using TAIL-PCR to amplify the *AcRbcS* gene promoter sequences, a total of 7 AD primers were screened (data not shown). Out of them, four primers were found to produce desired products. These AD primers were used in combination with a set of nested specific primers (RSP1, RSP2 and RSP3). Four groups of amplification products were obtained from three rounds of TAIL-PCR, among them, the products obtained using the AD7 primer were the best (fig. 4). As a result, the DNA fragment of approximately 1.2 kb in length (fig. 4, *lane 12*), obtained from the tertiary TAIL-PCR products amplified by the AD7 primer, was recovered, cloned into the pGEM-T Easy Vector and sequenced. After eliminating the unreadable sequences, a fragment of 813 bp was obtained and then aligned with the isolated *AcRbcS* promoter fragment as mentioned above using the Bioedit 7.0.5 Software [16]. The sequence alignment results showed the 606 bp sequence at 3'-end of the DNA fragment was in line with the whole sequence of *AcRbcS* promoter fragment obtained from ligation-mediated PCR. Herein, the 813 bp DNA sequence was regarded as the target *AcRbcS* promoter. This result indicated that the high specificity of the AD primer is certainly one of the keys to success. It becomes possible if conserved motifs are found in the homologies with a known sequence and a set of arbitrary degenerate primers is screened in order to determine the most specific ones. According to Sahdev et al. [17], it is difficult to amplify the GC-rich DNA sequences with GC content greater than 65% by common PCR, since GC-rich regions within a template generate a complex secondary structure that prohibits denaturing steps during the PCR amplification process. Therefore, denaturating promoter regions of such GC-rich genome in the green microalgae *A. convolutus* is not a simple task. In order to minimize this problem and ensure the greater success of using the TAIL-PCR technique on the isolation of *RbcS* promoter sequences, we

have optimized the conditions using different combinations of reagent concentration/denaturizing temperature. For this, optimization of the concentrations of AD primers and *Taq* DNA polymerase was performed and high denaturing temperatures were used. Once the conditions were optimized, the length of the final amplified product was remarkably improved compared with that of ligation-mediated PCR.

Taking all together, our results showed that the amplification efficiency of TAIL-PCR was higher than that of the ligation-mediated PCR method with the amplified promoter fragments of 1.2 and 0.8 kb in length corresponding to the promoter sequences of 813 and 606 bp after eliminating the unreadable sequences. The suitable restriction enzyme for the subsequent successful digestion was also selected for ligation-mediated PCR. The presence of undesirable amplification of PCR products flanked with the DNA cassette sequence at both ends is not favorable for amplification of the target sequence.

Although using of several commercial kits for the isolation of regions flanking to a known DNA sequence helps to minimize this problem by modifying the cassette structure [18], complete prevention of PCR amplification of non-target sequences is difficult. Although TAIL-PCR is one of the most common methods used for isolation of flanking regions from known sequences, success is not always guaranteed because of problems with non-specificity and/or low quantities of the amplified products that often occurs in organisms with high-complexity of the genome, such as green microalgae *Chlamydomonas* [19].

In conclusion, since its development, TAIL-PCR has become an extremely valuable and versatile tool in all research involving recovery of unknown genomic sequences adjacent to known sequences. It is not only utilized in functional genomics, detection of genetically modified material in food and screening of transgenic plants, but also in the isolation of promoter sequences. In comparison with the ligation-mediated PCR technique, TAIL-PCR has been shown to be an economical, efficient and rapid method for isolating the promoter region of the *RbcS* gene from *A. convolutus*. The results of the present study have shown that the advantages and efficiency of the TAIL-PCR method make it the preferable approach for isolating the promoter sequences of *A. convolutus*. This study has successfully demonstrated the efficiency and usefulness of TAIL-PCR and ligation-mediated PCR methods for isolating the promoter sequences from green microalgae *A. convolutus* and is the first report for this species. The isolated promoter is being used for our follow-up studies on the establishment of an alternative expression system using this microalga. Finally, this study suggests that it is possible to isolate promoter sequences from most algae species by using TAIL-PCR method. This contributed to the advancement of knowledge on isolation of promoter sequences from algae and other organisms which have no genome information.

## REFERENCES

1. Antal Z., Rascle C., Fèvre M., Bruel C. 2004. Single oligonucleotide nested PCR: a rapid method for the isolation of genes and their flanking regions from expressed sequence tags. *Curr. Genet.* **46**, 240−246.

2. Mueller P.R., Wold B. 1989. *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. *Science.* **246**, 780−786.

3. Rosenthal A., Jones D.S. 1990. Genomic walking and sequencing by oligo-cassette mediated polymerase chain reaction. *Nucl. Acids Res.* **18**, 3095−3096.

4. Terauchi R., Kahl G. 2000. Rapid isolation of promoter sequences by TAIL-PCR: the 5'-flanking regions of *Pal* and *Pgi* genes from yams (*Dioscorea*). *Mol. Gen. Genet.* **263**, 554−560.

5. Wang P., Sun Y., Li X., Zhang L., Li W., Wang Y. 2004. Rapid isolation and functional analysis of promoter sequences of the nitrate reductase gene from *Chlorella ellipsoidea. J. Appl. Phycol.* **16**, 11−16.

6. Leoni C., Gallerani R., Ceci L.R. 2008. A genome walking strategy for the identification of eukaryotic nucleotide sequences adjacent to known regions. *BioTechniques.* **44**, 229−235.

7. Shivji M.S., Rogers S.O., Stanhope M.J. 1992. Rapid isolation of high molecular weight DNA from marine macroalgae. *Mar. Ecol. Prog. Ser.* **84**, 197−203.

8. Varela-Álvarez E., Andreakis N., Lago-Lestón A., Pearson G.A., Serrão E.A., Procaccini G., Duarte C.M., Marbá N. 2006. Genomic DNA isolation from green and brown algae (*Caulerpales* and *fucales*) for microsatellite library construction. *J. Phycol.* **42**, 741−745.

9. Michiels A., Tucker M., van den Ende W., van Laere A. 2003. Chromosomal walking of flanking regions from short known sequences in GC-rich plant genomic DNA. *Plant. Mol. Biol. Rep.* **21**, 295−302.

10. Nichols H.W. 1973. Growth media-freshwater. In: *Handbook of Phycological Methods: Culture Methods and Growth Measurements*. Ed. Stein J.R. Cambridge: Cambridge University Press, pp. 7−24.

11. Thanh T., Chi V.T.Q., Abdullah M.P., Omar H., Noroozi M., Napis S. 2011. Cloning and characterization of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*) cDNA from green microalga *Ankistrodesmus convolutus*. *Mol. Biol. Rep.* **38**, 5297−5305.

12. Mazars G.R., Moyret C., Jeanteur P., Theillet C.G. 1991. Direct sequencing by thermal asymmetric PCR. *Nucl. Acids Res.* **19**, 4783.

13. Liu Y.G., Whittier R.F. 1995. Thermal asymmetric interlaced PCR automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics.* **25**, 674−681.

14. Liu Y.G., Mitsukawa N., Oosumi T., Whittier R.F. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**, 457−463.

15. Grachev M.A., Kuznetsova S.Y., Sherbakova T.A. 2006. A method for the isolation of pure DNA for PCR. *Mol. Biol. (Mosk.)* **40**, 159−161.

16. Hall T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95−98.

17. Sahdev S., Saini S., Tiwari P., Saxena S., Saini K.S. 2007. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. *Mol. Cell Probes.* **21**, 303−307.

18. Siebert P.D., Chenchik A., Kellogg D.E., Lukyanov K.A., Lukyanov S.A. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucl. Acids Res.* **23**, 1087−1088.

19. Silflow C.L. 1998. Organization of the nuclear genome. In: *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas.* Eds Rochaix J.D., Goldschmidt-Clermont M., Merchant S. Dordrecht: Kluwer Acad. Publ., pp. 25−40.