Pak. J. Biotechnol. Vol. 13 (3) 157-163 (2016)
www.pjbt.org
ISSN Print: 1812 - 1837
ISSN Online: 2312-7791

TRANSFORMATION OF *KAPPA (κ)-CARRAGEENASE* GENE FROM *Pseudoalteromonas* IN SEAWEED *Kappaphycus alvarezii* [DOTY]

Muh. Alias L. Rajamuddin[1], Alimuddin[2], Utut Widyastuti[3] and Enang Harris[2]

[1] Bogor Agricultural University, Bogor, Polytechnic Agricultural of Pangkep, South Sulawesi, Indonesia
e-mail : alimuddin@ipb.ac.id

[2]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, and [3)]Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia.

Article received 17.4. 2016; Revised 19.7.2016; Accepted 30.7.2016

ABSTRACT

Foreign gene transformation techniques have been applied to improve the valuable traits of farmed organism. As the first step towards increasing of kappa (κ)-carrageenan content, in this study *κ-carrageenase* gene (*κcar*) from *Pseudoalteromonas* was transformed into *K. alvarezii* mediated by *Agrobacterium tumefaciens* method. The pMSH-*κcar* binary plasmid construct was controlled by 35S CaMV promoter and Nos terminator. DNA analysis of transformants was conducted by polymerase chain reaction (PCR) method using two sets of primer. The result showed that 36% transformed explants were sprouted and 100% of them were having the *κcar* gene. The size of amplified DNA fragment from *K. alvarezii* transformants using 35S-F and tNos-R set primers was 2,000 bp, the same size as PCR product of the positive control of pMSH-*κcar* plasmid, while no amplification product in non-transformated (negative control) was found. Furthermore, PCR analysis using 35S-F and 35S-R set primers showed amplification product of 300 bp, the same size as 35S CaMV promoter sequence. Thus, *K. alvarezii* transformants carrying pMSH-*κcar* had been generated.

Keywords: *Kappaphycus alvarezii*, gene transformation, *kappa (κ)-carrageenase* gene

INTRODUCTION

Seaweed (*Kappaphycus alvarezii*) includes in red algae *Rhodophyceae* (Doty, 1987) group with the primary product is hydrocolloid kappa (κ)-carrageenan (Neish, 2005). The carrageenan has a wide utilization in industry (McHugh, 2003) it will be predicted that carrageenan requirement to continue in the future.

The κ-carrageenan of *K. alvarezii* is the final form synthesized from μ-carrageenan precursor (Campo *et al*., 2009). The enzyme involved in the κ-carrageenan biosynthesis is sulfohydrolase κ-car rageenase (Wong *et al*., 1979). Molecular study of κ-carrageenase in *K.alvarezii* remains to be perfor-med. However, *κ-carrageenase* gene has been successfully isolated from many marine organi-sms, such as *Pseudoalteromonas carrageenovora* (Michel *et al*., 2001), γ-Proteobacterium, *Pseudo-monas elongata* syn, *Microbulbifer elon-gatus* comb. Nov. (Khambhaty *et al*., 2007), *Pseudo-alteromonas*-like bacterium (Zhou *et al*., 2008), marine bacterium: *Alteromonadaceae*, *Marinimi-crobium*, and *Microbulbifer* (Tayco *et al*., 2013). Those microorganisms can be source of gene encoding κ-carrageenase enzyme.

Transgenic technology has been applied to modify the genome in order to improve the valuable traits of farmed organisms. A foreign gene is inser -ted in the genome that will appear in genetically modified organisms (Yuwono, 2006; Lutz, 2001). Application of transgenic technology in seaweed (macro algae) will show a big potency in the future (Hallmann, 2007; Walker *et al.*, 2005) for example: improving the resistance to an environ-mental stress and diseases. The gene transfor-mation in macro algae has been successful con-ducted, including: *Porphyra yezoensis* (Cheney *et al*., 2001) and *Laminaria japonica* (Li *et al.*, 2009) using *β-glucuronidase* (*GUS*) gene, and *Gracillaria* with *LacZ* gene (Huddy *et al*., 2012). Gene transformation in *K. alvarezii* has been done successfully such as *LacZ* gene (Wang *et al*., 2010); *PaCS* gene (signal of citratesyntase) (Daud *et al*., 2013); *green fluorescent protein* (GFP) gene (Rajamuddin *et al*., 2014); *lisozyme-c* gene (Handayani *et al.*, 2014); and *Methallothionein* type II gene (Fajriah *et al*., 2014).

Transgenic *K. alvarezii* is produced through different methods such as particle bombardment (Wang *et al*., 2010), electroporation method (Rajamuddin *et al*., 2014) and mediation of *Agrobacterium tumefaciens* method (Daud *et al*., 2014; Handayani *et al*.,2014; Fajriah *et al*., 2014). *A. tumefaciens* mediated transformation showed more advantage as gene replacement results are more reproducible (Hiei *et al*., 1997; de la Riva *et al*., 1998).

Therefore, this research was aimed to transform the gene encoding *kappa-carrageenase* using *A. tumefaciens* mediation towards improving the content of kappa-carrageenan in the *K. alvarezii*.

MATERIALS AND METHODS

Preparation of Steril *K. alvarezii* Explants

Explants source of *K. alvarezii* seaweed was collected from cultivaton in Pangkep coastal waters, South Sulawesi, Indonesia (Figure 1A). Seaweed was adjusted on culture condition (Figure 1B) at Genetic Laboratory of Research Center for Biological Resources and Biotechnology (PPSHB), Bogor Agricultural University. Explants were sterilized (Figure 1C) through following method by Reddy *et al*., (2003), Rajamuddin *et al*., (2010) and Sulistiani *et al*., (2012). Culture media used was sterile sea water with salinity level of 30-32 g/L, enriched with Provasoli enriched seawater (PES) (Provasoli, 1968).

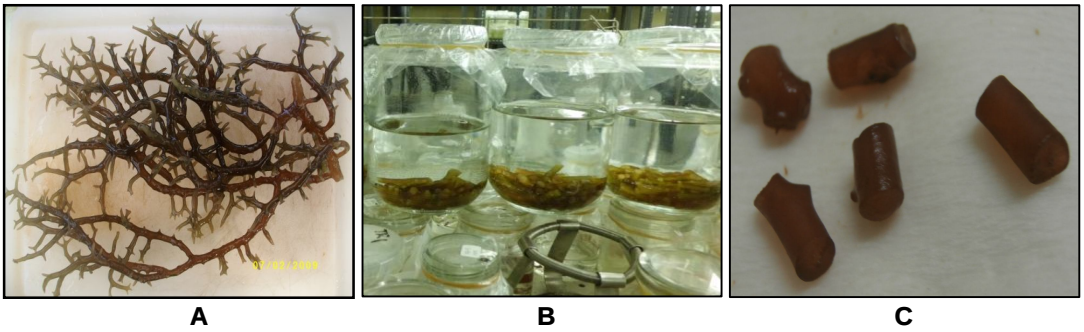

Figure 1 Collection of *Kappaphycus alvarezii* as explants source (A), culture of explants adjusted to laboratory condition (B), and sterile explants with 7-10 mm in length ready to use in transformation process (C).

Preparation of pMSH-*κcar* Expression Vector

κ-carrageenase (*κcar*) gene used in this research was obtained from Genetika Science service (1st BASE Pte Ltd. Singapore) based on sequence of *κ-carrageenase* gene that was originated from *Pseudo alteromonas* (Zhou *et al*., 2008) in gene bank. *κ-carrageenase* gene was a size of 1,300 bp and had constructed to pMSH binary plasmid (Nara Institute of Science and Technology (NAIST), Japan) as pMSH-*κcar* vector.

κ-carrageenase gene sequence was inserted in the site *Not*I and *Spe*I in location of multiple cloning sites (MCS) in pMSH plasmid, that was controlled by promoter 35S CaMV (size 300 bp) and terminator Nos (tNos; size 400 bp), resulting total size from promoter to terminator was 2,000 bp (Figure 2).

Gene construct pMSH-*κcar* was transferred in *Escherichia coli* DH5α for replication followed the methods of Sambrook *et al*., (1989) and Suharsono *et al*., (2002).

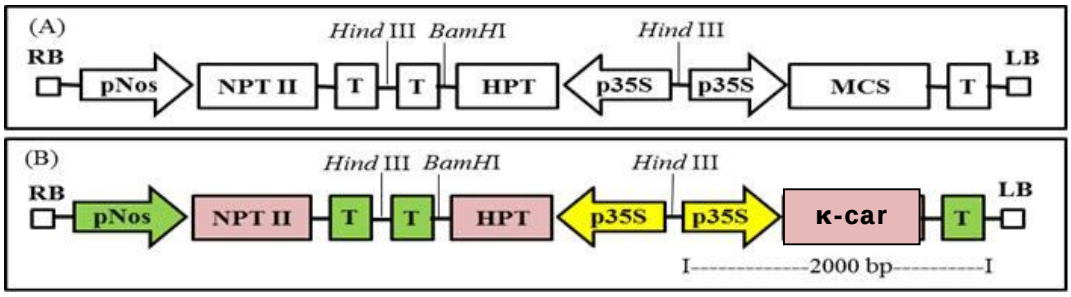

Figure 2 pMSH plasmid mapping (NAIST,Japan),(A):right border (RB), Nos promoter (pNos), marker selection gene: neomycin phosphotransferase II (NPT II) and hygromycin phosphotransferase (HPT), Nos Terminator (T), 35S promoter (p35S), multiple cloning site (MCS), left border (LB); (B): *κ-carrageenase* gene (*κ-car*) inserted on *Not*I and *Spe*I site in MCS.

Transformation of pMSH-*κcar* to *Agrobacterium tumefaciens*: The gene construct pMSH-*κcar* was transformed to *A. Tumefaciens* using triparental mating (TPM) method reported by Bevan, (1984) with some modifications Liberty *et al*., (2008) and Hannum, (2012). Transformation process was concerned with three bacteria namely *Escherichia coli* DH5α as donor that carried a plasmids of pMSH-*κcar* (kanamycin®, hygromycin®), bacteria

E. coli DH1 (pRK 2013) as a helper (kanamycin®), and *A. tumefaciens* LBA44 04 as recipient (streptomycin®). The success of transformation of pMSH-*κcar* to *A. tumefaciens* was verified using selective media and colony PCR analysis of *A. Tumefaciens* transformants using primers 35S-F: 5'-ATG GCT GGA GTA TTA GCT GGG-3' and tNos-R: 5'-CTC ATA AAT AAC GTC ATG CAT TAC A-3'(designed by Hannum, 2012).

Transformation of *κ-Carrageenase* Gene to *Kappaphycus alvarezii:* Transformation was conducted through following method of Cheney, (2000) with many modifications. A total of 50 sterile explants of *K. alvarezii* (size 7-10 mm) were injured by sterile needle. Explants were then inoculated on infection media which containing *A. tumefaciens* carrying pMSH-*κCar* (OD_{600}=0.5-1.0) and 100 µM acetosyringone for 30 minute with shaking of 100 rpm. Explants were dryed out with sterile tissues and moved to cocultivation media (solid media of PES and 100 µM aceto-syringone) for 3 days in dark area (Handayani *et al*., 2014). Explants from cocultivation treatment were cleaned with cefotaxim 200mg/L, rinsed with sterile sea water 3 times, dryed with sterile tissues and then cultured in the recovery media (PES without aceto-syringone) for 7 days. The putative transformed and untransformed control explants were cultured continuely on PES media until a new bud was exist. Buds would be used to confirm the transformed explants by PCR method.

DNA Analysis of *Kappaphycus alvarezii* Transformants by PCR Method: Two explants of *K. alvarezii* having bud were taken randomly to analyse DNA through PCR. New bud from two putative transformed explants were cut about 0.1 g to extract DNA using CTAB extraction buffer followed method of Doyle and Doyle, (1987).

The amplication of extracted DNA was done with PCR using primers 35S-F: 5'-ATG GCT GGA GTA TTA GCT GGG-3' and tNos-R: 5'-CTC ATA AAT AAC GTC ATG CAT TAC A-3' (designed by Hannum, 2012). For verification of successful transformation, PCR of promoter region of *K. alvarezii* transformants was also conducted using primers 35S-F: 5'-AAA CCT CCT CGG ATT CCA TT-3' and 35S-R: 5'-GAA GGG TCT TGC GAA GGA TA-3' (designed by Suharsono *et al*., 2002).

PCR program used for amplification was as follows; pre-denaturation on 95°C for 5 minutes during initial cycle only, followed by denaturation on 94°C for 30 seconds, annealing on 56°C for 1 minutes and extention on 72°C for 2 minutes. The amplification process was repeated for 30 cycles and final extention on 72°C for 5 minutes and cooling on 20°C for 10 minutes. About 10 µl PCR product was separated with 100 V electrophoresis for 30 minutes on 1% agarose in 1x TAE. DNA amplification product were visualized by using gel documentation system-UV transilluminator.

RESULTS AND DISCUSSION

Transformation of pMSH-κcar to *Agrobacterium tumefaciens:* Plasmid pMSH-*κcar* was successfully transformed to *A. tumefaciens* through tri-parental mating (TPM) method (Figure 3). It was observed that single culture bacteria showed no growth on selective media (kanamycin, hygromycin, streptomycin) (Figure 3A), while *A. tumefaciens* bacteria transformants which carried a plas- mid pMSH-*κcar* as result of TPM have capability of growth on selective media (Figure 3B).

A plasmid MSH-*κcar* showed successfull transformation to *A. tumefaciens* bactery. The capability of *A. tumefaciens* to grow in selective media was due to successful insertion of target gene along with marker selection gene of streptomycin, marker selection genes kanamycin (*NPT*II) and hygromycin (*HPT*) on pMSH- *κcar* construct.

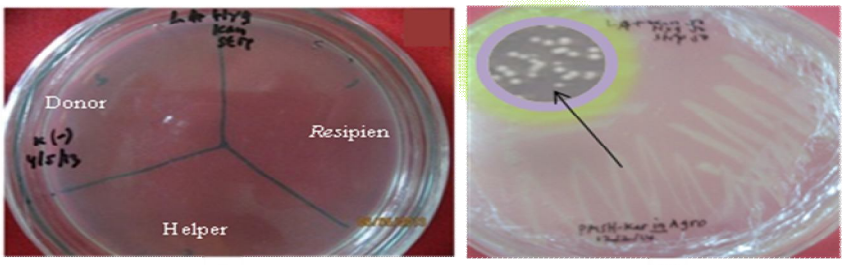

Figure 3 Transformation of pMSH-*κcar* used tri-parental mating method. Single culture of bacterial donor, helper, and recipient didn't grow on selective media (A) as negative control, and *Agrobacterium tumefaciens* colony transformants as conjugation result can grow, arrow (insert): colony transformants (B).

Conjugation mechanism of bacterial donor and recipient on TPM process: start from producted a pilus which is than attached to recipient cell. Plasmid was transferred directly

after cutting by endonuclease enzyme become single strand and moreover it was transferred to recipient cell. Both of cells synthesize their complementary strands to become double stranded forming a plasmid thus both of cells become a viabel donor (Clewell, 1993; Liberty *et al*., 2008).

The verification of successful transformation of pMSH-*κcar* to *A. tumefaciens* was conducted through PCR (Figure 4). Analysis result of PCR toward 5 colonies *A. tumefaciens* as result a TPM (A1, A2, A3, A4, A5) that were grown on selective media showed a amplificated product with size 2,000 bp that has a similiar with size pMSH-*κcar* plasmid as positive control (C+), while a ddH_2O as negative control (C-) didn't exist amflication product (Figure 4).

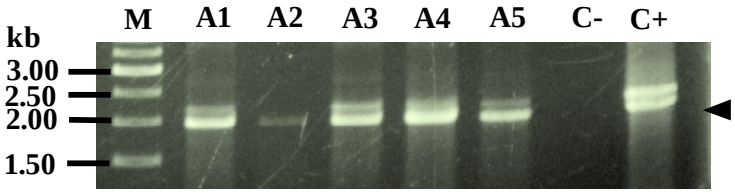

Figure 4 PCR Analysis from 5 colonies *Agrobacterium tumefaciens* transformants as result triparental mating (A1,A2,A3,A4,A5) used a primers 35S-F and tNos-R showed a fragments 2,000bp (arrow head), plasmid as positive control (C+), ddH2O as negative control (C-), M=Marker gene ruler 1KB DNA ladder (BioLabs, Inc. New England).

Transformation of *κ-Carrageenase* Gene to *Kappaphycus alvarezii:* Result from *K. alvarezii* explants of transformation treatment showed 36% bud explants were survived after transformation (Table 1). This was about 2.5 times lower than untransformed explants (Table 1). However, buds carrying pMSH-*κcar* (100%) were only found in transformation treatment (Table 1).

Table 1 Amount of explants grew bud and containing *κ-Carrageenase* gene analyzed by PCR method in the pMSH-*κcar* transformed *Kappaphycus alvarezii*

Indicator variable	Treatments	
	Transformation	Control (untransformed)
a. Initial amount of explants	50	50
b. Amount of explants survived post transformation	18	46
c. Percentage of explants having new bud (b/a* 100% = 1)	36	92
d. Amount of explant survived and grow new bud	16	40
e. Percentage of explant survived and grow new bud	88.9	87

The result showed that explants with transformation treatments have high mortality during infection process until cocultivation, while post infection survival of explants were similar to untransformation (control). Survived bud explants percentage in this research resulted 36% which is higher than reports as 27.4% (Fajriah *et al*., 2014), 23.6% (Handayani *et al*., 2014) and 7.5% (Daud *et al*., 2013).

The survival of bud explants is higher in our experiments which can be compared with the results of other researchers. This improvement may be due to modification in transformation method where cocultivated explants were screened on antibiotic media so the untransformed explants did not show impact on stress for long time. Moreover, buds that grew from transformed explants resulted, the size of 5-10mm during 2 months in culture (Fig. 5).

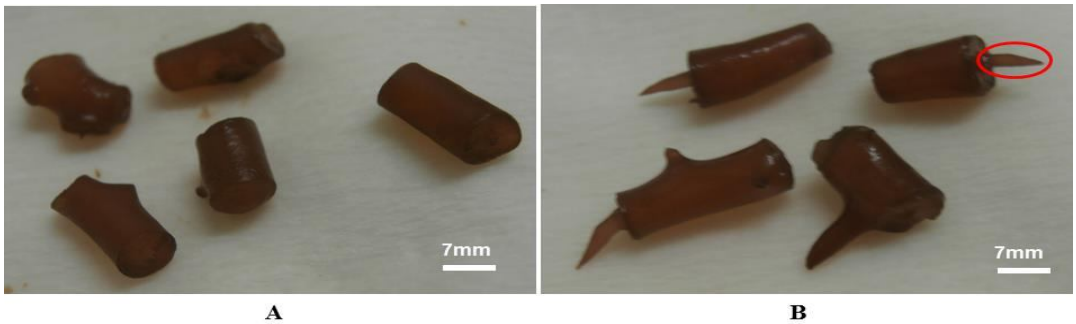

Figure 5 Initial visual morphology of *Kappaphycus alvarezii* explants (A), and explants at two months post-transformation that have a new bud with size 5-10 mm (marked by red circle) (B).

DNA Analysis of Transformants

Results of genome extraction of *K. alvarezii* explants were showed in Figure 6A. It showed that genome extractions of *K. alvarezii* trans-formants and control were successful with 30-40 ng/µl DNA if compared to λ (lambda) marker as 30 ng/µl (λ-3) and 50 ng/µl (λ-5). Moreover, results of PCR analyses on *K. alvarezii* trans-formants showed in Figure 6B and 6C. By using primers 35S-F and tNos-R, PCR resulted an amplified product of 2,000bp in *K. alvarezii* trans-formants (T1 and T2), while non-transformants (NT) as negative control failed to produce amplified product (Figure 6B). The size of PCR product is consistent with a pMSH-*κcar* plasmid (C+) as positive control. To confirm transfor-mation, PCR was conducted to amplify promoter region using a set primer of 35S-F and 35S-R, and amplification results showed a DNA band with size of 300 bp (Figure 6C). Both results of PCR (Figure 6B and 6C) indicated that transformation of *κ-carrageenase* gene was successfull to *K. alvarezii* genome.

In present study, 16 explants were survived and buded, out of which two samples (transfor-mants) were analyzed and showed PCR positive results with 100% transformation efficiency while other researchers such as Handayani *et al*., (2014) reported 3 from putative transformants (50%) PCR positive, and Fajriah *et al*., (2014) reported 13 PCR positive from 135 putative trans-formants (9.63%). The results of our study provides high confidence that the other sprout explants are also carrying the transgene.

Other researchers also reported gene transformation in *K. alvarezii*; Alizadeh *et al.,* (unpublish) used a similiar method and produced 20% transformants with *GUS* gene and 35S promoter. While Wang *et al*., (2010) reported higher percentage (33%) of transformation, but by microparticle bombardment method on 450 psi using *LacZ* gene and SV40 promoter.

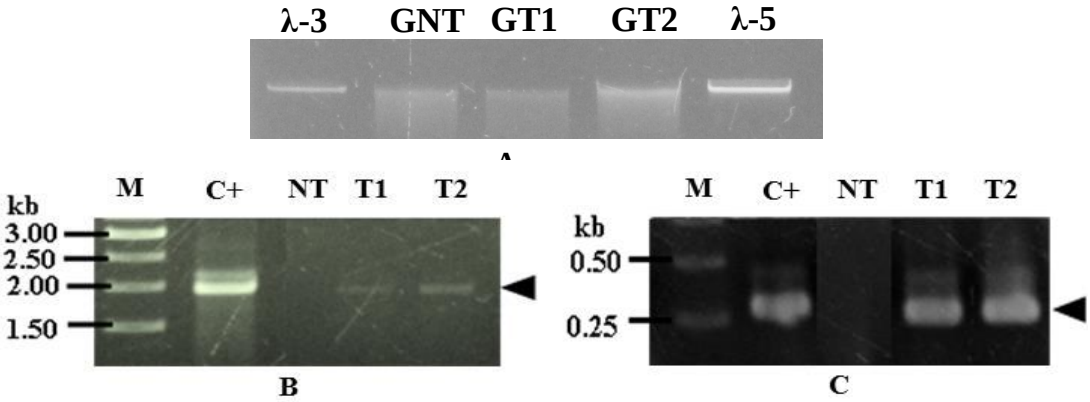

Figure 6 Extraction result of DNA from *Kappaphycus alvarezii* (A), PCR analysis using 35S-F and tNos-R primers with the size product of 2,000 bp (B), and PCR analysis using 35S-F and 35S-R primers with product of 300 bp (C). G: genome; λ-3 and λ-5: λ (lambda) marker with concentrations 30 ng/µl and 50 ng/µl (BioLabs, Inc.New England); T1 and T2: *K. alvarezii* transformants; NT: *K. alvarezii* non-transformed (negative control); C+: pMSH-*κcar* plasmid (positive control); M: marker gene ruler 1-kb DNA ladder (BioLabs, Inc.New England); arrow head showed a target PCR product.

In this study, we analyzed two buds of explant since those explants had achieved a size to minimum requirements of DNA extraction sample as 0.1 g (Doyle and Doyle, 1987). It referred on bud percentage of positive PCR samples, where from 2 samples and all positive PCR (transfor-mants), it showed a more high probability positive PCR on other buds.

In present research, only limited transformed buds were used for PCR analysis therefore, it was needed to culture explants continuously to get more buds to study the gene expression level and integration pattern as well as carregenane contents.

CONCLUSION

The κ-carrageenase gene has been successfully transformed in seaweed (*Kappaphycus alvarezii*) using *Agrobacterium tumefaciens* mediated transformation method. Percentage of explants survival and having new bud (36%) which is higher than previous reports with 100% PCR positive transformants.

ACKNOWLEDGEMENTS

This study was carried out at Research Center for Biological Resources and Biotechnology (PPSHB), Bogor Agricultural University, and Research Institute for Brackishwater Aquaculture (BPBAP), Maros, South Sulawesi.

REFERENCES

Bevan, M. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Research **12**: 8711–8721 (1984).

Campo, V.L., D.F. Kawano, J.D.B Silva and C.I. Ivone, Carrageenans: biological properties, chemical modifications and structural analysis. Carbohydrate Polymers **77**:167-180 (2009).

Cheney, D.P. *Agrobacterium*-mediated genetic transformation of multicellular marine algae, resultant strains and their products. North-eastern University. United States (2000). http://www.patentlens.net/patentlens/patents.htmL.patnums.

Cheney, D.P., B. Metz and J. Stiller, *Agrobacterium*-mediated genetic transformation in the macroscopic red alga *Porphyra yezoensis*. J. Phycol. **37**: 11 (2001).

Clewell, D.B. Bacterial Conjugation (Defense Research Series 4). Springer. ISBN: 03064 43767.WAPI(Tower ID): 101313984. (1993).

Daud, R.F., U. Widyastuti, Suharsono, E. Suryati and A. Parenrengi, Introduction of *Citrat Syntase* gene to *Kappaphycus alvarezii* using *Agrobacterium tumefaciens*. J.Ris. Akuakultur **8**(2): 201-208 (2013).

de la Riva, G.A., J.Gonzalez-Cabrera, R. Vazquez -Padron and C.Ayro-pardo, *Agrobacterium tumefaciens*: a natural tool for plant transformation. Electronic J. Biotech. 1(3):118-133 (1998).

Doty, M.S., The Production and Use of Eucheuma. In: Doty, M.S., J.F.Caddy and B. Santelices (editors). Case Studies of Seven Commercial Seaweed Resources. No.281. Roma (IT): FAO Technical Paper. pp 123-161 (1987).

Doyle, J.J. and J.L. Doyle, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem/ Bull. **19**: 11-15 (1987).

Fajriah, U., E. Suryati, A. Parenrengi, Suharsono and U. Widyastuti, Introduction of *Metallothionein Type II* gene to *Kappaphycus alvarezii* using *Agrobacterium tumefaciens*. J. Ris. Akuakultur. **9**(3): 377-385 (2014).

Hallmann, A., Algal transgenics and biotechnology. Trans Plant J. **1**(1): 81-89 (2007).

Handayani, T., Alimuddin, U. Widyastuti, E. Suryati, and A. Parenrengi, Binary vector construction and mediated *Agrobacterium tumefaciens* transformation of *Lysozyme* gene in seaweed *Kappaphycus alvarezii*. Biotropia **21**(2):80-90 (2014).

Hannum, S. Isolation, Cloning, and Expression Analysis of Gene Coding Copper/Zinc Superoxide Dismutase (CuZn-SOD) from *Melastoma malabathricum* L. PhD thesis. Bogor (ID): Institut Pertanian Bogor (2012).

Hiei, Y, T. Komari and T. Kubo, Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. **35**:205-218 (1997).

Huddy, S.M., A.E. Meyers and V.E. Coyne, Transformation of *lac*Z using different promoters in the commercially important red alga, *Gracilaria gracilis*. African J. Biotech.**11** (8): 1879-1885 (2012).

Khambathy, Y., K. Mody and B. Jha, Purification and characterization ofκ-carrageenase from a novel γ-Proteobacterium, *Pseudomonas elongata* (MTCC 5261) syn. *Microbulbifer elongatus* comb. Nov. Biotechnol Bioprocess Eng. **12**: 668-675 (2007).

Li, F., S. Qin, P. Jiang, Y. Wu and W. Zhang, The integrative expression of *GUS* gene driven by FCP promoter in the seaweed *Laminaria japonica* (Phaeophyta). J Appl Phycol. **21**: 287–293 (2009).

Liberty, M. H. and G.A. Wattimena, Construction of binary plasmids carrying *Cry1Ab* gene and binaryplasmid transformation by triparental mating method.Zuriat. **19**(2):130-139 (2008).

Lutz, C.G., Practical Genetics for Aquaculture. Fishing New Books, Blackwell Science Company, (USA), Pp 218-219 (2001).

McHugh, D.J., A Guide to the Seaweed Industry. FAO Fisheries Technical Paper, Rome (BR): FAO. No. 441, Pp105 (2003).

Michel, G., L. Chantalat, E. Duee, T. Barbeyron, B. Henrissat, B. Kloareg and O. Dideberg, The κ-carrageenase of *P. carrageenovora* featuresa tunnel-shaped active site: A novel insightin the evolution of clan-B glycoside hydrolases. Structure **9**: 513–525 (2001).

Neish, I.C., The Eucheuma Seaplant Handbook "Taxonomy, Reproduction, and Morfology". Matulessi, L.V. (Eds). SEA Plant Net Technical Monograph. Makassar (ID). Pp.48 (2005)

Provasoli, L., Media and prospects for the cultivation of marine algae. In: Watanabe, A. and A. Hattore [eds.] Culture and Collection of Algae.Tokyo (JP): Jap Soc. Plant. Physiol. Pp. 63–75. (1968).

Rajamuddin, M.A.L, Alimuddin, U. Widyastuti, E. Harris, and E. Suryati, Binary vector construction of *Kappa(K)-Carrageenase* gene and transformation to *Agrobacterium tumefaciens* as mediator for seaweed transgenic generation. J ilmu dan Teknologi Kelautan Tropis 8(1):345-354 (2016).

Rajamuddin, M.A.L., Alimuddin, U. Widyastuti, and I. Faizal., Evaluation of different promoters driving the GFP reporter gene in seaweed *Kappaphycus alvarezii*. *Indonesian Journal of Biotechnology* **19**(2):129-135 (2014).

Rajamuddin, M.A.L, A.A. Jaya, and R.E. Suryati, Callus induction of *Kappaphycus alvarezii* for somatic embryos production. J. Ris. Akuakultur. **5**(2): 211-219 (2010).

Reddy, C.R.K, G.R.K. Kumar, A.K. Siddhanta, and A. Tewari, *In vitro* somatic embryogenesis and regeneration of somatic embryos from pigmented callus of *Kappaphycus alvarezii* Doty (Rhodophyta, gigartinales). J. Phycol. **39**: 610–616 (2003).

Sambrook, J., E.F. Fritsch, and T. Maniatis, Molecular Cloning: A laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989).

Suharsono, U., Y. Fujisawa, T. Kawasaki, Y. Iwasaki, H. Satoh, and K. Shimamoto, The heterotrimeric G protein a subunit acts upstream of the small GTPase Rac in disease resintance of rice. Proc Natl Acad Sci. **99**: 13307-13312 (2002).

Sulistiani, E., D.T. Soelistyowati, Alimuddin and S.A. Yani, Callus induction and filaments regeneration from callus of cottonii seaweed *Kappaphycus alvarezii* (Doty) collected from Natuna Islands, Riau Islands Province. Biotropia. **19**(2): 103-114 (2012).

Tayco, C.C, F.A. Tablizo, R.S. Regalia, and A.O. Lluisma, Characterization of a κ-carrageenase-producingmarine bacterium, isolate ALA B-001. Philippine JSci. **142**(1): 45-54 (2013).

Walker, T.L, C. Collet, and S. Purton, Algal transgenic in the genomic era. J Phycol. **41**: 1077–1093 (2005).

Wang, J., P. Jiang, Y. Cui, X. Deng, F. Li, J. Liu, and S. Qin, Genetic transformation in *Kappaphycus alvarezii* using micro-particle bombardment: a potential strategy for germ plasm improvement. Aquacult Int. **18**(6): 1027-1034 (2010).

Wong, K.F. and J.S. Craigie, Sulfohydrolase Activity and Carrageenan Biosynthesis in Chondrus crispus (Rhodophyceae). Plant Physiol. **61**(4);663-666 (1979)

Yuwono, T., Biotechnology of Agricultural. Gadjah Mada University Press. Yogyakarta (ID), pp 284 (2006).

Zhou, M., J. Ma, J. Li, H. Ye, K. Huang and X. Zhao, A κ-carrageenase from a newly isolated *Pseudoalteromonas*-like Bacterium, WZUC10. Biotechnol Bioprocess Eng.**13**: 545-551 (2008).