Acclimatization of transformed seaweed *Kappaphycus alvarezii* carrying lysozyme gene in culture flask and the cultivation in floating net cage in Pangkep coastal

E Suryati ¹, A Tenriulo ¹, S Fadilah ²

¹Research Institute for Barchkishwater Aquaculture and Fisheries Extension (RIBAFE), Maros, Sulawesi Selatan Indonesia
²Research Institute for Seaweed Culture, Bualemo Gorontalo

E-mail: emmasuryati@yahoo.com

**Abstract.** The in vitro transformation of the lysozyme gene in seaweed *K. alvarezii* has been successfully executed to increase the viability against ice-ice disease. There were two major stages in this research; (1) transformation of lysozyme gene in seaweed *K. alvarezii* which was carried out on laboratory scale and the cultivation of gene-transformed explants in the culture flask stored in "culture chamber"; (2) the acclimatization in floating net cages of green nets (mesh size of 1 mm) with cage size of 50 x 50 x 50 cm, the population density of 200 explants and cultivated for two weeks. The explants were then transferred to blue nets (mesh size of 2 mm) with a cage size of 50 x 50 x 50 cm for four weeks of rearing. The plants were then enlarged using a long-line method in the floating net cage, by tying the seaweed using a double line with a gap of 15 cm each. The measurement of weight, bud lengths, and water quality was carried out within 2 weeks. The result shows that the daily growth rate of the transformed seaweed during the regeneration stage in the culture flask was around 0.33-0.4%/day, meanwhile during the acclimatization stage in the green nets the was 0.65-1.6%/day, and even more, increased during the acclimatization stage in the blue nets with DGR of 2.28-2.3%/day. During the enlargement stage in the floating net cages, the lysozyme-transformed seaweed showed an even higher DGR with a value of 3.2-8.2%/day. The results of the integration of the lysozyme gene in seaweed were indicated by the presence of a 670 bp of amplification products, that is the same total length of the 35 S-F promoter fragments and Nos T-R in the expression vector. Based on these results, the lysozyme gene was successfully transformed in *K. alvarezii* seaweed.

1. **Introduction**

The demand for seaweed worldwide keeps getting higher since 2010, with an escalation of 27.63% up until now. On the other hand, Indonesia as the main producer of seaweed with a production volume of 2.143.126 tonnes/year from 2005 to 2010 is still incapable of optimally supplying the world demands [1]. The internal consumption of seaweed as industrial raw materials itself reached 2.340.000 tonnes/year [2].

The family of algae *Kappaphycus alvarezii*, *Eucheuma cottonii*, and Rhodophyceae are the main commodities to produce carrageenan and are broadly cultivated in the coastal areas. However, the extreme changes in the environment might act as an inhibitor to seaweed growth, causing a lessening of productivity. An actual example is the ice-ice disease attack that was formed as a result of exposure...
towards municipal waste, agricultural waste, industrial waste, and other fishery activities (pearl cultivation and harbour activities) which also affect the ecological condition of waters. These conditions will disrupt the growth of seaweed and affect the quantity and quality of seaweed produced [3]. The disease indicates the organism's stress towards pathogen attacks. This was also supported by the fact that the ice-ice disease is more prominent in the environment with lower water quality, lesser water circulation, lower salinity, the temperature level of the waters, and also the higher level of heavy metal content in the waters [4]. One method to improve the genetic quality of the seaweed against this issue nowadays is through genetic engineering.

The successfully carried out researches have been promoting the GFP gene introduction using several promoters, which resulted in a healthy transgenic seaweed up until the filament development stage, and producing seaweed containing a high level of carrageenan and capable of tolerating environmental threats [5]. This was also supported by the successful regeneration from transgenic filament into seaweed thallus [6] shows a great sign to enhance the seaweed genetic through genetic engineering.

The activities to produce better quality carrageenan were successfully conducted by cloning the coding gene of carrageenan and building the expression vector. The transformation of a k-carrageenan gene in seaweed K. alvarezii has also been conducted [7]. The successful transformation also occurred for the gene introductions of PaCs, Mamt, MaSOD, and lysozyme into the thallus. This transformation has been applied to Kappaphycus alvarezii as well, using carrier Agrobacterium tumefaciens as in the higher-level plants, with a lower efficiency level of transformation around 20%. The in-vitro gene transformation of PaCs, MaMt, and lysozyme to enhance the seaweed viability in the harsh environment with heavy metal content and also towards ice-ice disease has been successfully carried out in the laboratory using culture medium and proper environmental condition [8], [9], [10], [11]. However, this result was unsatisfying, since some issues are persisted during the acclimatization stage in the culture flask and recirculation basin, especially regarding the improper quality of water and nutrients which cause death to the transformed explant of seaweed K. alvarezii. This research is focused on the acclimatization and regeneration stage of the transformed seaweed in the culture flask stored in the culture chamber, which was then acclimatized in a floating net cage and using the long line method to enhance the growth rate of the transgenic K. alvarezii. The objective of this research was to obtain a more viable transgenic K. alvarezii towards the environmental threats and ice-ice disease, which can help provision more viable seaweed seeds to support a more holistic aim towards increasing seaweed productivity.

2. Material and methods

2.1. Regeneration of seaweed K. alvarezii lysozyme-transformed explants in culture flask in the indoor laboratory

The transformed explant of seaweed carrying lysozyme gene was selected and transferred to sterile seawater with a salinity of 30 g/L, enriched with PES fertilizer, and added ZPT of Naphthalene Acetic Acid (NAA) and Benzyl Amino Purine (BAP) mixture with the ratio of 2:1, pH of around 6-7. The cultivation in a 2L culture flask was conducted with a population density of around 200 explants/flask equipped with aeration and stored in the culture chamber at 20OC, the light intensity of 1500 lux, and dark to the light ratio of 12:12. The cultivation period was 8 weeks, with medium reconditioning every week and the observation of bud growth and daily weight growth rate were conducted frequently every month.

2.2. Multiplication of transformed seaweed Kappaphycus alvarezii seed carrying lysozyme gene in floating net cages

The multiplication of transformed seaweed K. alvarezii seed carrying lysozyme gene which was cultivated in the culture flask in the laboratory was conducted by selection for cultivation and enlargement stage in the floating net cages. The floating net cage was a cubicle cage built from nets.
and stored under the water surface to avoid the explanations from being eaten by fish or falling into the deep ocean. The acclimatization stage was conducted in several steps: (1) transgenic seaweed explants and non-trans explants were stored in green net cages (mesh size of 1 mm) with the size of 50x50x50 cm and population density of 200 explants/cage. The cages were put in floating Cage Net with a gap distance of 50-70 cm, and a cultivation period of 2 weeks. The weight and bud length measurements were conducted every week, including the cleaning and reconditioning of the cages from muds and attached biofilm, to enable seawaters to flow freely inside out.

3. Results and discussion

3.1. The regeneration of lysozyme-transformed *K. alvarezii* in an indoor laboratory

The result from the regeneration of seaweed *K. alvarezii* carrying lysozyme gene in the culture flask for 2 months cultivation period showed that the putative bud growth for the three cultivating genes was insignificant. The putative buds from seaweed carrying the lysozyme gene showed relatively better DGR in the first month with a level of 0.32%/day and 0.41%/day in the following month (Figure 5).

![Figure 1](image.png)

**Figure 1.** The daily growth rate of the transformed seaweed *K. alvarezii* carrying lysozyme gene and non-trans in the culture flask in an indoor laboratory

The cultivation of transgenic seaweed *K. alvarezii* explant carrying lysozyme gene in the culture flask had a relaxed growth despite the maximum conditioning applied to it. This phenomenon has also been experienced by other researchers conducting similar observations. For example, in the cultivation of transgenic seaweed containing k-carrageenan, the growth rate was 5-10 mm in 4 months cultivation period [12], also in the cultivation of seaweed carrying PaCs gene, the growth rate was 2-4 mm in 2 months. This phenomenon was strictly correlated to the nutrient contents, oxygen supply, temperature, and light supply during the cultivation, as well as keeping the container free from contamination of disease.

3.2 Acclimatization and multiplication of transgenic seaweed *Kappaphycus alvarezii* seed carrying lysozyme gene in floating cage net

The acclimatization of transgenic seaweed *K. alvarezii* seed carrying lysozyme gene in KJA was conducted by using explants that had grown their putative buds in the culture flask. It was then selected for cultivation in the green net cages (mesh size of 1 mm), with a distribution density of 200 explants in every cage.

The daily growth rate (DGR) of the seaweed *K. alvarezii* in the green net cage for 2 weeks of cultivation is shown in Figure 2.
Figure 2. Daily growth rate (DGR) of transformed seaweed *K. alvarezii* carrying lysozyme gene and non-transgenic in green net cages (mesh size of 1 mm)

In the figure, it was shown that the DGR increased to 1.6%/day in the second week, relative to the first week, which was the effect of environmental conditions and water circulation. In the green net cages, the water circulation was inhibited due to the clogging of mud and causing the non-optimal growth of seaweed explants. The cultivation method was limited to 2 weeks only because the growth might stop due to this reason causing scarring and thallus decay in the seaweed.

On the other hand, the cultivation of transgenic seaweed carrying lysozyme genes in blue net cages (mesh size of 2 mm) was conducted for 4 weeks. The growth in this stage was observed to fluctuate but better than that in the previous green net cages (Figure 3).

Figure 3. Daily growth rate (DGR) of transformed seaweed *K. alvarezii* carrying lysozyme gene and non-transgene in blue net cages (mesh size of 2 mm)

The cultivation result of the transgenic seaweed in the blue net cages showed that the growth of seaweed carrying lysozyme genes had constant DGR from the initial week to the fourth week. This was strongly related to the environmental condition in the field as well as the gene function in the seaweed. According to [13], seaweed carrying the lysozyme gene contained a ubiquitin substance which is also a bactericidal agent capable to hydrolyse β-1, 4-glycosidic bond in peptidoglycan (polymer building up cell wall in positive-Gram bacteria) and in several cases, capable of the lysis cell wall in negative-Gram bacteria [14]. This was the reason that in a deficient environmental condition and under attack from bacteria and microorganisms, the seaweed might be able to protect itself and survive under those substandard conditions.
Figure 4. The transformed seaweed *K. alvarezii* carrying lysozyme gene and non-transgene in blue net cages (mesh size of 2 mm).

After 4 weeks of cultivation in blue net cages, the seaweed explants had developed primary and secondary branches, allowing them to be tied using a line. The result of the cultivation of transformed seaweed *K. alvarezii* carrying lysozyme gene using a long line method exhibited an increased growth (Figure 5).

Figure 5. Daily growth rate (DGR) of transformed seaweed *K. alvarezii* carrying lysozyme gene and non-transgene during cultivation using a long-line method.

Figure 6. The transformed seaweed *K. alvarezii* carrying lysozyme gene and non-transgene using a long line method.

The DGR of seaweed *K. alvarezii* carrying lysozyme gene was observed to increase from the initial week to the fourth week. The increased growth of seaweed was following the environmental condition...
during the first week of October 2016, in which the rain started to pour causing the seaweed to grow better up until the end of October 2016.

The results of the integration of the lysozyme gene in seaweed showed the band at position 670 bp by 35 S-F promoter fragments and Nos T-R in the 35S Ca MV vector. Based on these results, the lysozyme gene was successfully transformed in *K. alvarezii* seaweed

![Image of gel electrophoresis](image)

**Figure 7.** The results of the integration of the lysozyme gene in seaweed showed the band at position 670 bp 35 S-F promoter fragments and Nos T-R in the 35S Ca MV vector

The range of water quality parameters that might affect the viability and growth rate of transformed seaweed *K. alvarezii* was exhibited in Table 1.

**Table 1.** Water quality during the cultivation of transformed seaweed *K. alvarezii* carrying Lysozyme gene in the floating net cage

| Parameter     | Aug    | Sept   | Oct    | Nov    |
|---------------|--------|--------|--------|--------|
| Ammonia (ppm) | 0.072  | 0.0368 | <0.002 | 0.01   |
| Phosphate (ppm)| 0.1108 | 0.0813 | <0.0021| 0.0112 |
| Nitrate (ppm) | 0.0644 | 0.0008 | 0.088  | 0.092  |
| Nitrite (ppm) | 1.618  | 0.2741 | 0.6668 | 0.6543 |

The water quality data was observed to fluctuate from August to November 2016, in which during August to September, it was the dry season and continued to transition to the rain season. During these times, the seaweed growth was observed non-optimal as spoilage from disease or nutrient deficiency sometimes occurred. The result of the cultivation of seaweed *K. alvarezii* carrying lysozyme gene during these times exhibited better DGR relative to the other genes even though at the end of the research (November 2016), all seaweeds *K. alvarezii* carrying other different gene insertions exhibited optimum growth.

**4. Conclusion**

The regeneration of seaweed explants resulting from the transformation of lysozyme genes in flask culture in the laboratory showed a growth rate of 0.33-0.44 mm/day, after being transferred to green net cages net the growth rate increased to 0.65-1.6 mm/day, and in blue net cages growth rate was 2.28-2.3 mm/day. The highest rate of seaweed growth in the long line method is 3.2-8.2 mm/day. The results of the integration of the lysozyme gene in seaweed showed the band at position 670 bp by 35 S-F promoter fragments and Nos T-R in the 35S Ca MV vector. Based on these results, the lysozyme gene was successfully transformed in *K. alvarezii* seaweed.

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