Cultivation of Asparagopsis at Scale

The manual

Contents

1 Culturing the *Asparagopsis taxiformis* under controlled conditions. 2
1.1 Protocol for the Collection of *A. taxiformis* Reproductive Thalli 4
1.1.1 Laboratory Checklist 4
1.1.2 Protocol 4
1.1.3 Basic Concepts: 4
1.2 Protocol for extraction and cleaning cystocarps from *A. taxiformis* gametophyte. 5
1.2.1 Laboratory Checklist 5
1.2.2 Protocol 5
1.3 Protocol for the liberation and germination of carpospores. 8
1.3.1 Laboratory Checklist 8
1.3.2 Protocol 8
1.3.3 Key Concepts 9
1.4 Protocol for growth and culturing tetrasporophyte biomass. 10
1.4.1 Laboratory Checklist 10
1.4.2 Protocol 10
2 In-house biosynthesis of λ-Carrageenase 14
1. Protocol to Culture Transgenic E. coli expressing λ-Carrageenase. 14
2.1 Protocol to Culture of Transgenic E. coli expressing λ-Carrageenase. 15
2.1.1 Laboratory Checklist 15
2.1.2 Protocol – Generation of fresh culture via streaking on agar plates. 15
2.1.3 Protocol for Overnight Cell Culture 16
2.2 Protocol to Subculture Transgenic *E. coli* Strain & Induction of λ-Carrageenase Biosynthesis 17
2.2.1 Laboratory Checklist 17
2.2.2 Prepare uninduced SDS-PAGE sample. 17
2.2.3 Measure cell density of overnight culture. 17
2.2.4 Prepare a subculture with a starting OD600 of 0.3 18
2.2.5 Measure OD600 of subculture and induce GOI expression. 18
2.3 Protocol for Collecting Cell Pellets and Lysing Transgenic *E. coli* Cells. 19
2.3.1 Laboratory Checklist 19
2.3.2 Prepare Induced SDS-PAGE Sample 19
2.3.3 Pelleting-induced cells 19
2.3.4 Lysing-induced cells 19
2.4 Protocols for purification of λ-Carrageenase (from inclusion bodies) 21

2.4.1 Laboratory Checklist....21
2.4.2 Preparing Ni-NTA Column....21
2.4.3 Purification of λ-carrageenase under Denaturing Conditions....22
2.5 Solutions:....23
2.5.1 LB Agar Plates/Broth....23
2.5.2 Antibiotic Concentrations....23
2.5.3 Protein Purification Kits....23
3 Isolation of *A. taxiformis* protoplast....25
3.1 Protocol for Plasmolysis of *A. taxiformis* explants....26
3.1.1 Laboratory Checklist....26
3.1.2 Protocol....26
3.2 Protocol for protoplast isolation of *A. taxiformis.*....27
3.2.1 Laboratory Checklist....27
3.2.2 Protocol....27
3.3 Solutions:....28
3.3.1 Plasmolysis Solution....28
3.3.2 Enzymes Solution....28

Chapter 1

Culturing *Asparagopsis taxiformis* under controlled conditions

Chapter 1

1 Culturing the *Asparagopsis taxiformis* under controlled conditions.

A. taxiformis has a wide tolerance for changing environmental conditions. It is endemic to the cool temperate to tropical waters of Australia and is common in estuaries and bays, often found in subtidal areas less than 50 centimetres to several metres deep. It can be found either attached to rocks and colonising biogenic material, as an epiphyte on other algae and seagrasses, or free-floating. It is often found in high-energy environments and thrives in nutrient-rich waters. The species prefers salinities of about 35 parts per thousand (ppt) but has some tolerance for lower and higher salinities; for example, it grows in Shark Bay, Western Australia at salinities greater than 40 ppt. It is distributed from cool temperate to warm tropical waters, with an optimal temperature range of 15-28 °C.

A. taxiformis has a triphasic, diplohaplontic, heteromorphic life cycle with three morphologically distinct phases: the diploid tetrasporophyte, haploid gametophyte, and diploid carposporophyte stages. Initially, the haploid phase was misclassified as a separate species (*Falkenbergia hillebrandii*). The fully grown tetrasporophyte has sparse branches, long stolons, and erect shoots that ramify repeatedly, giving it a thallus-like appearance. The diploid tetrasporophyte produces haploid tetraspores via meiosis within tetrasporangia in the cortex. These spores, visible as red cross-shaped spots, are passively released into the water, settle on a substratum, and develop into multicellular discs. Each disc may produce multiple upright thalli that can grow free-floating. From the tetraspores arise male or female haploid gametophytes, indistinguishable until maturity. The gametophytes are pink, dense, and intertwining, forming clumps about 200 mm in height. Male gametophytes form spermatangia, while female gametophytes produce eggs (carpogonia) within the cortex. Fertilisation occurs when non-motile spermatia fertilize the eggs, forming a diploid zygote. The zygote develops into the carposporophyte phase, forming cystocarps on the female thallus. Within the cystocarp, diploid carpospores are produced through mitosis and released into the water. These spores settle, form multicellular discs, and develop into upright thalli, completing the cycle. Each disc can produce multiple thalli that may grow free-floating, perpetuating the species' unique reproductive strategy.

In addition to its complex three-phase sexual life cycle, *A. taxiformis* can reproduce asexually through vegetative propagation. This process enables a single individual to produce hundreds or thousands of genetically identical fragments, each capable of developing into a new blade. These blades can further fragment and proliferate, creating a simple and efficient means of propagation that ensures consistency within a culture environment. Such consistency is particularly advantageous when cultivating for specific traits, including bromoform production, growth rates, or biomass yields. However, vegetative propagation in *A. taxiformis* lacks an attachment stage, necessitating adaptations to cultivation techniques to suit the grower's needs.

Unlike *A. armata*, which propagates vegetatively via barbed branchlets, *A. taxiformis* does not possess this feature, making vegetative propagation more challenging. As a result, large-scale cultivation of *A. taxiformis* requires a closed-life cycle approach to ensure a reliable, year-round supply of young gametophytes, or germlings. Hatchery production hinges on inducing tetrasporangia formation to produce adequate spore quantities for seeding substrates, particularly when natural spore availability is inconsistent (Mihaila et al., 2023). Developing

effective protocols for tetrasporangia induction is thus a critical milestone for scaling ocean-based gametophyte farming (Zanolla et al., 2022).

In view of this, the following experiments focus on the vegetative culturing of *A. taxiformis* (under hatchery and nursery operations), covering key processes. The protocols stem from work performed in the SARaH lab's hatchery and nursery, and details procedures followed by lab personnel in starting cultures from carpospores and producing biomass within onshore facilities. The procedures were developed following a review of the literature and testing in the lab, and successful technique development in the lab, complemented by best professional judgment. Together, they represent a practical and successful methodology for culturing *A. taxiformis*.

Overall, the culturing of *A. taxiformis* can be divided into the following phases:

1. Protocol For the Collection of Reproductive Thalli.
2. Protocol For Extraction and Cleaning Cystocarps.
3. Protocol For the Liberation and Germination of Carpospores.
4. Protocol For Growth and Culturing Tetrasporophyte Biomass.

Chapter 1

1.1 Protocol for the Collection of *A. taxiformis* Reproductive Thalli

The protocols for the collection of *A. taxiformis* reproductive thalli are designed to ensure the successful harvest of high-quality material for cultivation and research from selected locations. These protocols focus on identifying and collecting mature reproductive gametophytes, which are critical for spore production and subsequent lifecycle propagation. By incorporating standardized methods for collection, handling, and transportation, these protocols aim to preserve the viability and integrity of the reproductive thalli, supporting efficient hatchery operations and sustainable seaweed cultivation practices.

1.1.1 Laboratory Checklist

- ☐ Catch bags
- ☐ Gametophyte holding system
- ☐ Esky

1.1.2 Protocol

1. Collects whole, healthy and reproductively fecund gametophyte thalli-bearing mature cystocarps from selected locations.
2. Keep the thalli in catch bags and immersed in seawater to avoid temperature shocks.
3. Transfer the thalli to an esky or cooler with site seawater for transportation to the marine research facility.
4. In the lab, store the thalli within a holding system with a temperature that matches that of the collection site, and held under white light at an intensity of 80-100 µmol photons m-2 s-1 and 10:14 photoperiod, and with aeration.



Figure 1. Wild gametophytes in holding tanks at SARaH lab. The gametophyte is held under white light at an intensity of 80-100 µmol photons $m^{-2}s^{-1}$ and 10:14 photoperiod, and with aeration. Under ideal conditions, the gametophyte can be held for 3-4 days.

1.1.3 Basic Concepts:

Healthy gametophyte: 'Healthy and reproductively fecund' gametophytes are defined as those with deep red colouration and minimal bleaching and with each thalli bearing at least 10-20 mature cystocarps.

Mature cystocarps: 'Mature' cystocarps are defined as those with deep red colouration and that are approximately 1 mm in diameter. The carposporangium (ball of spores) is visible within the semi-transparent cystocarp.

1.2 Protocol for extraction and cleaning cystocarps from *A. taxiformis* gametophyte.

The protocol for extracting and cleaning cystocarps from *A. taxiformis* gametophytes is designed to isolate viable reproductive structures for spore release and cultivation. This process involves carefully separating cystocarps from the gametophyte tissue while minimizing damage to ensure high viability. Standardized cleaning methods are employed to remove debris, epiphytes, and contaminants, ensuring the purity of the extracted cystocarps. These steps are critical for optimizing the efficiency of spore production and supporting consistent hatchery operations.

1.2.1 Laboratory Checklist

- Three plastic trays (30 cm)
- Two containers (1 L)
- Petri dishes (90 mm)
- Petri dishes (90 mm) with 1% agar
- Tweezers with serration
- Transfer pipettes
- 200 µL pipette and tips for 100 µL
- 1 L culture vessels
- Seawater (filtered and UV-treated if possible)
- 4 L UV-filtered and sterilised seawater (19 °C prepared the day before)
- 1 L Cell-Hi® F2P (stock solution 30 g L-1 at 0.1 g L-1 basal concentration, 19 °C prepared the day before) or alternative nutrient media (see nutrient protocols)

1.2.2 Protocol

1. Thoroughly clean the bench area and instruments with ethanol 70% before starting.
2. Collect a few A. taxiformis thalli from the holding tank and clean them by physically removing any large epiphytic algae or debris, as well as any small macro-invertebrates. Wash off any finer particles and algae using filtered seawater (19 °C). Repeat the cleaning and washing steps until no macroinvertebrates or epiphytic algae are visible. Use a dissecting microscope to assist in visual inspections after each rinse.
    - ❖ Discard the heavily contaminated parts of the gametophytes.
3. Submerge and spread out 2-3 of clean A. taxiformis thalli in a plastic tray filled with sufficient clean seawater (Figure 2a). Visually inspect the thallus of the gametophytes for fertilised cystocarps.
    - ❖ The fertilized look like small pink-to-white-coloured balls approximately 1 mm in diameter (Figure 2b).

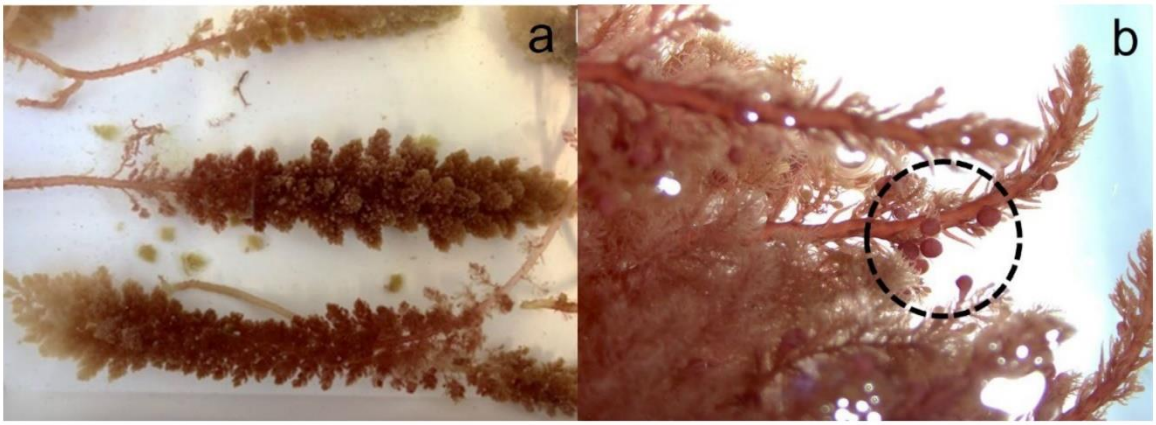


Figure 2. (a) *Asparagopsis taxiformis* thalli spread out in tray for inspection and cleaning; (b) *A. taxiformis* thalli with mature cystocarps (small pinkish balls).

4. Look at the thalli under the microscope; identify and mark the healthy and larger cystocarps; free from any visible contamination and epiphytic growth (Figure 3a). Under the microscope, look for fertilised cystocarps. These will be round and swollen, and the carposporangium (ball of spore) will be visible (Figure 3b). Identify healthy cystocarps and transfer these to the petri dish.
    - ❖ Discard malformed or dark red cystocarps (Figure 3c) – these have not been fertilised.
    - ❖ Avoid all other debris, cystocarp husks and cystocarps that are damaged.

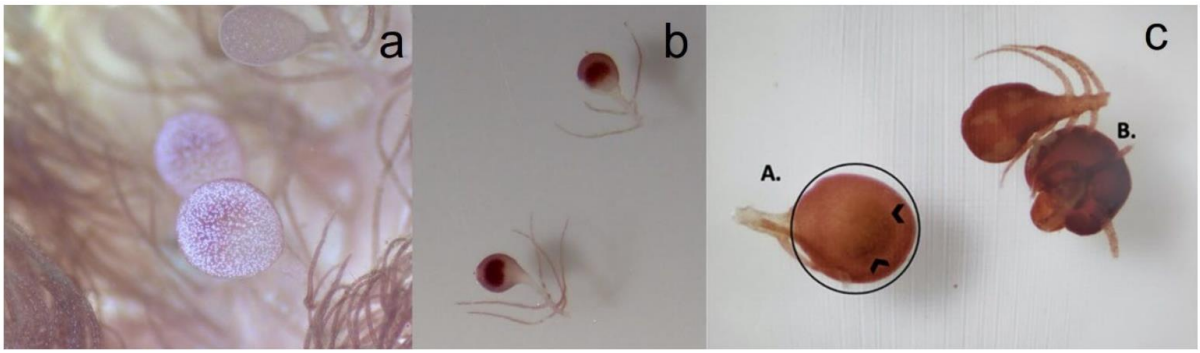


Figure 3. (a) *A. taxiformis* cystocarps attached to the gametophyte thallus; (b) *A. taxiformis* cystocarps after excision from the thallus to allow for cleaning using the agar drag method; (c – A) *A. taxiformis* fertilised cystocarp with arrows highlighting sporangium; (c – B) malformed and dark red cystocarps that are unfertilised.

5. Remove the individual cystocarps from the thallus using a pair of fine forceps, and transfer them to a petri dish containing filtered, sterilised seawater. Carefully cut the cystocarps leaving a small thallus attached to the cystocarps to allow for agar dragging.
    - ❖ The following steps should be performed as quickly as possible as as cystocarps will begin releasing carpospores immediately after they have been excised from the thallus.
6. Prepare four petri dishes with filtered and sterilised seawater next to a dissecting microscope for disinfection and a three-stage washing of cystocarps after selection. Using a 3 mL pipette, transfer cystocarps across the three petri dishes of filtered and sterilised seawater before placing them in a final petri dish with filtered and sterilised seawater. Cystocarps can be also 'washed' with small jets of filtered and sterilised seawater from a 3 mL pipette.
7. Now, drag an individual cystocarp through the agar plate by using forceps for further cleaning. Aim to maximise contact of the cystocarp with the agar, so rotate the cystocarp after each drag. Repeat the dragging step 3-4 times. Ensure the cystocarp structure does not break as small quantities of agar may get trapped inside.
    - ❖ If the thallus breaks, cystocarps can be 'pushed' through the agar using a pair of fine forceps.

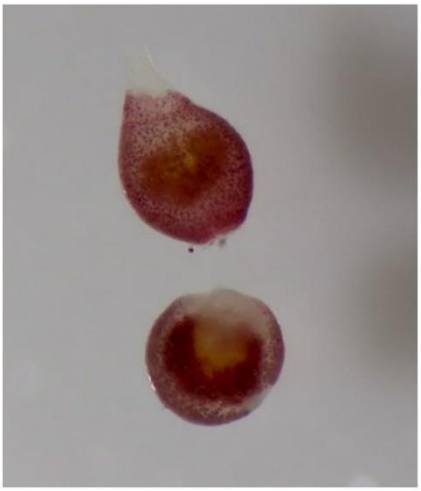


Figure 4. Cleaned and healthy *A. taxiformis* cystocarps with excess thallus removed. The top cystocarp has begun releasing spores.

8. After dragging, clean each cystocarp with a three-stage rinse. Pipette each cystocarp into a clean dish and repeat the process two more times to remove agar and any other contamination.
9. Using a dissecting microscope, ensure all agar and excess thallus are removed (e.g. Figure 4). If necessary, use a scalpel blade to remove any excess thallus (as shown in Figure 3c).
10. Transfer the clean cystocarp to a culture petri dish containing 30 mL filtered and sterilised seawater and 100 µL Cell-Hi® nutrient solution (0.1 g L-1 working concentration) and GeO2 at a concentration of 0.1 g L-1 to prevent diatom growth. Close the petri dish, seal it with plastic film and label it accordingly.
11. Repeat steps 4-10 to achieve the desired number of petri dish cultures.
12. Store the culture petri dishes in an illuminated incubator held at 19 °C. The initial light intensity should be 5-20 µmol photons m-2 s-1 and a photoperiod of 10:14 (light: dark).
    - ❖ Ensure the minimum light intensity is not below 5 µmol photons m-2 s-1 otherwise germination, growth and survival may be impacted.
    - ❖ Ensure the maximum light intensity does not exceed 20 µmol photons m-2 s-1 to minimise stress to the carpospore.

1.3 Protocol for the liberation and germination of carpospores.

The protocol for the liberation and germination of carpospores outlines the steps to release viable carpospores from cleaned cystocarps and promote their successful germination. This process involves carefully controlled environmental conditions, such as light, temperature, and salinity, to mimic natural cues and optimize spore release. Germination techniques focus on creating favourable conditions for the carpospores to adhere to substrates and develop into multicellular structures. This protocol is essential for generating healthy juveniles for large-scale cultivation efforts.

1.3.1 Laboratory Checklist

- Three plastic trays (30 cm)
- Two containers (1 L)
- Petri dishes (90 mm)
- Petri dishes (90 mm) with 1% agar
- Tweezers with serration
- Transfer pipettes
- 200 µL pipette and tips for 100 µL
- 1 L culture vessels
- Seawater (filtered and UV-treated if possible)
- 4 L UV-filtered and sterilised seawater (19 °C prepared the day before)
- 1 L Cell-Hi® F2P (stock solution 30 g L-1 at 0.1 g L-1 basal concentration, 19 °C prepared the day before) or alternative nutrient media (see nutrient protocols)

1.3.2 Protocol

1. Cystocarps release over 80% of the carpospore within the first 48 hours. Each cystocarp can release between 50 and 400 carpospores. To reduce overcrowding in cultures, transfer cystocarps to a new dish after 24 hours.
    - ❖ This process will need to be systematic, a few dishes at a time to minimise the exposure of carpospores to different light and temperatures. That is, for each batch of cystocarps, you will have ‘A’ and ‘B’ isolates.
    - ❖ Cystocarps release carpospores almost immediately after the excision from the thallus and continue to release carpospores for several days.
2. Prepare a petri dish with 30 mL of filtered and sterilised seawater and nutrient solution (100 µL Cell-Hi® 30 g L-1 at 0.1 g L-1 working concentration, and GeO2; at a concentration of 10 mg L-1 to prevent diatom growth; all liquids should be 18-19 °C) to transfer cystocarps from isolate ‘A’ and inoculate isolate ‘B’.
3. Check the health of the cystocarps under the dissecting microscope; remove and dispose of broken and/or empty cystocarps (husks). Only transfer cystocarps with carposporangium visible inside (red ball of carpospores; see Figure 3) to the new dish. Close both dishes (‘A’ and ‘B’), seal with plastic film and label accordingly.
    - ❖ Repeat the process for all dishes from isolate ‘A’ & return ‘A’ and ‘B’ isolates to the incubator, keeping the light, temperature and photoperiod the same.
4. After the ‘B’ isolate has liberated carpospores for 24 hours, it is time to discard all cystocarps from ‘B’. Select all isolate ‘B’ petri dishes from the incubator and remove and discard old cystocarps.
5. After 24 hours, the carpospores begin to swell, and germination occurs between 24-48 hours (observing a small transparent rhizoid and a bunch of dividing cells or early-stage filament protruding from the carpospore (Figure 5a).

6. After germination, a rhizoid begins to develop, which anchors the carpospore (now tetrasporophyte) to the petri dish (Figure 5b). Once the rhizoid has been observed (48 hours), on day three the culture dishes can have a water change to flush out cystocarp debris and metabolites released into the petri-dish water, and to replenish nutrients.
    1. To change the water in a petri dish, use a pipette to remove the old petri-dish water. Choose a location on the petri dish where tetrasporophytes are not visible to prevent damage to tetrasporophytes.
    2. Alternatively, tetrasporophytes can anchor to the petri dish by the holdfast. In that case, prepare a new culture medium for each culture (i.e. 30 mL of 19 °C with Cell-Hi® nutrient solution at 0.1 g L-1 working concentration and GeO2) and pour the petri dish out into an empty container for later disposal.
    3. Quickly refill the petri dish with the prepared 30 mL culture medium. Replace with 30 mL of 19 °C filtered and sterilised seawater and 100 µL 19 °C Cell-Hi® nutrient solution (0.1 g L-1 working concentration) and 10 mL of GeO2 (see Appendix A). Wrap petri dishes and return them to the incubator.

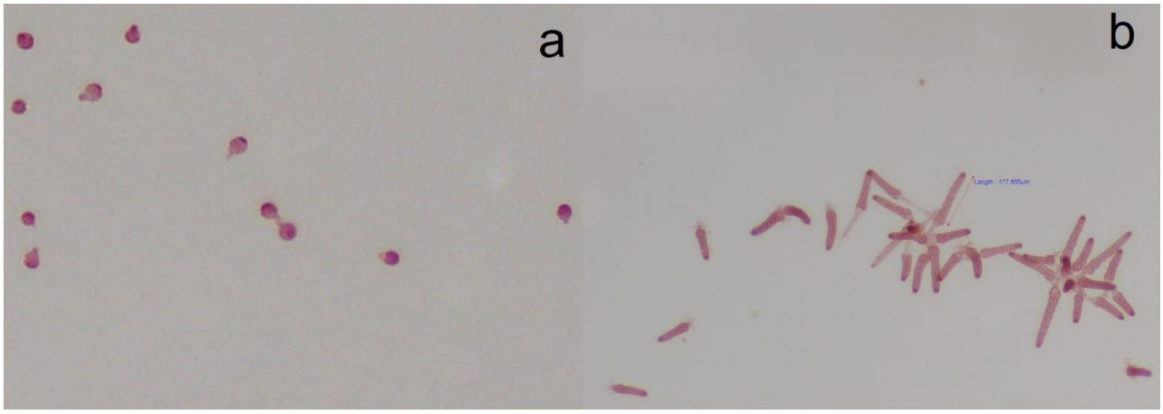


Figure 5. (a) Petri dish cultures of *A. taxiformis* after 24 hours with germinated carpospores (30 µm diameter); (b) 48-to-72-hour-old carpospores developing a rhizoid (light pink tail) and filament (vivid red filament 60-200 µm long).

7. Monitor isolates daily to check for tetrasporophyte survival, health, growth and contamination. If there are signs of contamination, discard the petri dish.
    - ❖ For the first four days, there should be lateral elongation of the sporeling filament, and it should be a red-to-pinkish-red colour.
8. The tetrasporophytes begin to produce their first branch after five or six days (Figure 6). When this happens, the light intensity can be increased to 10 µmol photons m-2 s-1 and a photoperiod of 10:14 (light: dark).

1.3.3 Key Concepts

A rhizoid is a transparent finger-like projection from the basal end of the tetrasporophyte.

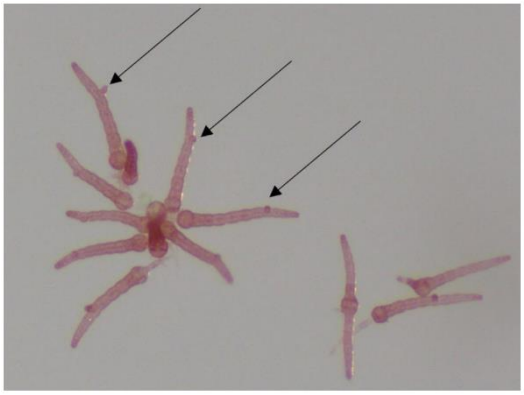


Figure 6. *A. taxiformis* tetrasporophytes showing branching after six days in petri dish culture.

1.4 Protocol for growth and culturing tetrasporophyte biomass.

The protocol for the growth and culturing of *A. taxiformis* tetrasporophyte biomass focuses on establishing optimal conditions for its successful development and propagation. It outlines key steps, including the preparation of cultivation systems, nutrient management, and environmental parameter control, such as light intensity, temperature, and salinity. By maintaining ideal growth conditions, this protocol ensures the production of robust tetrasporophyte biomass, which serves as a vital stage in the species' life cycle and supports further cultivation and research efforts.

1.4.1 Laboratory Checklist

- Three plastic trays (30 cm)
- Two containers (1 L)
- Petri dishes (90 mm)
- Petri dishes (90 mm) with 1% agar
- Tweezers with serration
- Transfer pipettes
- 200 µL pipette and tips for 100 µL
- 1 L culture vessels
- Seawater (filtered and UV-treated if possible)
- 4 L UV-filtered and sterilised seawater (19 °C prepared the day before)
- 1 L Cell-Hi® F2P (stock solution 30 g L-1 at 0.1 g L-1 basal concentration, 19 °C prepared the day before) or alternative nutrient media (see nutrient protocols)

1.4.2 Protocol

1. Once there is branching visible over >90% of the petri dish (about day 8-10), the tetrasporophytes can be scraped from the petri dish and transferred to tumble (aerated) culture.
    - ❖ To transfer tetrasporophytes in the petri dish to tumble culture (Figure 7), tetrasporophytes need to be scraped and suspended in the petri dish using a bacterial loop and a 3 mL pipette and transferred to a 1 L vessel containing 19 °C filtered and sterilised seawater and 10 mL Cell-Hi® at 0.3 g L-1 production concentration (if alternative production nutrient media are to be used, consult the nutrient media standard operating procedures for those media and relevant working concentrations) and 10 mg L-1 of GeO2 to prevent diatom growth.



Figure 7. *A. taxiformis* tetrasporophytes in 1 L culture stock culture vessels with filtered aeration lines.

2. Transfer the young tetrasporophytes in the 1 L culture vessel to a constant environment room at 19 °C with approximately 10-15 µmol photons m-2 s-1 and a photoperiod of 10:14 (light:dark). Then, add gentle aeration to circulate the tetrasporophytes and prevent the freshly liberated holdfasts from sticking to the culture vessel. Cap the culture vessels to minimise the risk of contamination and reduce evaporative loss of water (Figure 7).
3. Over the next six days, incrementally increase the light each 48-hour period to 15, 30 and 50 µmol photons m-2 s-1, respectively. The photoperiod should remain at 10:14 (light:dark).
4. Monitor tumble cultures daily to check for tetrasporophyte survival, health, growth and contamination. If there are signs of contamination, discard the culture.
5. Water changes occur weekly using the following procedure.
    i. Strain the culture vessel water and tetrasporophytes through a 25 µm micro-filter and then suspend the tetrasporophytes in a new, sterile 1 L vessel containing 19 °C UVSW and 10 mL Cell-Hi® nutrient solution at 0.3 g L-1 production concentration (if alternative production nutrient media are to be used, consult the nutrient media standard operating procedures for those media) and 10 mg L-1 of GeO2 to prevent diatom growth.
    ii. Transfer the young tetrasporophytes in the 1 L culture vessel back to the constant environment room at 19°C, 50 µmol photons m-2 s-1 and a photoperiod of 10:14 (light: dark) and add gentle aeration to circulate the tetrasporophytes. Cap the culture vessels to minimise the risk of contamination and reduce evaporative loss of water.
6. The production life of a tetrasporophyte is four weeks from the time it is growing under 50 µmol photons m-2 s-1 in tumble culture. Harvest size at maturity was 8-10 mm for our strains in Perth.
7. To generate commercial quantities of seaweed juveniles to build culture biomass for production, mature tetrasporophytes can be rapidly fragmented. Fragments from 0.1-1 mm in length are viable options for generating new tetrasporophytes and are essentially at the same stage as tetrasporophyte sporelings that are transferred from a petri dish to a tumble culture (8-14 days old).
    i. To fragment one or many mature tetrasporophytes, place the tetrasporophytes in a 50-100 mL beaker with 10-20 mL filtered and sterilised seawater at 19 °C.
    ii. Using a hand-held milk frother or another device that doesn't result in the tetrasporophytes being emulsified, agitate the tetrasporophytes for 20-30 seconds or until the tetrasporophytes are completely fragmented and no large (>1 mm) fragments are observed.
    iii. One mature tetrasporophyte (8-10 mm in diameter) can generate 500-700 viable juveniles; 500-700 tetrasporophytes are required to populate a 1 L vessel at a final density of 5 g L-1 (Figure 8a). A 1 L vessel with fragments generated from a 5 g L-1 mature tetrasporophyte culture (Figure 8b) could then populate a >100 L production system.
    iv. After four to six weeks, a density of 5 g L-1 could be achieved in the >100 L production system, which in turn can populate tens of thousands of litres of production systems. Production systems can be wide and varied, with simple production systems (e.g. as shown in Figure 9) generating biomass.

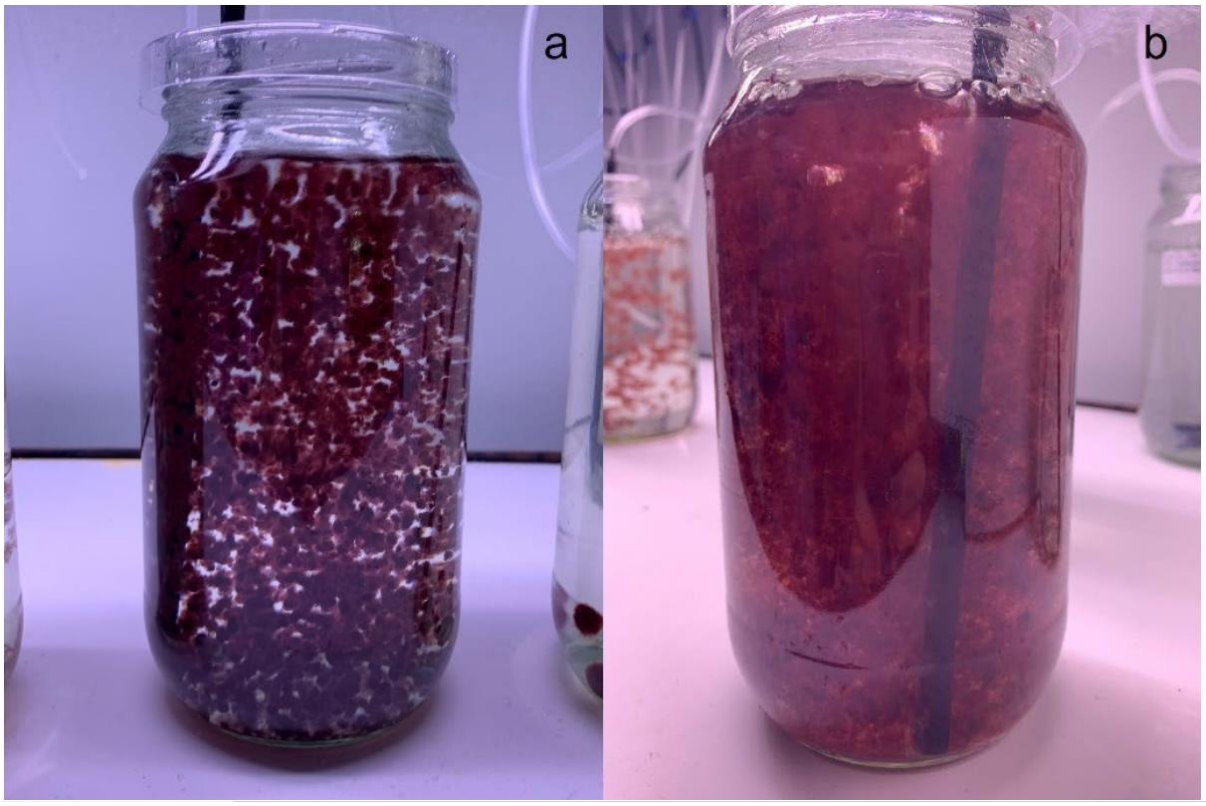


Figure 8. (a) Asparagopsis taxiformis mature tetrasporophytes (8-10 mm in diameter) with a density of 5 g L-1 in 1 L culture vessel; (b) using our rapid fragmenting technique, 5 g L-1 is fragmented to generate tens of thousands of seaweed juveniles with >95% viability.

Chapter 2

In-house biosynthesis of λ-carrageenase

2 In-house biosynthesis of λ-Carrageenase

The cell wall of *A. taxiformis* consists primarily of λ-carrageenan and β-agaran, necessitating the inclusion of λ-carrageenase and β-agarase in the enzyme cocktail for successful protoplast isolation. While these enzymes have been identified and characterized by various research groups, they are not commercially available, particularly λ-carrageenase. This scarcity prompted a detailed search for enzyme manufacturers offering custom biosynthesis services.

λ-Carrageenase, identified by a single research group, has not been widely studied or synthesized by commercial manufacturers, as confirmed by public databases. This limited availability led to hesitancy among manufacturers to undertake custom biosynthesis, given the low success rate and high upfront costs associated with heterologous expression and enzyme purification. The manufacturers cited technical challenges, including the lack of precedent for λ-carrageenase production, which required extensive email communication over three months to ensure all necessary information was provided. Despite initial agreements, the projected two-month production timeline experienced an additional four-week delay, highlighting the complexities and uncertainties of outsourcing this process.

Given these challenges, we decided to pursue in-house biosynthesis of λ-carrageenase (for this purpose, we requested the transgenic E. coli strain developed by the commercial manufacturer who provided us with 2 lots of λ-carrageenase). This approach offers significant savings in both time and capital but necessitates the development of comprehensive protocols. These include creating a transgenic E. coli strain expressing the λ-carrageenase gene, culturing and sub-culturing the strain to produce sufficient biomass, inducing protein expression, and purifying the target enzyme.

During in-house biosynthesis, λ-carrageenase was expressed as inclusion bodies, requiring the establishment and optimization of protocols for their purification and refolding. To streamline the purification process, a commercially available Ni-NTA Purification System (ThermoFisher Scientific) was utilized, enabling the recovery and refolding of the enzyme to achieve functionality. This chapter outlines the stepwise protocols developed for the in-house biosynthesis and purification of λ-carrageenase, addressing the unique challenges encountered during the process.

Overall, the in-house biosynthesis of λ-Carrageenase can be divided into the following phases:

1. Protocol to Culture Transgenic E. coli expressing λ-Carrageenase.
2. Protocol to Subculture Transgenic E. coli Strain & Induction of λ-Carrageenase Biosynthesis
3. Protocol for Collecting Cell Pellets and Lysing Transgenic E. coli Cells.
4. Protocols for purification of λ-Carrageenase

Chapter 2

2.1 Protocol to Culture of Transgenic E. coli expressing λ-Carrageenase.

This protocol outlines the steps for culturing transgenic *E. coli* strains engineered to express λ-carrageenase. Optimal conditions for growth, including the choice of media and incubation parameters, are specified to promote robust bacterial proliferation. The protocol ensures that sufficient biomass is produced to proceed with downstream processes such as subculturing and protein induction.

2.1.1 Laboratory Checklist

Chemicals

- LB-Kanamycin plates
- Glycerol stock of transgenic E. coli

Equipment

- Incubator set at 37°C
- Marking pen
- 2-20ul Adjustable volume micropipette and tips
- Basic microbiology lab & equipment

2.1.2 Protocol – Generation of fresh culture via streaking on agar plates.

The agar streaking technique generates single colonies from a concentrated suspension of bacteria. A minute amount of the suspension may contain hundreds of thousands of bacterial cells. Under favourable conditions, one cell multiplies to become millions of genetically identical cells in just 24 hours. A single 1 mm bacteria colony can have millions of individual bacteria which are progeny of a single cell, and they are now labelled as fresh and pure culture of that parental cell. Below are the steps describing how to generate fresh and pure culture via streaking on an agar plate.

1. Pipet 10 µl of *E. coli* cells from stock culture using a sterile pipet tip onto an LB-Kanamycin agar plate. Use a sterile loop to perform the streaking. Streaking takes place sequentially in four quadrants. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in one quadrant.
    a. In subsequent quadrants the cells become more and more dilute, increasing the likelihood of producing single colonies.
2. For subsequent streaks, the goal is to use as much of the surface area of the plate as possible. Rotate the plate approximately 45 degrees (so that the streaking motion is comfortable for your hand) and start the second streak.
    a. Do not dip into the rehydrated bacteria a second time.
    b. Go into the previous streak about two times and then back and forth for a total of about 10 times.
3. Rotate the plate again and repeat streaking.
4. Rotate the plate for the final time and make the final streak.
    a) When you are finished streaking the plate, cover it immediately to avoid contamination.
    b) Label the plate with your initials.
5. Place the plate upside down inside the incubator overnight at 37°C. Once colonies have grown to 1 mm in diameter, they may be stored in a sealed bag upside down at 4ºC until ready to use for up to one month.
6. *E. coli* forms off-white colonies that are uniformly circular with smooth edges. Avoid using plates with contaminant colonies.

2.1.3 Protocol for Overnight Cell Culture

Chemicals

- LB-Kanamycin plates streaked with transgenic *E. coli*
- 2% Sterile glucose (300ul)
- Sterile LB-Kanamycin broth

Equipment

- Incubator set at 37°C
- Marking pen
- 50 mL conical tubes
- 2-20ul Adjustable volume micropipette and tips
- Basic microbiology lab & equipment

Protocol

1. Label the 50 ml conical tube containing 3 ml of sterile LB-Kanamycin broth.
2. Add 150 µl of 20% sterile glucose to the 3 ml of sterile LB-Kanamycin broth to make a final 1% glucose in the LB-Kanamycin broth solution.
3. Pick a single colony from the starter plate using a micropipette with a sterile inoculating loop and inoculate the 3 ml of 1% glucose, LB-Kana broth by twirling the pipet tip vigorously in the broth to dislodge the cells.
4. Recap the tube. Incubate/shake the culture at 37°C for 12–18 hours.

2.2 Protocol to Subculture Transgenic *E. coli* Strain & Induction of λ-Carrageenase Biosynthesis

Subculturing and induction are critical for initiating λ-carrageenase production in transgenic *E. coli*. This protocol describes transferring bacterial cultures to fresh media and inducing protein biosynthesis using IPTG as a chemical inducer. Careful monitoring of growth and induction conditions ensures maximum expression of the target enzyme.

2.2.1 Laboratory Checklist

We require the following items to prepare a sample of unindicted cells and induce the cells for SDS-PAGE analysis.

Chemicals

- Overnight culture
- 100mM IPTG
- LB-Kanamycin broth
- Sterile conical tube (100ml)
- Laemmli sample buffer

Equipment

- Microcentrifuge tubes: 1.5 & 2 ml
- Microcentrifuge
- Cuvettes
- Spectrophotometer
- Parafilm squares
- Adjustable-volume micropipette and tips
- Shaking incubator set to 37°C
- Dry bath set to 95°C
- Marking pen.

2.2.2 Prepare uninduced SDS-PAGE sample.

1. Label a 1.5 ml screwcap microcentrifuge tube “Uninduced PAGE” with your initials.
2. Aliquot 100 µl of the overnight culture into the tube.
3. Centrifuge the tube at 16,000 x g for 2 minutes.
4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.
5. Add 100 µl of lysis buffer and 1X Laemmli sample buffer to the pellet and fully resuspend the pellet by vertexing. Heat the tube at 95°C for 5 minutes.
6. Store the sample at –20°C until ready to analyse via SDS-PAGE analysis.

2.2.3 Measure cell density of overnight culture.

1. Prepare a 1:10 dilution of your overnight culture by combining 900 µl of sterile LB broth with 100 µl of overnight culture in a clean semi-micro volume cuvette.
2. Cover the cuvette with parafilm and gently invert the cuvette several times to mix the diluted overnight culture.
3. Set a spectrophotometer to read absorbance at 600 nm and then blank the instrument using 1 ml LB broth in a cuvette. (Note: Save this cuvette containing LB broth to use as a blank for subsequent readings.
4. Measure the absorbance at 600 nm (OD600) of the 1:10 dilution of overnight culture cells. Use the following formula to calculate the OD of overnight culture.
5. OD600 of 1:10 dilution of overnight culture: _____
6. OD600 of overnight culture = OD600 of 1:10 dilution x 10 : _____

2.2.4 Prepare a subculture with a starting OD600 of 0.3

1. Prepare a 100 ml culture with an OD600 of 0.3 by combining the correct volumes of overnight culture and sterile LB broth that has been warmed to 37°C.

$$\boldsymbol{X} \text{ ml of overnight culture} = \frac{100\text{ml} * 0.3}{OD\ 600\ of\ overnight\ Culture}$$

2. Calculate the amount of overnight culture that you will need to prepare a subculture with an OD600 of 0.3 and record the value below.
   _____ ml of overnight culture
3. Retrieve your Erlenmeyer flask tube containing 100 ml of LB-Kanamycin broth that has been warming to 37°C and label it.
4. Remove and discard a volume of LB-Kanamycin broth, equivalent to the volume you calculated in step 1, from the 100 ml flask that you would need for overnight culture using a sterile 10 ml serological pipet.
5. Gently swirl your overnight culture to resuspend all of the cells and then add the volume you calculated of overnight culture in step 1 to the remaining LB-Kanamycin in the Erlenmeyer flask using a sterile 10 ml serological pipet.
6. Cover the flask with foil and place the 100 ml culture in an incubator shaker at 37ºC with shaking at 250–275 rpm for one hour.
7. Record the time that you start your subculture below.
   Subculture Start Time: _____
8. Record the time you stop your subculture below.
   Subculture End Time: _____

2.2.5 Measure OD600 of subculture and induce GOI expression.

1. After four hours of incubation measure the OD600 of your subculture.
2. Blank a spectrophotometer at 600 nm with 1 ml LB broth.
3. Add 1 ml of subculture to a semi-micro volume cuvette and measure the OD600 of the undiluted subculture and record below.

   OD600 of subculture cells: _____

4. Add ----µl of 1 M IPTG to the subculture in the 500 ml Erlenmeyer flask and continue incubating and shaking the culture at 20ºC for 4–24 hours. During this time, most of the resources of the *E. coli* will go toward producing the λ-Carrageenase protein rather than cell division, though cell division does still occur.
5. Record the time that you start and end your induction below.
   Induction Start Time: _____
   Induction End Time: _____

2.3 Protocol for Collecting Cell Pellets and Lysing Transgenic *E. coli* Cells.

This protocol focuses on harvesting *E. coli* cells post-induction and lysing them to extract intracellular λ-carrageenase. It details centrifugation steps to collect cell pellets and the use of lysis buffers to release the expressed protein. Proper cell disruption is critical to recovering the enzyme for subsequent purification.

2.3.1 Laboratory Checklist

We require the following items to prepare a sample of unindicted cells and induce the cells for SDS-PAGE analysis.

Chemicals

- Induced cell culture 100ml
- Laemmli sample buffer
- Lysis buffer (1:10)

Equipment

- Microcentrifuge tubes: 1.5 & 2 ml
- Adjustable-volume micropipette and tips
- Microcentrifuge
- Shaking incubator set to 37°C
- Vortexer
- Dry bath set to 95°C
- Sonicator
- Marking pen.
- Ni-NTA Purification System (ThermoFisher Scientific, Cat# K95001)

2.3.2 Prepare Induced SDS-PAGE Sample

1. Label a 1.5 ml screwcap microcentrifuge tube “Induced PAGE” with your initials.
2. Aliquot 100 µl of the induced culture into the tube.
3. Centrifuge the tube at 16,000 x g for 2 minutes.
4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.
5. Add 100 µl lysis buffer and 1X Laemmli sample buffer to the pellet and fully resuspend the pellet by pipetting up and down and vertexing.
6. Heat the tube at 95°C for 5 minutes.
7. Store the sample at –20°C until ready to analyze via SDS-PAGE.

2.3.3 Pelleting-induced cells

1. Place the 100 ml of induced culture in a 250 ml centrifuge bottle that will fit into your benchtop or floor model centrifuge.
2. Centrifuge the cell pellet at 4,500 x g for 10 minutes.
3. When the centrifugation is done the supernatant should look transparent. If it is not transparent centrifuge the bottle for another 10 minutes. Carefully decant or pipet the supernatant making sure not to disturb the pellet and to dispose of the supernatant properly. Store the cell pellet at –20°C or continue on to lysing-induced cells.

2.3.4 Lysing-induced cells

Use the following procedure to prepare bacterial cell lysate under denaturing conditions (The following protocol has been adopted from the manual of ThermoFisher Scientific’s Ni-NTA Purification System for purification of polyhistidine-containing recombinant proteins):

1. Equilibrate the Guanidinium Lysis Buffer, pH 7.8 (supplied with the system) to 37°C.
2. Harvest cells from a 50 mL culture by centrifugation (e.g., 5000 rpm for 5 minutes).

3. Resuspend the cell pellet in 8 mL of Guanidinium Lysis Buffer from Step 1.
4. Slowly rock the cells for 5–10 minutes at room temperature to ensure thorough cell lysis.
5. Sonicate the cell lysate on ice with three 5-second pulses at high intensity.
6. Centrifuge the lysate at 3000 × g for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
7. Remove 5 µL of the lysate for SDS-PAGE analysis. Store the remaining lysate on ice or at –20°C (When ready to use, proceed to the denaturing protocol stated next).

Note: To perform SDS-PAGE with samples in Guanidinium Lysis Buffer, you need to dilute the samples, dialyze the samples, or perform TCA precipitation prior to SDS-PAGE to prevent the precipitation of SDS.

2.4 Protocols for purification of λ-Carrageenase (from inclusion bodies)

Purification protocols are essential for isolating λ-carrageenase from crude cell lysates. This section describes affinity-based purification techniques (Ni-NTA chromatography), to achieve a high-purity enzyme. Optimization steps for the inclusion body solubilization and refolding are also included to recover the biologically active form of λ-carrageenase.

Instructions to perform purification using denaturing conditions with prepared denaturing Buffers, columns, and cell lysate are described below. Be sure to check the pH of your buffers before starting.

2.4.1 Laboratory Checklist

You will need the following items:

- Denaturing Binding Buffer
- Denaturing Wash Buffer, pH 6.0
- Denaturing Wash Buffer, pH 5.3
- Denaturing Elution Buffer
- Prepared Ni-NTA Agarose with denaturing buffers
- Lysate prepared under denaturing conditions.

Important Note

Be sure to check the pH of your buffers before starting. Note that the denaturing buffers containing urea will become more basic over time.

Preparing the Denaturing Wash Buffer pH 5.3

Using a 10 mL aliquot of the kit-supplied Denaturing Wash Buffer (pH 6.0), adjust the pH to 5.3 using HCl. Use this for the Denaturing Wash Buffer pH 5.3 in Step 5.

2.4.2 Preparing Ni-NTA Column

When preparing a column as described below, make sure that the snap-off cap at the bottom of the column remains intact.

To prepare a column:

1. Resuspend the Ni-NTA Agarose in its bottle by inverting and gently tapping the bottle repeatedly.
2. Pipet or pour 2 mL of the resin into a 10-mL Purification Column supplied with the kit. Allow the resin to settle completely by gravity (5–10 minutes) or gently pellet it by low-speed centrifugation (1 minute at 800 × g). Gently aspirate the supernatant.
3. Add 6 mL of sterile, distilled water and resuspend the resin by alternately inverting and gently tapping the column.
4. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
5. For purification under Denaturing Conditions, add 6 mL of Denaturing Binding Buffer.
6. Resuspend the resin by alternately inverting and gently tapping the column.
7. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant. Repeat Steps 5 through 7.

2.4.3 Purification of λ-carrageenase under Denaturing Conditions

Using the denaturing buffers, columns, and cell lysate, follow the provided procedure to purify proteins under denaturing conditions:

1. Add 8 mL lysate to a prepared Purification Column.
2. Bind for 15–30 minutes at room temperature using gentle agitation (e.g., using a rotating wheel) to keep the resin suspended in the lysate solution. Settle the resin by gravity or low-speed centrifugation (800 × g), and carefully aspirate the supernatant.
3. Wash the column with 4 mL Denaturing Binding Buffer by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low-speed centrifugation (800 × g), and carefully aspirate the supernatant.
4. Save supernatant at 4º C for SDS-PAGE analysis. Repeat this step one more time.
5. Wash the column with 4 mL Denaturing Wash Buffer (pH 6.0) by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low-speed centrifugation (800 × g), and carefully aspirate the supernatant. Save supernatant at 4º C for SDS-PAGE analysis. Repeat this step one more time.
6. Wash the column with 4 mL Denaturing Wash Buffer pH 5.3 by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low-speed centrifugation (800 × g), and carefully aspirate the supernatant. Save supernatant at 4º C for SDS-PAGE analysis. Repeat this step once more for a total of two washes with Denaturing Wash Buffer pH 5.3.
7. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein by adding 5 mL of Denaturing Elution Buffer. Collect 1 mL fractions and monitor the elution by taking OD280 readings of the fractions. Pool fractions that contain the peak absorbance and dialyze against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. Concentrate the dialyzed material by any standard method (i.e., using 10,000 MW cut-off, low protein binding centrifugal instruments or vacuum concentration instruments).
8. If you wish to reuse the resin to purify the same recombinant protein, wash the resin with 0.5 M NaOH for 30 minutes and equilibrate the resin in a suitable binding buffer.

2.5 Solutions:

2.5.1 LB Agar Plates/Broth

Sr. #	Chemical	For 1000 mL
1	Yeast extract	5.0 g
2	Peptone	10.0 g
3	Sodium chloride	10.0 g
4	Agar-agar	12.0 g
	Adjust the pH to 7 and sterilize.	

2.5.2 Antibiotic Concentrations

Antibiotic	Recommended Stock Concentration	Recommended Working Concentration
Kanamycin	50 mg/m	50 µg/mL

2.5.3 Protein Purification Kits

Sr. #	Product	Provider	Cat log
1	Ni-NTA Purification System	ThermoFisher Scientific	K95001
2	Bolt™ Bis-Tris Plus Welcome Pack and Mini Blot Modules, 4–12%		NW0412G

Chapter 3

Isolation and culturing of *A. taxiformis* protoplasts

3 Isolation of *A. taxiformis* protoplast

A. taxiformis, a red macroalga, has garnered significant attention for its ecological and biotechnological potential. Renowned for its distinctive triphasic heteromorphic life cycle, *A. taxiformis* exhibits morphological adaptations that contribute to its resilience in marine environments. Its utility extends beyond its ecological role, as it has been identified as a promising candidate for methane mitigation in livestock, attributed to its production of bioactive compounds such as bromoform. However, the complex cell wall composition of *A. taxiformis*, primarily consisting of λ-carrageenan and β-agarans, presents challenges for biotechnological applications, including tissue culture, genetic engineering and metabolic studies via protoplast technology.

Protoplasts—cells devoid of their rigid cell wall—offer an invaluable platform for studying cellular processes and enabling genetic modifications. By removing the cell wall, protoplasts provide direct access to the plasma membrane, simplifying molecular manipulations such as transformation, fusion, and metabolite uptake. Protoplast technology has proven to be a versatile tool in algal research, enabling advancements in asexual propagation, strain improvement, gene editing, and physiological studies.

Isolation of protoplasts typically involves enzymatic digestion of the cell wall, leveraging specific hydrolytic enzymes tailored to the cell wall composition. For *A. taxiformis*, enzyme cocktails containing λ-carrageenase and β-agarase are essential due to the polysaccharide-rich nature of its cell wall. Optimization of these enzymes, along with supporting components such as cellulase and macerozyme, is critical to achieving effective wall degradation and high protoplast yield.

The isolation of *A. taxiformis* protoplasts is pivotal for advancing its biotechnological applications. Protoplasts enable genetic engineering approaches to enhance bromoform production and investigate stress tolerance mechanisms. Moreover, they provide a model system for understanding the cell biology of *A. taxiformis*, laying the groundwork for innovations in sustainable seaweed cultivation and methane mitigation strategies. This chapter outlines the protocols and considerations for successful protoplast isolation, offering a comprehensive guide for researchers and industry stakeholders.

3.1 Protocol for Plasmolysis of *A. taxiformis* explants.

Plasmolysis is a process that involves a plant/algal cell that loses water content and results in contracting and shrinking its cytoplasm and plasma membrane, separating it from its cell wall. Plasmolysis happens when the solution surrounding the cell is hypertonic or has a high concentration of solutes.

3.1.1 Laboratory Checklist

Chemicals

□ Mannitol	□ KCl	□ Proteinase P
□ NaCl	□ $MgCl_2$	□ ASW

Equipment

□ Shaker incubator	□ 0.22 filtration assembly	□ Alcohol lamp
□ Microscope	□ Razor blade	□ 35mm Petri Dish
□ Aluminium foil	□ Forceps	□

3.1.2 Protocol

1. Cut 2-3 fronds from the gametophyte. Remove the leaflets.
2. Cut the fronds into small pieces i.e. up to 2-3 mm (horizontally)
3. Prepare the plasmolysis solution as stated in the heading 3.3.1.
4. Dispense 2-3 mL of plasmolysis solution into a Petri dish.
5. Prepare a control plate containing autoclaved seawater.
6. Add sufficient small pieces of explant into both plates.
7. Wrap both plates with aluminium foil.
8. Incubate the plates at 25C, for 30-45min and 70 rpm.
9. Following the incubation, analyse the explants under the microscope (40x)

3.2 Protocol for protoplast isolation of *A. taxiformis.*

The protoplast isolation protocol for Asparagopsis taxiformis is designed to effectively remove the algal cell wall, granting access to intact, wall-free cells for downstream applications. This process involves the use of a carefully optimized enzymatic cocktail, including λ-carrageenase and β-agarase, tailored to degrade the polysaccharide-rich composition of the A. taxiformis cell wall. This protocol provides a systematic approach to achieving high protoplast yield and viability.

3.2.1 Laboratory Checklist

Chemicals

□ Mannitol	□ NaCl	□ MES
□ Dextran sulphate	□ ASW	□ λ-Carrageenase
□ β-Agarase	□ Cellulase	□ Macerozyme
□ Pectolyase Y-23	□	□

Equipment

□ Shaker incubator	□ 0.22 filtration assembly	□ Alcohol lamp
□ Microscope	□ Forceps	□ 35mm Petri Dish
□ Aluminium foil	□	□

3.2.2 Protocol

If starting without the plasmolysis step:

1. Cut 2-3 fronds from the gametophyte. Remove the leaflets.
2. Cut the fronds into small pieces i.e. up to 2-3 mm (horizontally)
3. Prepare the enzyme solution as stated in the heading 3.3.2.
4. Dispense the required volume of protoplast isolation enzyme solution into a Petri dish.
5. Prepare a control plate containing autoclaved seawater.
6. Add sufficient small pieces of explant into both plates.
7. Wrap both plates with aluminium foil.
8. Incubate the plates at 25C, for 2h and 70 rpm.
9. Following the incubation, analyse the explants under the microscope (40x)

If starting with/after the plasmolysis step:

1. Prepare the enzyme solution as stated in the heading 3.3.2.
2. Dispense the required volume of protoplast isolation enzyme solution into a Petri dish.
3. Transfer all pieces of the explant from the plasmolysis plate into the plate containing the enzyme solution.
4. Keep the control plate as it is.
5. Wrap the plates with aluminium foil.
6. Incubate the plates at 25C, for 2h and 70 rpm.
7. Following the incubation, analyse the explants under the microscope (40x)

3.3 Solutions:

3.3.1 Plasmolysis Solution

Sr. #	Solutes	Stock Sol.	Working Sol.		Osmolarity	10 ml
		Moles	mMoles	Moles	mOsmol	(ml or g)
1	Mannitol	-	800	0.8	800	1.46g
2	NaCl	3	150mM	0.15	300	0.50
3	MgCl2	1	50mM	0.05	150	0.50
4	KCl	1	50mM	0.05	100	0.50
5	Proteinase P (Amano) 6SD		1%	-	-	0.1g
6	Solvent (ASW)	-	-	-	1000	Up to 10ml
	Total osmolarity				2350	
	Adjust the pH between 6.5-7					

3.3.2 Enzymes Solution

Sr. #	Solutes	Stock Sol.	Working Sol.		Osmolarity	10ml
		Moles	mMoles	Moles	mOsmol	(mL or g)
1	NaCl	3	172 mM	0.172	344	0.57mL
2	MES	0.10	25 mM	0.025	25	2.50mL
3	Mannitol	-	800 mM	0.8	800	1.458g
4	Dextran sulphate	-	1%	0	0	0.1g
5	λ-Carrageenase	-	-	-	100ul from stock solution per 1ml enzyme solution	
6	β-Agarase	-	-	-		
7	Cellulase	-	2%	-	-	0.2g
8	Macrozyme	-	2%	-	-	0.05g
9	Pectolyase Y-23	-	0.5%	-	-	0.05g
10	Solvent (ASW)				1000	Up to 10 mL
	Total osmolarity				2169	
	Adjust the pH between 6.5-7					

Note: Prepare the enzymes solution with all components except λ-Carrageenase and β-Agarase in 10mL final volume. Adjust the pH and take 800ul from them into a 35mm Petri dish. Add 100ul of each λ-Carrageenase (0.89mg/mL stock solution, provided by private enzymes manufacturer) and β-Agarase (1mg/mL stock solution, provided by private enzymes manufacturer) into the Petri Dish. Finally, add 35ul of each Calcofluor white (0.01% diluted in ASW) and Toluene Blue O (0.01g in 50ml ASW) into the plate. Finally, transfer explants into the plate and place it in a shaker incubator.