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Protoplast isolation and regeneration from the potential economic brown alga *Petalonia fascia* (Ectocarpales, Phaeophyceae)

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Abstract

Petalonia fascia is a widespread brown alga with economic potential due to its use as raw or dried powder, and in the biomedical field. Protoplasts are living plant cells devoid of cell wall with a wide range of applications in basic and applied research, especially in crop improvement, and as a seedstock in seaweeds. Protoplasts have been previously isolated from *P. fascia*, but, their regeneration ability, an important prerequisite for protoplast applications, has not been explored. In this work, we report the protoplast isolation and successful regeneration from *P. fascia* using the commercially available cellulase "Onozuka" RS (1%) and alginate lyase (4 U mL^{-1}). Protoplast production was enhanced under increased osmolarity (2512 mOsm L^{-1} H_2O), with chelation pre-treatment, and short incubation time (4 h). Our protocol produced more than 14 times the number of protoplasts obtained using previously reported protocols. After 4 weeks in culture, protoplasts developed into prostrate and discoid and mixed thallus, as well as cell clumps. Blades mostly emerged from prostrate thalli. Antibiotics were not crucial for improving protoplast regeneration, and temperature did not affect the development of the morphological forms and blades. Our results show that high yields of protoplasts (10^7 protoplasts g^{-1} FW) with good regeneration ability can be obtained from *P. fascia* using a simple mixture of commercial enzymes. This represents the first report of successful protoplast regeneration in *P. fascia*.

Keywords Brown algae · Commercial enzymes · *Petalonia fascia* · Protoplast isolation · Protoplast regeneration

Introduction

Petalonia fascia (O.F.Müller) Kuntze is a brown alga distributed along temperate coasts worldwide. Its thallus is mostly epiphytic and shows a polystichous structure, with blades up to 30 cm high attached to the substratum by small discoid holdfasts (Boo 2010). Cultivation of *P. fascia* was proposed by Lee et al. (2003) in Korea as a new economic species. They stated that *P. fascia* could be easily cultivated on ropes and that its market value was promising, either as raw or dried powder. In addition, chemical compounds or extracts from *P. fascia* has been extensively investigated for biomedical applications and protected with nine patent applications. For example, fucoidan from this species has been used for enhancing stem cell mobilization and proliferation, elastase inhibitor for obtaining a synergy effect with fish-derived collagen, and an extract for enhancing bone mass (Kim and Jung 2019). Thus, *P. fascia* represents a potential economic brown alga with multiple uses.

Protoplasts are naked living plant cells that can be obtained by enzymatic digestion of the cell walls. These are widely used for studying plant genetics, for breeding, and, more recently, for genome-editing and gene silencing technologies (Davey et al. 2005; Burris et al. 2016). In seaweeds, protoplasts can be also used as seeds for large-scale farming, offering the advantage of continuous supply of plantlets without a waiting period (Gupta et al. 2018). Isolation and regeneration of brown algal protoplasts have been accomplished in 33 species (Fisher and Gibor 1987; Chen and Shyu 1994a; Matsumura 1998; Reddy et al. 2008; Avila-Peltroche et al. 2019; Avila-Peltroche and Won 2020). Considering the economic potential of *P. fascia*, this species is a good candidate for protoplast

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technology. High yields of protoplasts have been obtained from *P. fascia* using a combination of cellulase "Onozuka" RS and macerozyme R-10 (Chen and Shyu 1994a). However, their regeneration ability, an important prerequisite in protoplast technology, has not been assessed.

In this study, we report the protoplast isolation and successful regeneration from *P. fascia* using a simple mixture of commercial enzymes. We tested the effect of osmolarity, driselase inclusion, chelation pre-treatment, incubation time, and previous isolation protocols on protoplast production. Also, we assessed the effect of antibiotics and temperature in protoplast cultures to determine the best conditions for regeneration.

Materials and methods

Isolation and culture of the strain

Petalonia fascia was isolated from crude cultures of filamentous brown algae collected in Geoje Island, Gyeongsangnam-do, Korea, on March 16, 2018. Young germlines were cultured in 60×15 mm Petri dishes containing PES medium (Provasoli 1968) under 14:10-h light/dark photoperiod at 20 °C with light intensity 40 µmol photons m^{-2} s^{-1} of blue LED (DyneBioCo. Korea). The medium was renewed weekly. In this condition, prostrate thalli were obtained. Erect thalli (blades) developed upon cultivation in 1 L flat-bottomed round flasks filled with 1 L PES medium under aeration with a light intensity of 40–72 µmol photons m^{-2} s^{-1}. Temperature and photoperiod were the same as indicated above. The air was sterilized using 0.22 µm SFCA syringe filters (Corning, Germany). The medium was renewed every 2 weeks. After a month in culture, blades reached about 8 cm in length before starting the formation of plurilocular sporangia. Representative specimen was deposited in the herbarium of Chosun University (CUK19258, =MBRB0029TC19258).

Identification of the culture strain

Taxonomic identification was performed using morphological characters (Boo 2010) from blades maintained in 1 L flat-bottomed round flasks with aeration. Photomicrographs were taken using a Leica inverted microscope (DMi8; Leica, Germany) equipped with a Leica DFC450C camera. Genomic DNA extraction, PCR amplification, DNA purification, and sequencing were performed according to Bustamante et al. (2016) using cultured samples. The plastid *rbc*L was amplified using the primer combinations described by Kogame et al. (1999). The amplified gene sequences were compared to the GenBank nucleotide database using the BLAST program (Altschul et al. 1997).

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study included cellulase "Onozuka" RS (Yakult Co. Ltd., Japan), alginate lyase, and driselase (Sigma-Aldrich, USA). Different enzyme combinations and conditions are shown in Table 1.

Protoplast isolation was carried out as previously described (Coelho et al. 2012) with some modifications. Briefly, approximately 100–300 mg of explants of 4–6 mm^2 from cultured blades of *P. fascia* (about 8 cm in length) were incubated in a 0.22 µm filter-sterilized enzymatic solution (400 mM NaCl, 130 mM $MgCl_2 \cdot 6H_2O$, 22 mM $MgSO_4$, 160 mM KCl, 2 mM $CaCl_2$, and 10 mM MES; pH 6; 1570 mOsm L^{-1} H_2O) containing cellulase "Onozuka" RS and alginate lyase, either with or without driselase, at 20 °C with shaking at 70 rpm in the dark. Vegetative blades were used in all experiments. The osmolarity of the enzymatic solution was tested in two levels: normal osmolarity (1× = 1570 mOsm L^{-1} H_2O) and increased osmolarity (1.6× = 2512 mOsm L^{-1} H_2O). Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. The inclusion of driselase was assessed together with osmolarity. Also, we tested the effect of chelation pre-treatment and incubation time on protoplast yields. Pre-treatment, which was conducted with a calcium-chelating solution [665 mM NaCl, 30 mM $MgCl_2 \cdot 6H_2O$, 30 mM $MgSO_4$, 20 mM KCl, and 20 mM ethylene glycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt (EGTA-Na_4) as the calcium chelator; pH5.5] for 20 min prior to enzymatic digestion (Coelho et al. 2012). Protoplast isolation was repeated four times in each treatment.

Protoplast isolation was also performed following the Chen and Shyu's (1994a) with 4% cellulase "Onozuka" RS and 2% macerozyme R-10 and Kevekordes' (1993) non-enzymatic method (only chelation pre-treatment; Kevekordes et al. 1993) in order to determine the best protocol under

Table 1 Combinations and concentrations of enzyme mixtures for protoplast isolation from *Petalonia fascia*

Commercial enzymes	Composition of enzyme mixtures			
	A	B	C	D
Cellulase "Onozuka" RS (%)	1	1	1	1
Alginate lyase (U mL^{-1})	4	4	4	4
Driselase (%)	1	-	1	-
Osmolarity	1×	1×	1.6×	1.6×

1×, 1570 mOsm L^{-1} H_2O; 1.6×, 2512 mOsm L^{-1} H_2O

the same laboratory conditions. Their protoplast yields were compared to the values obtained using our protocol under optimal conditions.

Protoplast purification was performed according to Avila-Peltroche and Won (2020). Protoplast yield was estimated by using a hemocytometer (Marienfeld, Germany) and expressed as protoplasts g^{-1} fresh weight (FW). Protoplast size was calculated by using ImageJ 1.46r software (Abràmoff et al. 2004) based on 100 cell measurements for each repetition.

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as previously described (Avila-Peltroche et al. 2019).

Protoplast regeneration

Cells were dispensed into 1 mL of regeneration medium (PES + 285 mM NaCl + 5 mM $CaCl_2$; Mejjad et al. 1992) in 24-well tissue culture test plates, and cultured in the dark at initial protoplast density of 9×10^3 protoplasts mL^{-1}. After 2 days in the dark, osmotic pressure was reduced slowly by adding PES medium (1/5 the volume of the initial regeneration medium). Osmotic pressure was further reduced during the next 2 days by adding PES (2/5 the volume of the initial regeneration medium each day). Protoplasts were exposed to 2–4 µmol photons m^{-2} s^{-1} by the second day of osmolarity reduction. Light intensity was increased to 20–25 µmol photons m^{-2} s^{-1} by the end of the osmolarity reduction. PES medium was renewed once a week. White fluorescent light was used in all the cultures.

The addition of an antibiotic mixture (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin, and 5 mg L^{-1} chloramphenicol; Coelho et al. 2012) and three temperatures (10 °C, 16 °C, and 20 °C) was evaluated in three repetitions. The percentage (%) of dividing cells was calculated after 3 weeks in culture. Morphological forms (i.e., prostrate thallus, discoid thallus, mixed thallus and cell clumps) obtained in each treatment combination were recorded as the number of forms per well after 4 weeks in culture. The regeneration rate (%) was calculated at this point by counting the number of blades in each well.

Statistical analysis

Normality and homoscedasticity were examined by using the Shapiro-Wilk and Levene tests, respectively, prior to conducting parametric tests. Two-way analysis of variance (ANOVA) was used for the comparison of protoplast yield under driselase inclusion and osmolarity. One-way ANOVA was performed to examine the effect of chelation pre-treatment, incubation time, and isolation protocols on protoplast yields. Effect sizes (Sullivan and Feinn 2012) were presented as ω^2 (Field 2009; Lakens 2013) in case of significant results. All these analyses were performed using "car" (Fox and Weisberg 2019) and "userfriendlyscience" (Peters 2018) packages in R.

Tukey's post hoc test was used when the results were significant. Post hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.

The percentage (%) of dividing cells and regeneration rate (%) were analyzed as proportions with beta regressions, since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari and Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto and Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson and Verkuilen 2006).

The number of each morphological form per well was analyzed using either negative binomial or Poisson regression model. Likelihood ratio test was used for deciding which count regression model to use. If zeros were present, Voung test for non-nested data was carried out to check if a zero-inflated regression was needed (Elhai et al. 2008). The analyses were performed using "pscl" (Jackman 2015) and "MASS" (Venables and Ripley 2002) packages in R (R Core Team 2016).

The significance threshold was set at $p = 0.01$ in order to reduce the true Type I error rate (at least 7%, but typically close to 15%) (Sellke et al. 2001). All graphs were created in Graphpad Prism 6.0 (GraphPad Software, USA).

Results

Identification

The vegetative characteristics of the cultured blades matched with the description of *Petalonia fascia* (Boo 2010), although they were somewhat twisted due to culture conditions (suspension cultures; Fig. 1a). Our morphological identification of *P. fascia* was also confirmed by molecular analysis. A 1333-bp portion of the 1476-bp *rbc*L gene was sequenced from our strain of *P. fascia* (Genbank accession number, MW810430). The *rbc*L sequence of our strain was 99% similar to field samples of *P. fascia* reported from Japan by Matsumoto et al. (2014).

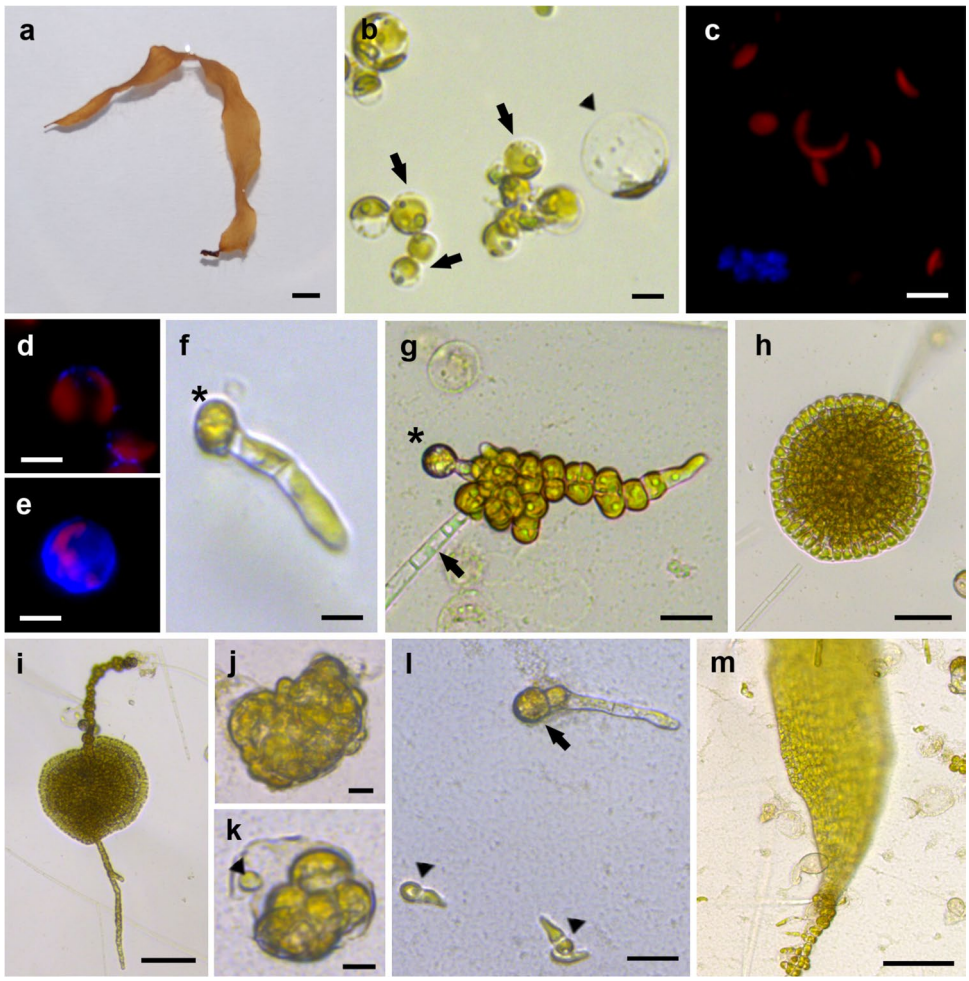

Fig. 1 Protoplast isolation and regeneration from *Petalonia fascia*. **a** A blade from suspension culture in 1 L flat-bottomed round flask with aeration. **b** Protoplasts from the cortex (arrows) and medulla (arrowhead) **c** True protoplasts showing red chlorophyll autofluorescence. The blue fluorescence indicates cell wall material. **d** A protoplast starting cell wall formation after 24 h of culture. **e** Complete cell wall regeneration after 4 days of culture. Bright blue fluorescence indicates cellulose deposition. **f** Three-celled stage after 3 weeks of culture. **G** Prostrate thallus with a phaeophycean hair (arrow) after 4 weeks of culture. The asterisks in **f** and **g** indicate the initial protoplast. **h** Discoid thallus. **i** Mixed thallus showing prostrate filaments and disc thallus. **j** A cell clump. **k** A "spore" (arrowhead) being released from a group of cells derived from a protoplast-cell. **l** Germination of "spores" (arrowheads) and germination of a protoplast (arrow). **m** A blade formed from prostrate thallus after 4 weeks of culture. Scale bars: **a** = 1 cm; **b**, **f**, **j**, **k** = 10 µm; **c**, **d**, **e** = 5 µm; **g**, **l** = 25 µm; **h** = 50 µm; **i**, **m** = 100 µm

Protoplast isolation using enzymes

Protoplast yields ranged from 28–85 × 10^6 protoplasts g^{-1} FW. Mixture C (cellulase 'Onozuka" RS, alginate lyase and driselase with 1.6× osmolarity) produced the highest number of protoplasts (85.16 ± 29.62 × 10^6 protoplasts g^{-1} FW), followed by mixture D (cellulase "Onozuka" RS and alginate lyase with 1.6× osmolarity) with 48.80 ± 12.45 × 10^6 protoplasts g^{-1} FW. The effect of osmolarity and driselase inclusion is shown in Fig. 2a. Osmolarity increase had a positive effect on protoplast production ($p = 0.008$; $\omega^2 = 0.36$). Under increased osmolarity (1.6×), protoplast yields were around 1.8 times higher than in normal osmolarity (1×). Although there was a tendency toward higher protoplast yields when driselase was included, this was not statistically significant ($p = 0.011$). The interaction between both factors did not have a significant effect ($p = 0.999$).

In an effort to simplify our protoplast isolation protocol, chelation pre-treatment and different incubation times were tested. Our experiments showed that pre-treatment had a significant effect on protoplast yield ($p = 0.002$; $\omega^2 = 0.77$; Fig. 2b). Also, incubation time could be reduced to 4 h without compromising the protoplast numbers (Fig. 2c).

When comparing protoplast protocols, 98.58 ± 10.27 × 10^6 protoplasts g^{-1} FW were isolated from our protocol 1% cellulase "Onozuka" RS and 4 U mL^{-1} with chelation

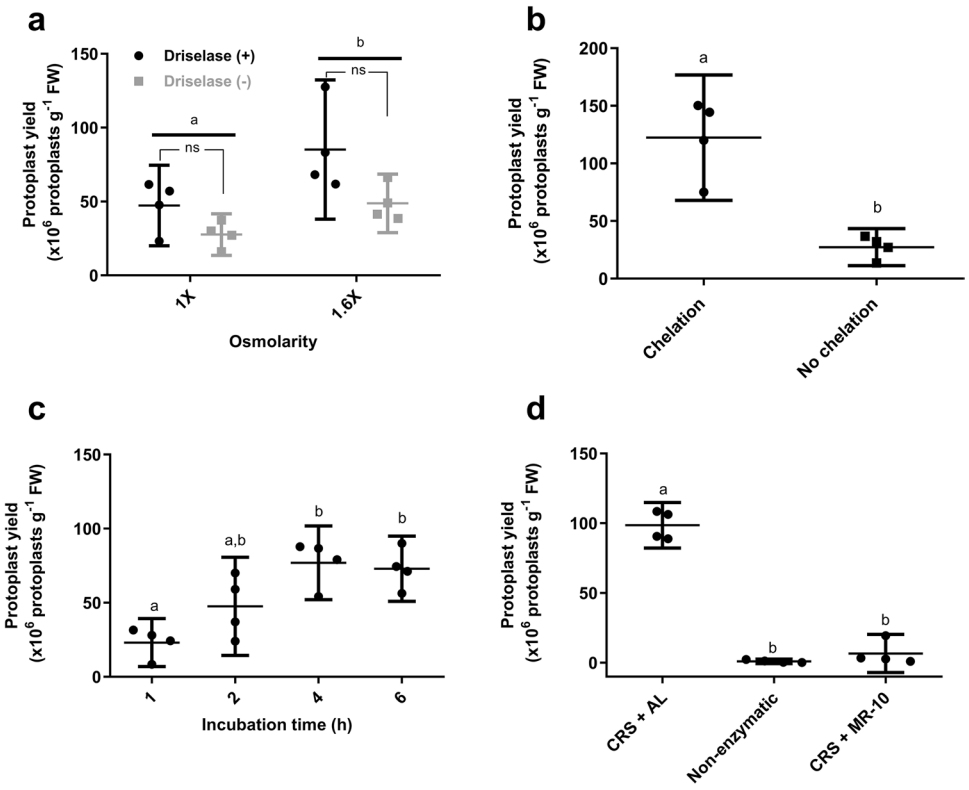

Fig. 2 Effects of different isolation conditions on protoplast yield from *Petalonia fascia*. **a** Effect of osmolarity and driselase inclusion. **b** Effect of chelation pre-treatment. **c** Effect of incubation time. **d** Effect of three different isolation protocols: CRS+AL (this study); non-enzymatic (Kevekordes et al. 1993); and CRS+MR-10 (Chen and Shyu 1994a). Independent data points and averages (horizontal lines) are shown ($n = 4$). Error bars represent 95% confidence intervals. Different letters indicate significant differences between means ($p < 0.01$). CR, cellulase "Onozuka" RS; AL, alginate lyase; MR-10, macerozyme R-10; ns, no significant difference ($p > 0.01$)

pre-treatment). In contrast, $6.66 \pm 8.55 \times 10^6$ protoplasts g^{-1} FW were isolated from Chen and Shyu's (1994a) protocol (4% cellulase "Onozuka" RS and 2% macerozyme R-10), and $1 \pm 0.99 \times 10^6$ protoplasts g^{-1} FW from Kevekordes' (1993) non-enzymatic method (Fig. 2d).

Explants were totally digested after 2–4 h in the enzymatic mixture. Numerous protoplasts were isolated from the cortex and medulla of the blades (Fig. 1b). Protoplasts were pale yellow-brown, spherical shape with a single discoid chloroplast. They were 14.43 ± 5.91 µm (range, 7–36 µm) in diameter. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% (Fig. 1c).

Protoplast culture and regeneration

Staining with calcofluor white revealed that protoplasts started regenerating their cell walls as soon as 12–24 h in culture (Fig. 1d). Some protoplasts showed complete re-synthesis of their cell walls after 4 days in culture (Fig. 1e). However, complete cell wall regeneration was extensively observed during first two weeks in culture. A week later, protoplasts underwent first asymmetric cell division and progressed into a 3-celled stage (Fig. 1f).

The addition of antibiotic mixture at the beginning of protoplast culture showed significant effect on cell division after 3 weeks in culture ($p = 0.002$, PRE = 0.01). Also, it presented a significant interaction with temperature ($p = 0.002$, PRE = 0.05). The highest value of dividing cells was found at 10°C with antibiotics (11.66 ± 1.91%). After 4 weeks in culture, four main morphologies were observed: (1) prostrate thallus; (2) discoid thallus; (3) mixed thallus; and (4) cell clumps (Fig. 1g–j). Antibiotic mixture and temperature did not have significant effect on the formation of these morphological forms ($p > 0.01$). Prostrate thallus was the predominant form in all the cultures and blades arose almost exclusively from this one (Fig. 1m). The highest regeneration rate was found at 20°C without antibiotics (8.45 ± 7.35%). However, the regeneration values were not significantly affected by the antibiotic mixture and temperature ($p > 0.01$; Table 2). In one culture, we could observe successive cell divisions within some spherical protoplast-cells, and the subsequent formation of "spores" that were later released (Fig. 1k). This "spores" were 4.85 ± 0.97 µm (range, 3–6 µm), 3 times smaller than protoplasts (Fig. 1l). They were able to germinate but their further development was not followed.

Discussion

The present study reports the isolation of high amounts of protoplasts (10^7 protoplasts g^{-1} FW) from *Petalonia fascia* using commercial enzymes. Also, this work is the first presenting the successful regeneration of protoplasts from *P. fascia*.

Table 2 Effect of the addition of antibiotics and temperature on protoplast regeneration of *Petalonia fascia* after 3 and 4 weeks in culture. Superscript letters indicate significant differences among treatments ($p < 0.01$) at each response variable (i.e., dividing cells, morphological forms and regeneration rate). Values are presented as mean ± SD ($n = 3$)

Antibiotic mixture*	Temperature	3 weeks in culture	4 weeks in culture				
			Morphological forms				Regeneration (%)
		Dividing cells (%)	Prostrate thalli (thalli well^{-1})	Discoid thalli (thalli well^{-1})	Mixed thalli† (thalli well^{-1})	Cell clumps (clumps well^{-1})	
With	10 °C	11.67 ± 1.91^{a}	7 ± 5^{a}	2 ± 2^{a}	4 ± 4^{a}	0.3 ± 1^{a}	3.53 ± 0.40^{a}
	16 °C	4.55 ± 1.15^{b}	17 ± 13^{a}	3 ± 4^{a}	1 ± 1^{a}	1 ± 1^{a}	7.08 ± 5.67^{a}
	20 °C	6.17 ± 3.57^{a,b}	3 ± 2^{a}	1 ± 2^{a}	2 ± 2^{a}	1 ± 1^{a}	3.70 ± 2.09^{a}
Without	10 °C	4.33 ± 2.08^{b}	13 ± 12^{a}	1 ± 2^{a}	1 ± 1^{a}	0^{a}	4.89 ± 3.20^{a}
	16 °C	5.27 ± 2.64^{b}	6 ± 2^{a}	6 ± 10^{a}	2 ± 3^{a}	0.3 ± 1^{a}	7.23 ± 1.74^{a}
	20 °C	4.38 ± 0.98^{b}	6 ± 4^{a}	0^{a}	0^{a}	1 ± 1^{a}	8.45 ± 7.35^{a}

*Antibiotic mixture was added once at the beginning of the culture. It consisted of penicillin G, streptomycin, and chloramphenicol at a final concentration of 50 mg L^{-1}, 25 mg L^{-1}, and 5 mg L^{-1}, respectively (Coelho et al. 2012). †Mixed thalli consisted of prostrate and discoid thalli arising from one single spot

A simple mix of commercial cellulase "Onozuka" RS and alginate lyase could completely digest *P. fascia* explants releasing high yields of protoplasts. The inclusion of driselase showed a positive effect on protoplast production; however, this was not statistically significant. Driselase is a natural mixture of enzymes (e.g., cellulase, hemicellulase, and pectinase) that can cleave mixed-linked glucan (MLG), also known in fungi as lichenan (Thibault and Rouau 1990). Although the presence of MLG in brown algal cell walls has been reported (Salmeán et al. 2017), our results suggest that driselase might not be crucial for improving protoplast yields.

Increased osmolarity of the enzymatic solution favored the protoplast release in *P. fascia*. Possible reasons for this effect are (1) stimulation of alginate lyase activity by increasing salt concentrations (Huang et al. 2013) or (2) protection of the protoplast membrane (Xiaoke et al. 2003). Similar results were found on protoplast isolation from *Undaria pinnatifida* gametophytes and *Sphacelaria fusca* (Avila-Peltroche and Won 2020; Avila-Peltroche et al. 2020).

In our experiments, chelation pre-treatment improved the protoplast yield of *P. fascia.* This positive effect has also been reported in protoplast isolation from Ectocarpales (Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989). However, in a previous report, this pre-treatment did not enhance protoplast release from *P. fascia* (Chen and Shyu 1994a). It is known that the pH of the EGTA solution used for pre-treatment is a critical factor. Butler et al. (1989) indicated that values over pH 5.5 caused extensive tissue damage in the brown alga *Laminaria*. The pH for the pre-treatment used in our work and the previous report was 5.5 and 6.5, respectively. This could explain the difference in the effectiveness of the pre-treatment in *P. fascia*. Our results also showed an optimal incubation time of 4 h, which is in the range of values reported for *Petalonia* and other blade forms (2–5 h; Chen and Shyu 1994a; Matsumura 1998).

Our new protocol (1% cellulase "Onozuka" RS and 4 U mL^{-1} alginate lyase with chelation) produced 15 and 100 times higher than the ones obtained following Chen and Shyu's (1994a) protocol and Kevekordes' (1993) method under the same laboratory conditions, respectively.

Also, we obtained 10^6 protoplasts g^{-1} FW from cultured samples of *P. fascia* using Chen and Shyu's protocol, while Chen and Shyu (1994a) reported 10^8 protoplasts g^{-1} FW from field material of this species. Also, we obtained 10^6 protoplasts g^{-1} FW from *P. fascia* using the non-enzymatic method, while Kevekordes et al. (1993) reported 10^7 protoplasts g^{-1} FW from kelps (Laminariales).

Protoplast regeneration of *P. fascia* involved the formation of prostrate and discoid and mixed thalli, as well as cell clumps. Among the four forms, the first one was the predominant in cultures. Erect thalli (blades) arose usually from the prostrate thalli after 4 weeks of culture. Discoid thalli and cell clumps have been also found in protoplast cultures from *Petalonia binghamiae*. In this species, blades only emerged from discoid thalli formed by protoplasts from immature blades and young plantlets (Chen and Shyu 1994b). Prostrate and discoid thalli have been reported in the life cycle of *P. fascia* (Hsiao 1969; Kogame 1997; Lee et al. 2003). The occurrence of "spores" from group of cells formed within the spherical protoplast-cell has not been reported in protoplast regeneration of brown algae. Chen and Shyu (1994b) indicated that outer cells in clumps detached in later stages of protoplast cultures from *P. binghamiae*; however, this does not seem to be the case in our cultures, as these "spores" were smaller than the other cells in the group. Protoplast-derived sporangia have been only reported in the

green seaweed *Ulva lactuca* (Gupta et al. 2018). A detailed examination of this process is needed for understanding the nature of these "spores".

Temperature did not have a significant effect on the development of the morphological forms and blades. A similar trend was found by Hsiao (1970), who reported that prostrate and discoid thalli, as well as blades, were present in cultures from 6 to 20 °C. The addition of antibiotics at the beginning of the culture did not enhance the regeneration in *P. fascia*. This suggests that protoplasts from this species are not very sensitive to microbial contamination, which differs from those ones of kelps (Benet et al. 1997).

In conclusion, high amounts of protoplasts could be obtained from *P. fascia* using a simple mix of commercial enzymes (cellulase "Onozuka" RS + alginate lyase), short incubation time (4 h), chelation pre-treatment, and increased osmolarity. Protoplasts regenerated into blades through the formation of prostrate thalli after 4 weeks in culture. Other forms were also observed but in less frequency. Antibiotics were not needed for improving regeneration and temperatures from 10 °C to 20 °C were suitable for protoplast culture.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Jose Avila-Peltroche. The first draft of the manuscript was written by Jose Avila-Peltroche and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author, Tae Oh Cho, upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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