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Factors affecting yield and gelling properties of agar

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Abstract Agar, a gelatinous polysaccharide in the cell wall of many red algal species, is widely used as a gelling, thickening and stabilizing agent. The commercial value of seaweed is judged by their agar content and gel quality. Seaweed materials with higher agar yield and better gelling properties are desired due to the growing demand for agar in the global market. Agar biosynthesis in seaweeds is affected by genetic variations, developmental stages and environmental conditions, while different agar extraction techniques can also affect the yield and quality of agar. In this paper, the effects of different physiological states of seaweed, abiotic and biotic factors, seaweed storage and agar extraction techniques on the agar yield and gelling characteristics, are reviewed. This information is important as a guide for marine aquaculture of potential agarophytes and the possible effects of climate change on the stock of this natural resource.

Keywords Agar · Agarophyte · Agar yield · Cultivation · Gel strength · *Gracilaria*

Introduction

Agar was first prepared from seaweed unintentionally by a Japanese innkeeper, Minoya Tarazaemon, in the winter of 1658 (Armisen 1995). Agar, also known as 'Kanten' in Japanese (which means 'frozen sky') is associated with the freeze-thawing process discovered by Minoya Tarazaemon (Armisen 1991). The word 'agar', originated from a Malay word 'agar-agar', was first used to describe a jelly-like product extracted from *Eucheuma* (Tseng 1944). In Taiwan, agar is often translated into 'vegetable swiftlet' as it shares a similar texture with the swiftlet nest, which is used to make birds' nest soup (Imeson 2009). The US Pharmacopeia and the Food Chemicals Codex defines agar as a hydrocolloid which is soluble in boiling water with a clear aqueous solution at 1.5% (*w*/*v*) and forms a gel between 32 and 43 °C which does not melt below 85 °C.

The phycocolloid agar is uniquely produced in the cell wall of red algal species in the Gracilariaceae, Gelidiaceae, Pterocladiaceae and Gelidiellaceae (Armisen and Galactas 1987). The sugar monomers, D- and L-galactose, are joined by a glycosidic bond to form an alternating chain of polysaccharide. The addition of ester sulphate, methoxyl and pyruvate ketal groups to the polysaccharide chain leads to the formation of agaropectin with poor gelling ability (Araki 1966). Sulphation at the C-6 of the L-galactose unit can be hydrolysed enzymatically into 3,6-anhydro-L-galactose. These repeating D-galactose and 3,6-anhydro-L-galactose are called agarose, which forms a three-dimensional helix structure that triggers the gelation of agar (Morris 1986; Norton et al. 1986; Murano 1995).

There is a high market demand for agar, with a global production of 9600 t in 2009 (Bixler and Porse 2011). Agar has a higher retail price (US$18 kg^{-1}), compared to other seaweed hydrocolloids, such as alginates (US$12 kg^{-1}) and carrageenans (US$10.4 kg^{-1}) (Rhein-Knudsen et al. 2015). Due to its physicochemical and gelling characteristics, agar has been commercially used as a gelling and thickening agent in the food, pharmaceuticals and cosmetic industries, as well as a solid medium for bacteria growth in biomedical and

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biotechnological research. Over the years, natural stocks of agarophytes have been exploited extensively leading to a shortage of algal materials for agar production (Armisen 1995). As an alternative, aquaculture of agar-producing seaweeds especially *Gracilaria* spp. was initiated in many countries, such as Thailand (Trono 1989), Chile (Santelices et al. 1993), Vietnam (Tran 1993), China (Wu et al. 1993), Portugal (Matos et al. 2006), Australia (Cordover 2007), Brazil (Bezerra and Marinho-Soriano 2010) and India (Ganesan et al. 2011).

The commercial value of agar in the phycocolloid market depends largely on the agar yield and quality, which can be affected by many factors. The last comprehensive reviews on this topic date back to the 1980–1990s, focusing on the chemical structure and quality of agar (Armisen and Galactas 1987; Lahaye and Rochas 1991; Murano 1995). In this review, some recent updates on the latest technology to evaluate agar quality and industrial requirement for agar quality are discussed. In addition, the effects of different factors such as physiological states of seaweed, abiotic and biotic factors, seaweed storage and agar extraction techniques on agar yield and quality of gel, which have not being discussed in the past, are reviewed.

Yield and quality of agar

Agar yield is the percentage of the algal dry weight of extracted as crude agar. The agar yields of seaweeds range from 6 to 71% of the total dry weight of seaweeds, with the majority of the agarophytes having an agar yield of 20–30% (McLachlan and Bird 1986). The exceptionally high agar yields reported (e.g. >50%) could due to contamination by floridean starch extracted together with the agar. However, the agar yield varies greatly among species and under different environmental conditions or developmental stages (Armisen 1995). Agar yield is important for seaweed farmers, as the economic return on the seaweed crop largely depends on the yield of seaweed per dollar of production cost and yield of agar per kg of seaweed (Cordover 2007).

The quality of agar is reflected by its physical characteristics (e.g. gel strength, gel syneresis, viscosity, gelling and melting temperatures) and chemical properties (e.g. content of sulphate and 3,6-anhydrogalactose). The gel properties/relative proportions of algal constituents are highly dependent on the amount and position of sulphate groups as well as the amount of 3,6-anhydrogalactose fraction of the phycocolloid (Duckworth et al. 1971). Gel strength is the main indicator for agar quality. It refers to compressive force (expressed in g cm^{-2}) required to fracture an agar gel of a standard concentration of 1.5% (*w*/*v*). The Nikan-Sui method is traditionally used to measure agar gel strength, in which 1 cm^2 load is added one by one to the Nikan-Sui apparatus made by Kiya Seisakusho Ltd. and the gel strength is measured by calculating the amount of loads (equivalent to force applied) needed to break the gel in 20 s. Other instruments with higher sensitivity and accuracy than the Nikan-Sui apparatus, such as food-grade texture analysers, have been used in recent studies (Marinho-Soriano and Bourret 2003; Romero et al. 2008; Villanueva et al. 2010; Sousa et al. 2013a, b; Yarnpakdee et al. 2015; Lee et al. 2016). The gelling properties of an agar depend on the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues, which constrain the molecule to form a helix, and the interaction of these helices resulting in gel formation (Rees 1961). When the 3,6-anhydro-L-galactose residue is replaced with galactose-6-sulphate, kinks are formed in the helix, thus inducing the formation of an agar with lower gel strength.

Agarose, a linear polymer of repeating units of D-galactose and 3,6-anhydro-L-galactopyranose, is the neutral and gelling fraction of the agar, which has the ability to form a firm gel. Pure agarose measured by texture analyser has a reference gel strength of 950 g cm^{-2} (Marinho-Soriano 2001). The international market usually follows the criteria set by the Japanese Specifications for Processed Agar (JSPA), which is 350 and 600 g cm^{-2} for 1.5% (*w*/*v*) of the first-grade food agar and superior grade agar, respectively, as measured by the Nikan-Sui method (Skriptsova and Nabivailo 2009). Different gel strength measurement methods (e.g. instrument, diameter and operating speed of the probe) and preparation of agar gel (e.g. gel depth and gel surface area) can produce different gel strength values (de Castro 1993); thus, a standardised method is required for valid comparison across experiments.

Gel syneresis is a phenomenon of water losing from the agar hydrogel over the time (Sanderson 1990). Aggregation of double helices in agar gel causes contraction of the polymer network, which decreases the interstitial space available to hold the water (Whytock and Finch 1991). Several factors can affect gel syneresis, such as agar concentration, storage time, sulphate content, agar gel strength and pressure (Matsuhashi 1990). Agar is converted to sol form when heated above its melting point (usually 80 °C and above), and the viscosity of the sol is considered as one of the criteria evaluated for agar quality. Viscosity of the sol is directly proportional to its molecular weight and is greatly dependent on seaweed species, extraction conditions, surrounding temperatures and agar concentration (Guiseley 1972; Murano 1995; Praiboon et al. 2006). A low-viscosity agar gel has a lower substitution of charged groups on its polysaccharide chain, which makes the gel structure less hydrophilic and traps the water in a three-dimensional network (Stanley 2006). For industrial processing, agar gel with lower syneresis shrinkage is desirable, since it is easier to filter during the extraction process (Istini et al. 1994; Murano 1995; Mao et al. 2001).

The gelation mechanism of agarose involves conversion of fluctuating extended helices in solution to compact and ordered helical structures and also aggregation of helical structures (Rees et al. 1982). The sol-gel transition of agar results in hysteresis at 40–60 °C, with the melting temperature higher

than the gelling temperature (Stanley 2006). A high gel hysteresis is due to formation of large aggregates, which dissociate at higher temperature compared to the individual helices. A higher degree of sulphation or methoxylation on the agar, which inhibits aggregation of large helices and interferes with the intermolecular hydrogen bonding, was found to decrease its thermal hysteresis (Guiseley 1970; Murano 1995). Thus, a good-quality agar should have fewer side-chain substitutions and a wide hysteresis range. The gelation process is reversible, in which agar gel can melt at high temperature and solidify when cooled for many times without any significant effects on the gelling properties (Imeson 2009). Seaweeds producing agar with lower gelling temperature are desired to make bacteriological agar as it prevents heat damage to the materials (e.g. antibiotics) added into hot agar solution (McHugh 2003).

Factors affecting agar yield and gelling properties

Agar extraction method and storage

The process of agar extraction from seaweeds is divided into five steps: (1) washing, drying and chemical treatment; (2) aqueous extraction of agar by heating; (3) filtration to remove seaweed residues; (4) cooling, freezing and thawing of agar gel; and (5) washing, bleaching and desiccation of the formed solid agar (Coppen and Nambiar 1991).

Aqueous extraction of agar from seaweeds, traditionally performed by autoclaving at high temperature and pressure or using a water bath at boiling point, was found to give variable agar yield. Buriyo and Kivaisi (2003) reported 7% (*w*/*v*) higher agar yield extracted by autoclaving as compared to water bath. In addition, the agar extracted by autoclaving was cleaner and clearer than that extracted in the water bath. Autoclaving which couples high pressure and temperature may be more effective in breaking and softening the cell wall of seaweeds to release agar as compared to water bath extraction, which only uses high temperature. Newer methods for agar extraction such as microwave-assisted extraction (MAE) (Sousa et al. 2013a, b) and cold extraction (Maciel et al. 2008) have also been developed. However, the cold extraction process was found to contribute to a low agar yield of *Gracilaria birdiae* (Maciel et al. 2008), while the MAE method contributed to a 40.9% higher agar yield and 62.6% stronger gel as compared to traditional heat extraction method in a 85 °C hot water bath for 2 h (Sousa et al. 2010). These results indicate that the agar yield depends on extraction temperatures as reported by Melo et al. (2002).

Treatment of seaweeds with alkali (usually sodium hydroxide) was first reported by Funaki and Kojima (1951). Alkali treatment was shown to reduce the sulphate content and increase the 3,6-anhydrogalactose content of agar, improving the gel strength of *Pterocladia* species (Lemus et al. 1991), *Gelidiella* species (Meena et al. 2011), *Gelidium* species (Matsuhiro and Urzda 1990; Lemus et al. 1991; Meena et al. 2011) and *Gracilaria* species (Freile-Pelegrin and Robledo 1997; Montano et al. 1999; Rath and Adhikary 2004; Praiboon et al. 2006; Arvizu-Higuera et al. 2008; Meena et al. 2008; Li et al. 2008; Orduna-Rojas et al. 2008; Mehta et al. 2010; Vergara-Rodarte et al. 2010; Ahmad et al. 2011; Yarnpakdee et al. 2015). However, a few studies have found that alkali treatment failed to improve agar gel strength (Freile-Pelegrin and Murano 2005; Meena et al. 2008), possibly due to the presence of a high number of alkali-stable sulphate groups (e.g. galactose-2-sulphate and galactose-4-sulphate) in the agar (Lahaye et al. 1986; Murano et al. 1992). The persistence of these sulphate groups in the alkali-treated agar could cause poor gelling ability, suggesting that sulphate groups at other positions apart from the C-6 of galactose sugar could also affect the agar gel strength (Murano 1995).

The agar yield of alkali-treated seaweeds from temperate countries is generally lower compared to that of native agar (Lemus et al. 1991; Freile-Pelegrin and Robledo 1997; Montano et al. 1999; Rath and Adhikary 2004; Freile-Pelegrin and Murano 2005; Praiboon et al. 2006; Arvizu-Higuera et al. 2008; Li et al. 2008; Meena et al. 2008; Orduna-Rojas et al. 2008; Vergara-Rodarte et al. 2010; Ahmad et al. 2011; Meena et al. 2011), although some have reported higher or no significant changes in agar yield after alkali treatment (Matsuhiro and Urzda 1990; Montano et al. 1999; Praiboon et al. 2006; Meena et al. 2008; Yarnpakdee et al. 2015). The alkali-treated agar might diffuse into the alkali treatment solution or be degraded in the process under high temperature (Freile-Pelegrin and Robledo 1997; Praiboon et al. 2006; Arvizu-Higuera et al. 2008; Ahmad et al. 2011). However, several species such as *Gracilaria tenuistipitata*, *Gracilaria fisheri* and *Gracilaria edulis* from tropical regions such as Thailand and the Philippines (Montano et al. 1999; Praiboon et al. 2006; Yarnpakdee et al. 2015) and *Gelidium rex* from Chile (Matsuhiro and Urzda 1990) achieved higher yield after alkali treatment, suggesting that the denser and complex cell wall components of seaweeds grown in hot regions may require harsher extraction method such as alkali treatment to achieve higher yield.

Optimum treatment conditions such as the alkali concentration, extraction temperature and treatment duration are species dependent (Villanueva et al. 1997). The optimal alkali concentration ranged from 3 to 10% (*w*/*v*) for *Gracilaria* species. Most *Gracilaria* species treated for a short duration (0.5 to 3 h) at high temperatures (80 to 90 °C) were found to produce agar with a higher gel strength (Arvizu-Higuera et al. 2008; Vergara-Rodarte et al. 2010) as compared to long treatment duration (16 to 24 h) at room temperatures (27 to 33 °C) (Orduna-Rojas et al. 2008), with for the exception of some species such as *Gracilariopsis longissima*, *G. cervicornis*, *G. blodgettii*, *G. verrucosa* (currently known

as *Gp. longissima*), *G. fisheri*, *G. edulis*, *Gracilaria* sp., *G. foliifera* and *G. corticata* in which only a slight increase in gel strength was reported after alkali treatment for a short duration at high temperature. Short treatment time with high temperature may reduce the agar loss by diffusion while promoting breakage of seaweeds and release of agar from cell wall.

Although alkali treatment seems to be a promising way in improving agar quality, it may produce toxic wastewater, which could become an environmental issue if not properly treated (Villanueva and Montano 2014). Thus, a more eco-friendly alternative method is needed to improve the gel strength of agar. Enzymatic treatment on commercial agar using sulphatase/sulphohydrolase (50 U) purified from *Gracilaria dura* was shown to be able to decrease the sulphate content and increase both the 3,6-anhydrogalactose content and gel strength of agar (Shukla et al. 2011). However, the commercial feasibility of sulphatase/sulphohydrolase is questionable, based on yield of pure enzyme obtained per kg of seaweed and production cost compared to alkaline treatment. In addition, agar gel strength can be improved by adding sugar in high concentration (usually more than 50% *w*/*w*) during preparation of agar gel from most *Gracilaria*, *Gelidium* and *Gelidiella* species (Armisen and Galactas 1987; Matsuhashi 1990; Romero et al. 2000; Meena et al. 2006).

Bleaching of seaweeds prior to agar extraction is a common industrial practice, which aims to produce pure white agar with a higher aesthetic property. Bleaching is carried out using chemical or photobleaching (Li et al. 2008). Photobleached alkali-treated agar was found to contain less sulphate, higher 3,6-anhydro-galactose content and higher gel strength as compared to chemical-bleached alkaline-treated agar and alkaline-treated agar. Li et al. (2008, 2009) reported that the agar yield was not affected by bleaching. In contrast, bleaching was shown to decrease the agar yield of *G. edulis* and the yield of alginates, although the gel strength was increased (Durairatnam 1987; Istini et al. 1994). Photobleaching has advantage over the traditional chemical bleaching using sodium hypochlorite; the production of chlorine gas and effluents in the latter method is a threat to the environment and workers' health (Li et al. 2008).

The agar quality was also influenced by the conditions and the storage duration of *Gracilaria* samples before agar extraction. Polysaccharides of stored seaweeds are susceptible to agarase degradations by endogenous enzymes and bacteria such as *Bacillus cereus* and *Pseudomonas atlantica* (Armisen 1991). Postharvest treatment of *Gracilaria* using chemicals such as acid, alkali and formaldehyde is necessary to prevent enzymatic and microbial degradation (Freile-Pelegrin and Robledo 1997; Ganesan et al. 2004; Freile-Pelegrin and Murano 2005; Arvizu-Higuera et al. 2008). Treatment using either acid or alkali, or both, before storage enhanced the agar yield and physical properties of *Gelidiella acerosa* and *G. edulis* (Ganesan et al. 2004). Generally, long-term storage of seaweed starting materials before agar extraction decreased the gel strength, as shown in *G. cornea* and *Gracilaria eucheumatoides* after 6 months of storage (Freile-Pelegrin 2000; Romero et al. 2008). In these reports, agar yield was significantly affected by long-term storage (Freile-Pelegrin 2000; Romero et al. 2008).

Physical parameters

Agarophytes have a cosmopolitan distribution, covering both temperate and tropical regions, with a broad range of salinities and temperatures (Yokoya and Oliveira 1992; Praiboon et al. 2006). Seasonal variabilities in the yield and quality of agar extracted from wild seaweeds had been well documented for *Gracilaria*, *Gracilariopsis*, *Gelidiella*, *Gelidium* and *Pterocladiella* species (Table 1; Online Resource 1). Most of the previous studies reported a higher agar production for seaweeds growing in spring and/or summer compared to those growing in winter (Carter and Andersen 1986; Price and Bielig 1992; Chirapart and Ohno 1993; Yenigul 1993; Freile-Pelegrin et al. 1995; Oliveira et al. 1996; Marinho-Soriano and Bourret 2003; Vergara-Rodarte et al. 2010; Martin et al. 2013b), except for a few which showed other results (Rodriguez-Montesinos et al. 2013). Most studies conducted on seaweeds in tropical region showed that those growing in the rainy season have a higher agar yield compared to those growing in the dry season (Luhan 1992; Roleda et al. 1997; Villanueva et al. 1999; Ganesan et al. 2008; Bezerra and Marinho-Soriano 2010), except for the study by Phang et al. (1996) which reported a higher yield for seaweeds growing in the dry season. The discrepancy in the results could be primarily due to the different agar extraction methods (e.g. alkaline treatment, extraction time and temperature) used by different researchers (Table 1), possible regional effects (e.g. geographical locations, habitats and environmental parameters) (Table 1 and Online Resource 1) and/or genetic variations of the seaweeds.

Multiple environmental factors, such as nutrient status, solar radiation, day length, water temperature and developmental stages of the seaweed, could contribute to differences in the agar extracted from two different seasons (Onraet and Robertson 1987). However, some of these data were not available in the literature mentioned previously (Online Resource 1), thus making the comparison difficult. The agar gel strength depends mainly on species and locality and might be complicated by different environmental parameters, as well as different agar extraction methods, or treatments (e.g. alkaline treatment), and gel strength measurement methods. Hence, it is difficult to investigate the effects of a single factor on the yield and quality of agar extracted from seaweeds grown in the natural environment.

Table 1 Seasonal effects on agar yield and gel strength of agarophytes

Publication	Species	Locality	Agar extraction method	Agar yield		Method of gel strength measurement	Gel strength		Duration of study
				Highest	Lowest		Highest	Lowest	
Martin et al. (2013b)	*Gracilaria gracilis*	Argentina	70 °C in water bath, 4 h	Summer	Winter	Instron Universal Testing Machine (1 mm s^{-1}, 30-mm plunger)	Spring	Winter	9 months (samples collected in May, Aug, Oct and Jan)
Marinho-Soriano and Bourret (2003)	*Gracilaria gracilis*	France	110 °C in autoclave, 1 h	Spring	Autumn	Texture analyser (1 mm s^{-1} 10-mm plunger)	Summer	Winter	1 year (seasonal sampling)
	Gracilaria bursa-pastoris	France	110 °C in autoclave, 1 h	Summer	Winter	Texture analyser (1 mm s^{-1} 10-mm plunger)	Autumn	Summer	1 year (seasonal sampling)
Rodriguez-Montesinos et al. (2013)	*Gracilaria veleroae*	Mexico	85 °C in water bath, 3 h (PT 3% NaOH, overnight)	Autumn	Summer	Nikansui Shiki gel strength meter	Spring	Summer	5 seasons (seasonal sampling)
	Gracilaria vermiculophylla	Mexico	85 °C in water bath, 3 h (PT 3% NaOH, overnight)	Winter	Summer	Nikansui Shiki gel strength meter	Winter	Summer	4 seasons (seasonal sampling)
Vergara-Rodarte et al. (2010)	*Gracilaria vermiculophylla*	Mexico	100 °C in water bath, 1.5 h	Summer	Winter	Nikan-Sui gelometer	Summer	Autumn	1 year (seasonal sampling)
			100 °C in water bath, 1.5 h (PT 7% NaOH, 12 h)	Summer	Spring	Nikan-Sui gelometer	Spring	Autumn	1 year (seasonal sampling)
Yenigul (1993)	*Gracilaria verrucosa* (= *Gracilariopsis longissima*)	Turkey	100 °C in water bath, 3 h	Summer	Winter	Instron Universal Testing Machine (50 mm min^{-1} 1-cm^2 plunger)	Autumn	–	2 years (monthly sampling)
Price and Bielig (1992)	*Gracilaria edulis*	Australia	100 °C in water bath, 1 h	Summer	Winter	–	–	–	19 months (monthly sampling)
Chirapart and Ohno (1993)	*Gracilaria* sp. (chorda type)	Japan	100 °C in water bath, 3 h (PT 5% NaOH at 70, 80, 90 °C)	Spring–summer	Autumn	Nikansui Shiki gelometer (1-cm^2 plunger)	Winter	Spring	11 months (bimonthly sampling)
Carter and Andersen (1986)	*Gelidium pristoides*	South Africa	100 °C in water bath, 1 h	Summer	Winter	–	–	–	26 months (monthly sampling)
Freile-Pelegrin et al. (1995)	*Gelidium canariensis*	Spain	120 °C in autoclave, 2 h (PT 0.5% Na_2CO_3, 85–90 °C, 30 min)	Summer	Autumn–winter	Unknown instrument (1-cm^2 plunger)	Winter	–	12 months (monthly sampling)
Oliveira et al. (1996)	*Pterocladia capillacea* (=*Pterocladiella capillacea*)	Brazil	100 °C in glycerol bath, 3 h, with 2.5 g of Celite	Spring–summer	Winter	–	–	–	11 months (monthly sampling)
Phang et al. (1996)	*Gracilaria changii*	Malaysia	121 kPa in autoclave, 15 min	Dry	Rainy	Nikansui Shiki gel strength tester	Rainy	Dry	14 months (monthly sampling)
Bezerra and Marinho-Soriano (2010)	*Gracilaria birdiae*	Brazil	110 °C in autoclave, 1 h (PT 0.6% NaOH, 85 °C, 30 min)	Rainy	Dry	Unknown instrument (1-cm^2 plunger)	Rainy	Dry	7 months (monthly sampling)
Luhan (1992)	*Gracilaria heteroclada* (=*Gracilariopsis heteroclada*)	Philippines	100 °C water bath (PT 5% NaOH, 90 °C, 1 h)	Rainy	Dry	Marine Colloid Gel tester (1.05-cm plunger 16–18 cm min^{-1})	Dry	Rainy	1 year (monthly sampling)
Villanueva et al. (1999)	*Gracilaria eucheumatoides*	Philippines	100 °C water bath, 1 h (PT 10% NaOH, 90 °C 2 h)	Rainy	Dry	Marine Colloids Gel Tester, Model GT-1 (1-cm^2 plunger 25 mm s^{-1})	–	–	10 months (bimonthly sampling)
	Gelidiella acerosa			Rainy	Dry		Rainy	Dry	10 months (bimonthly sampling)

Table 1 (continued)

Publication	Species	Locality	Agar extraction method	Agar yield		Method of gel strength measurement	Gel strength		Duration of study
				Highest	Lowest		Highest	Lowest	
			121 °C and 15 psi in autoclave, 1 h			Marine Colloids Gel Tester, Model GT-1 (1-cm^2 plunger 25 mm s^{-1})			
Roleda et al. (1997)	*Gelidiella acerosa*	Philippines	15–20 psi in pressure cooker (PT 0.5% acetic acid 16–20 °C, 1 h)	Rainy	Dry	Marine Colloids Gel Tester, Model G141-2	Dry	Rainy	1 year (monthly sampling)
Ganesan et al. (2008)	*Gelidiella acerosa*	India	Under pressure (15 Pa) for 2–3 h	Rainy	Dry	Unknown instrument with 1-cm^2 plunger (mercury beads used as load)	Dry	Rainy	2 years (monthly sampling) except for station 3 (1 year with monthly sampling)

PT pre-treatment

Agarophytes are generally found in marine waters with salinity ranging from 15 to 38‰ (McLachlan and Bird 1986). A high salinity in summer (43.8‰) was found to be negatively correlated with agar yield but positively correlated with gel strength for the natural populations of *G. verrucosa* (currently known as *Gp. longissima*) (Sasikumar et al. 1999). However, the results could be masked by other physical factors, such as a longer day length, higher water temperature and illumination. In a controlled laboratory experiment, the agar production of *G. tenuistipitata* was found to be the highest at 0‰, compared to 25 and 31‰, which is close to the salinity in the natural environments for most red seaweeds (Bunsom and Prathep 2012). In another report, *Gracilaria changii* was shown to have a 2-fold increase in agar yield when grown under high salinity (50‰) compared to the seaweeds grown at normal salinity (30‰), while seaweeds grown at low salinity (10‰) conditions showed no significant differences in agar yield compared to that at 30‰ (Teo et al. 2009; Siow et al. 2012). The gel strength was slightly lower for agar extracted from seaweeds treated under both conditions (10 and 50‰) compared to that in the normal condition.

In general, osmotic pressure and salinity stresses have been found to cause significant reduction in the growth of red seaweeds (Dawes et al. 1998; Wong and Chang 2000; Phooprong et al. 2007) and decreased agar content due to decreased photosynthesis, carbon fixation and prioritisation of resources for ion homeostasis (Macler 1988; He et al. 2002). However, under these circumstances, seaweeds might need to provide structural support to turgid and flaccid cells by modifying their agar content and composition, possibly through floridean starch degradation by alpha-glucosidase/1,4-glucan phosphorylase (Yu and Pedersen 1990; Ekman et al. 1991; Rincones et al. 1993). The enzymatic degradation of floridean starch produces glucose-6-phosphate and/or glucose-1-phosphate, which can be further converted into uridine diphosphate (UDP)-glucose and/or UDP-galactose as building blocks of agar (Manley and Burns 1991). Thus, the amount of initial floridean starch storage in the seaweeds may explain the discrepancy in the published data in agar content during salinity stresses between species and experiments.

The quality and intensity of light are important for carbon fixation in agarophytes. The agar yield of *Gracilaria* and *Gracilariopsis* species is known to increase under low/dim light condition, whereas their starch contents have been found to be positively correlated with light intensity (Rotem et al. 1986; Bird 1988; Rincones et al. 1993). Under light deprivation, the enzyme activities of alpha-glucosidase and alpha-1,4-glycan phosphorylase were increased (Rincones et al. 1993), channelling carbon from starch to agar. However, the content of L-galactose-6-sulphate was reduced significantly in *Gracilaria chilensis* cultured for 30–44 days in the dark in both the field and laboratory experiments, while the agar gel strength was increased (Hemmingson and Furneaux 2000). In

this experiment, when dark treatment was coupled with an increase in water temperature (from 18 to 20 to 29 °C), a marked reduction in L-galactose-6-sulphate content and increase in agar gel strength were observed within a short period (13 days). This suggested that enzymatic conversion of L-galactose-6-sulphate to 3,6-anhydrogalactose could be faster at higher water temperatures, correlated with a lower L-galactose-6-sulphate content in summer (12–13%) compared to the winter (16–17%). However, the effects of high water temperature (>30 °C) on agar quality are not known.

Gracilaria sordida (currently known as *G. chilensis*) grown under light deprivation and altered salinity (3 days at 70 ppt in darkness followed by 5 days at 10‰ in darkness) (Ekman et al. 1991) and *G. cornea* grown at 50‰ in darkness for 4 days followed by 25‰ in darkness for 4 days (Freile-Pelegrin et al. 2002) were reported to have a higher agar yield compared to the controls grown under illumination (800 and 100 μmol photons m^{-2} s^{-1}, respectively) and normal salinity (33‰). The results suggested that agar biosynthesis was regulated by degradation of floridean starch, which increased under light deprivation and altered salinities. However, agar gel strengths were either increased or decreased slightly, with no pronounced changes after light deprivation and altered salinity, suggesting that modification of agar composition could also be due to other factors apart from the previously mentioned abiotic factors.

Temperature can be an important factor affecting the yield and quality of agar, as differences in agar yield and gel strength have been noted between summer/dry and winter/rainy samples in natural seaweed populations (Table 1). Daugherty and Bird (1988) found that lower agar gel strengths were recorded for seaweeds cultivated at 23 ± 3 and 31 ± 1 °C, compared to those cultured at normal temperature (29 ± 1 °C), while the agar yield increased with increased water temperature. Friedlander (1991) found that both agar yield and gel strength of *Gracilaria conferta* cultured in tanks were positively correlated with water temperature. In contrast, a few studies have reported that agar yield declined during high seawater temperature in summer or during periods of rapid growth (Christiaen et al. 1987; Christeller and Laing 1989; Bird and Ryther 1990). Thus, increasing water temperature due to climate change (Harley et al. 2006) may affect algal growth and their agar production.

Sedimentation was found to be an important factor that affects marine aquaculture and seaweed exploitation, as it reduces underwater irradiance and gaseous exchange by covering the seaweeds (Chapman and Fletcher 2002; Airoldi 2003), which subsequently decreases photosynthesis and produces lower agar yield (Bunsom and Prathep 2012). In addition, the depth of seawater in which the seaweeds grow also affects the agar yield, as significantly higher agar yield was observed for *Gracilariopsis lemaneiformis* cultivated at 3.5-m depth compared to 0.5-m depth (Xu and Gao 2008). Reduction in light absorption was associated with reduced cell growth and accumulation of agar in the seaweeds, suggesting the impact of light on the agar yield.

Although the effects of major abiotic factors on seaweeds had been investigated in the past, the effects of pH on agar yield and gelling properties are unknown, with only physiological responses documented for *G. tenuistipitata* var. *liui* (Israel et al. 1999). The amount of dissolved oxygen (DO) and carbon dioxide (DC) in the seawater may change drastically due to global climate change (Harley et al. 2012), causing ocean acidification, which may affect seaweed populations and their agar production. Different types of natural habitats and their interactions with different environmental factors might cause variation in agar content and gel quality (Oyieke 1994; Lee et al. 2016). Thus, a regional survey on the type of habitats suitable for seaweed mariculture is important. In addition, agarophytes may modify their agar composition in response to tidal changes, dessication and strong waves, as evidenced by a higher agar content found in *Pterocladia capillacea* (currently known as *Pt. capillacea*) exposed to strong waves, compared to those found in a sheltered area and an area with moderate wave action (Oliveira et al. 1996). Thus, carefully designed indoor experiments are important to investigate the effects of these abiotic factors.

Nutrient status

Macronutrients such as phosphate, nitrate and sulphate are essential for the growth of seaweeds and may affect agar production in agarophytes. The effects of nitrogen concentration on the growth and agar yield of agarophytes had been explored (Christeller and Laing 1989; Buschmann et al. 1994; Martinez and Buschmann 1996; Troell et al. 1997), but the effects of phosphorus and sulphur nutrition on the agar production are less thoroughly studied in agarophytes.

Nitrogen is one of the essential nutrients which often limits the growth of seaweeds in natural ecosystems (Hanisak 1990). Seaweeds are often cocultivated with fish and marine crustaceans in aquaculture farms, where the nutrient feeds and nitrogenous wastes released from the farms increase the nitrogen concentration of seawater. Studies have shown that *Gracilaria* species cultured in tanks with fish effluents or in areas nearer to salmon cages exhibited lower agar yield, when compared to their respective controls growing in tanks with seawater or in culture areas that were away from the salmon cages (Buschmann et al. 1994; Martinez and Buschmann 1996; Troell et al. 1997). In general, the agar content of *Gracilaria* and *Gelidium* species is known to be negatively correlated with the nitrogen level of the growth environment (Christeller and Laing 1989) and nitrogen content in the seaweed tissue (Hoyle 1978; Bird et al. 1981; Carter and Andersen 1986; He et al. 2002; Marinho-Soriano and Bourret 2003). However, *G. fisheri* and *G. tenuistipitata* var.

liui cultured in ponds with shrimp farm effluents and ambient seawater did not show significant differences in terms of agar yield, but their agar yields were positively and negatively correlated with the total dissolved inorganic nitrogen (ammonia, nitrite and nitrate) (Chirapart et al. 2006).

Under high nitrogen availability, seaweeds have the ability to assimilate and store excess nitrogen (Hanisak 1990). Bird et al. (1981) found a positive correlation between nitrate concentration in seawater and thallus nitrogen content in cultured *Gracilaria tikvahiae* but an inverse correlation between thallus nitrogen concentration, protein content and protein/carbohydrate ratio with agar content. These results suggest that an increased uptake of nitrogen into the seaweed cells favours protein synthesis instead of biosynthesis of polysaccharides (e.g. agar) (Fogg 1964; Mshigeni 1974). The increase of nitrogen content in the thallus was also associated with an increase in gel strength and melting temperature (Bird et al. 1981; Lewis and Hanisak 1996), which could be due to longer agar polymer and greater agar molecular weight (Selby and Wynne 1973). From the commercial point of view, the reduction in agar content due to nitrogen enrichment can be compensated by an increase of growth rate and productivity of the seaweeds, as well as a better gel quality. However, the nitrogen concentration needs to be optimised, as overfertilisation might promote the growth of opportunistic epiphytes, which are considered a major problem in marine aquaculture (Fletcher 1995; Veeragurunathan et al. 2015).

Seawater is rich in sulphate, with molar concentrations ranging from to 25 to 28 mM, which is 1000 times higher than that in the freshwater systems (Bochenek et al. 2013). Although sulphate ester is the major side chain substituent of agar polysaccharide, with the commercially important

Table 2 Effects of different life stages of agarophytes on their agar yield and gel strength

Publication	Species	Locality	Life stages	Findings
Kim and Henriquez (1979)	*Gracilaria verrucosa* (=*Gracilariopsis longissima*)	–	Carposporophyte Tetrasporophyte	Higher agar yield but lower gel strength was found in carposporophyte compared to tetrasporophyte.
Yao et al. (1984)	*Gracilaria verrucosa* (=*Gracilariopsis longissima*)	China	Carposporophyte Tetrasporophyte	Agar yield was slightly higher in carposporophyte compared to tetrasporophyte but no significant difference in gel strength between the two stages.
Marinho-Soriano et al. (1999)	*Gracilaria bursa-pastoris*	France	Carposporophyte Tetrasporophyte Vegetative	No difference in agar yield among the three stages, but the gel strength from vegetative plants was significantly higher than carposporophyte and tetrasporophyte.
Durairatnam and Nascimento (1985)	*Gracilaria cylindrica*	Brazil	Carposporophyte Tetrasporophyte Vegetative	No difference in agar yield was found among the three stages, but gel strength was higher in tetrasporophyte than cystocarpic and vegetative plants.
Penniman (1977)	*Gracilaria foliifera*	USA	Carposporophyte Tetrasporophyte Vegetative	Little variation in the agar yield among the carposporophyte, tetrasporophyte and vegetative plants.
Pickering et al. (1990)	*Gracilaria sordida* (=*Gracilaria chilensis*)	New Zealand	Carposporophyte Tetrasporophyte Male gametophyte	Agar gel strength was lower in male gametophyte plants than those from carposporophyte and tetrasporophyte.
Hoyle (1978)	*Gracilaria bursa-pastoris* *Gracilaria coronopifolia*	Hawaii	Tetrasporophyte Male gametophyte Female gametophyte	No significant change was found in the yield and gel strength of agar extracted from seaweeds of different life stages.
Gupta et al. (2011)	*Gracilaria dura*	India	Tetrasporophyte Male gametophyte Female gametophyte	No difference in agar yield was found between tetrasporophyte and female gametophyte, but their agar yields were significantly higher than male gametophyte. Agar gel strength was the highest in tetrasporophyte, followed by that in female gametophyte and the lowest in male gametophyte.
Roleda et al. (1997)	*Gelidiella acerosa*	Philippines	Tetrasporophyte Vegetative	Higher agar yield and gel strength were found in vegetative plants compared to tetrasporophyte.

Gracilaria genus having higher sulphate content compared to other genera (Murano 1995), studies on the effects of sulphate concentration on the agar yield and quality are limited in the literature. Friedlander (2001) reported that sulphate deprivation significantly reduced the daily agar yield (mg tank^{-1} day^{-1}) of *G. conferta* tips (cultured for 3 weeks in aerated indoor tanks at 25 °C) by 1.5- to 2.0-fold. However, Lee et al. (2014) reported that sulphate deprivation did not significantly alter the agar yield and gel strength of the sulphate-deprived *G. changii* and *Gracilaria salicornia* cultured in two different seasons for 5 days, except for *G. salicornia* collected during the rainy season where its agar yield increased significantly. Their results suggested that sulphate availability did not have immediate effects on agar quality, but more experimental data, especially on long-term treatments, are necessary to understand better the relationship between sulphate availability and agar quality.

Phosphorus is generally not a limiting factor in the marine environment, but its varying concentrations could affect the growth of seaweed and their agar production. A clonal culture of *Gelidium robustum* had a higher agar content when cultured in culture media supplemented with 20 μM sodium phosphate for 4 weeks, compared to that cultured at lower phosphate concentrations (0–10 μM) (Sousa-Pinto et al. 1996). The authors also found that the gel strength of *Ge. robustum* was significantly lower under low-phosphate conditions (0–5 μM). In contrast, Lewis and Hanisak (1996) reported a lower agar yield in *Gracilaria* strain G-16S when cultured in high-phosphate media (15–30 μM), compared to that cultured at lower phosphate media (0–5 μM). However, they also found that agar gel strength was low in the low-phosphate concentration (0 μM) cultures.

Biotic factors

Epibiont and epiphytes are generally found on seaweeds in natural environment and cultivation conditions. Some marine bacteria are able to produce agarase to degrade agar (reviewed in Fu and Kim 2010). However, no experiments have been conducted to determine the effects of these agarolytic bacteria and other epibionts on agar yield and gelling properties. Most epiphytes have found to be detrimental to seaweeds by competing for nutrients, sunlight, dissolved gases and space, while their attachment to hosts increased dragging and caused thallus breakage (Buschmann et al. 1990; Kuschel and Buschmann 1991; Buschmann and Gomez 1993; Fletcher 1995; Martin et al. 2013a; Ganesan et al. 2015). To counter the damages caused by epiphytes, algae have developed defence or escape mechanism which may alter the cell wall structure and, consequently, the agar composition (Dawes et al. 2000; Weinberger and Friedlander 2000; Friedlander et al. 2001). Epibionts (e.g. mussel and bivalve) and epiphytes may cause slower growth rate in agarophytes by interfering with light availability, but they may induce stronger gel strength by providing nutrients through their excretory products (Cancino et al. 1987; Friedlander 1991). The agar yields of *Pt. capillacea* collected from a region with a high number of epiphytes were slightly lower, but the gel strength was not affected (Freile-Pelegrin et al. 1996). However, due to lack of

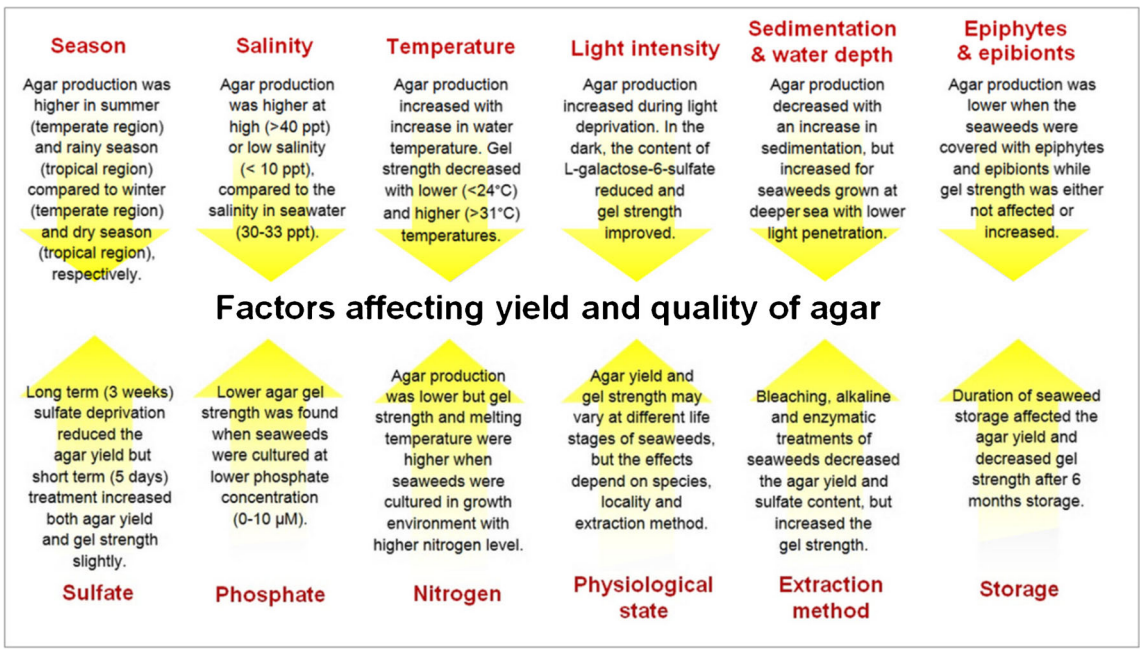

Fig. 1 Factors that affect yield and quality of agar

literature in this area, the beneficial or deleterious effects of epiphytes on agar composition could not be determined.

Physiological state of seaweeds

Gracilaria species have a triphasic life cycle, which is characterised by carposporophyte, gametophyte and tetrasporophyte (Kim and Henriquez 1979). Different developmental stages have been shown to affect the agar yield and gel strength, but the effects were mainly dependent on species and locality, with no conclusive information on which life stage produced the highest agar yield and gel strength (Table 2). In addition, several studies demonstrated that the yield and strength of agar from different life stages of *Gracilaria* spp. were subjected to seasonal variation (Whyte and Englar 1981; Onraet and Robertson 1987; Penniman and Mathieson 1987; Munoz and Fotedar 2011).

Concluding remarks

For years, studies on agarophytes have focused on algal growth, yield and gelling quality of agar under various environmental conditions, physiological factors and extraction procedure (Fig. 1). However, the results are not conclusive and hampered by species-specific effects, genetic factors, lack of information on life stage of seaweed, effects of locality and differences in experimental design. While laboratory experiments help to elucidate the effect of single environment factors on agar production and expand our knowledge on marine aquaculture of seaweeds, optimisation of agar production in indoor and outdoor cultivation systems which involve multiple factors is necessary for commercial purposes. Genetic effects on agar production and gel quality should also be considered in future research and selection of seaweed materials for aquaculture. Recent development of genetic markers for several agarophytes (Ayres-Ostrock et al. 2016; Boo et al. 2016) and gene expression studies (Chang et al. 2014) should assist this research. Studies on the effects of different environmental factors (e.g. temperature and pH) on seaweeds and agar are also important for us to predict the possible effects of future climate changes on the natural stock of seaweeds. Postharvest modifications of seaweeds such as alkali and enzymatic treatments can improve the quality of agar to meet the industrial requirements.

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