Journal of Environmental Biology **July 2008,** 29(4) 621-627 (2008)

Comparison of the effectiveness of four organic chemoattractants towards zoospores of *Ulva pertusa* and macrofouling

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(Received:September 04, 2007 ; Revised received: June 07, 2008; Accepted: June 09, 2008)

Abstract: *Algal spores respond to many environmental variables, especially, to chemical "cues". This chemotactic response can be utilized to attract spores, thereby colonization of a new substrata is possible to be influenced. In this attempt, four chemoattractant candidates were screened against spores of Ulva pertusa to reveal their efficiencies. Attachment and subsequent germination of Ulva spores were effectively influenced by these chemoattractant candidates. In particular, 100 µg cm^{-2} of D-glucose coating was found to enhance spore attachment by >150%. Furthermore, field investigations carried out with test panels, clearly indicate the chemoattractive properties of test coatings. In recent years, various anthropogenic activities and natural hazards cause detrimental impacts on the benthic algae and other fishery resources. Artificial reefs have been laid on many coastal regions to increase or restore marine resources. Chemoattractant coatings can be applied on artificial surfaces to increase the colonization of benthic forms. It also can be used in the mariculture devices. Influence of chemoattractants on Ulva spores and fouling biomass estimated on test panels are discussed.*

Key words: *Organic chemoattractants, Fouling biomass, Germlings, Spore attachment, Germination, Ulva pertusa*
PDF of full length paper is available with author ***(*pander27@gmail.com)***

Introduction

A feature common in many benthic marine plants are the release of propagules that serve as the organism's only mechanism of dispersal (Abelson and Denny, 1997). Successful dispersal depends to a large extent on the process of settlement. The delivery of motile propagules to the substratum, and the subsequent establishment of the spores/gametes are recognized as critical stages for the successful colonization.

Densely growing macroalgae provide food and shelter to diversified group of benthic communities. Where marine productivity is less or natural disasters and man-made pollution affected the growth of marine organisms, certain strategic maricuture practices are needed. Therefore, propagation of macroalgae is an important step to increase as well as restore the marine productivity.

The great majority of blooms are reported to consist of members of the family Ulvaceae (Fletcher, 1996; Sidharthan *et al*., 2004). These are among the world's most common fouling algae, which are used as model organisms in studies conducted with spore attachment (Stanley *et al*., 1999; Callow *et al*., 2000; Shin and Smith, 2001a; Sidharthan *et al*., 2007). In these green algae, colonization of substrata occurs mainly through the production and release of enormous numbers of motile spores into the water column. The critical event involved in colonization of substrata is the transition from a motile cell to an attached, nonmotile settled cell that develops a cell wall and germinates to produce a new plant.

A number of studies have been concerned with the response of spores to light, thigmotactic and chemotactic stimuli, and the role of other surface properties such as surface free energy (Fletcher and Callow, 1992; Callow and Callow, 2000).

In recent years, apart from industrial pollution (Chung and Lee, 1989; Chung and Kim, 1992) natural hazards, such as forest fire pollution (Shin *et al*., 2002; Kim *et al*., 2003) and yellow sand deposition were also drastically affecting the Korean coastal environment.

The phenomenon known as algal whitening which is associated with decreases in the seaweed flora of some rocky areas is associated with few species of crustose algae. One of the most dominant species causing algal whitening in Korea and Japan is *Lithophyllum yessoense* Foslie (Tokuda *et al*., 1994). Herbivores also contribute to the devastation of seaweed beds by overgrazing (Watanabe and Harrold, 1991; Agateuma *et al*., 1997). Certain algal species produce allelopathic substances that destroy or inhibit the spores of other algae (*e.g.* coralline algae, Suzuki *et al*., 1998). In addition, toxic antifouling compounds released into the coastal waters are also reported to be affecting the marine algae (Owen *et al*., 2001; Shin and Smith, 2001b; Negri *et al*., 2002; Jones, 2007).

In mariculture practices, to enhance the seaweed cultivation chemoattractants are used. Artificial reefs deployed on coastal regions are also being coated with chemoattractants to facilitate increased algal spore attachment. In order to increase marine productivity or to restore the marine communities that are affected by environmental degradation, algal spore attachment enhancing organic chemical substances were screened.

In organic substances used for chemoattraction purposes may cause severe environmental problems. Because inorganic

substances are easily bioconcentrated into micro- and macro-organisms, moreover at excessive levels they become toxic. It was reported that *Macrocystis pyrifera* spores were repelled by ferrous ion concentrations ≥45 µm (Amsler and Neushul, 1989).

The green tide alga *Ulva pertusa* is abundantly occurring on the upper intertidal zone of Korean coast. Therefore, *U. pertusa* was used in this study as test organism. The main purpose of this study was to develop organic chemoattractant coatings for mariculture practices and as well as for artificial surfaces.

Materials and Methods

Four chemoattractants, choline, gly-gly, D-glucose and poly-L-lysine were procured from Sigma Co. USA. D-glucose and poly-L-lysine were previously demonstrated as chemoattractants (Taylor and Panasenko, 1984; Santelices and Aedo, 1999). Other two chemoattractants, choline and gly-gly were selected from preliminary screening experiments conducted with *Ulva* spore attachment.

Sample collection: *Ulva pertusa* was collected during low tide from the rocky shores of Ayanjin on the east coast of Korea (May and September 2005). The algal materials were transported in plastic bags to the laboratory using icebox without seawater.

Spore release: In laboratory, epiphytes were removed from *Ulva* plant surface and washed twice with tap water followed by filtered (0.45µm) seawater. They were spread on paper towels and kept in room temperature. After 12 hr, selected *Ulva* plants were transferred into a beaker containing filtered seawater (0.45 µm) to facilitate the release of spores (Fletcher, 1989). After 5-10 min when spores released into seawater, *Ulva* plants were removed and the beaker containing spore solution was wrapped with aluminum foil and kept under room temperature. Initial spore density was calculated using a hemocytometer under microscope (Olympus, CK2, Japan). Density of spore solution was then adjusted to 4×10^5 ml^{-1}.

Screening of chemoattractants: In order to find optimum concentrations, all the chemoattractant-candidates were diluted in methanol (1 mg ml^{-1} or 100 mg ml^{-1}). Aliquots of diluted test solutions were applied into 24 well plate (3524: Corning Incorporated, USA) to make 0.5, 1, 10, and 100 µg cm^{-2} coatings except poly-L-lysine which was made with 0.1, 1, 10 and 100 µg cm^{-2} and then dried in oven at 35°C for 12 hr.

In each coated well, 1×10^5 spores were inoculated along with 1 ml of PES medium (Provasoli, 1968) and 10 µl of ampicillin (350 mg l^{-1}). Well plates were kept in darkroom at 20°C for 24 hr. Then medium in the well plate was discarded and gently washed with sterile seawater (0.45 µm) to remove the loosely attached or unattached spores. Later, wells were refilled with 1 ml of PES medium. After 9 day, spores attached and successfully germinated were counted under microscope (200x). Six or ten microscopic fields were counted per well. Results were expressed as mean number of spores cm^{-2} ± SD.

Table - 1: Composition of soluble matrix coating with chemoattractant

Ingredients	%
Chemoattractant	28.6
Wood rosin	27.1
Vinyl resin	8.6
Xylene (solvent)	28.6
MIBK (solvent)	7.1
Total	100.0

Table - 2: DO evolution (mg ml^{-1}) during 72, 114 and 216 hr *Ulva* spore culture in the presence of three selected chemoattractants

Chemoattractant	Conc. µg cm^{-2}	Culture duration		
		72 hr	144 hr	216 hr
Choline	0.5	6.0 ± 1.5	7.3 ± 1.2	7.5 ± 0.6
	1	6.3 ± 1.4	7.6 ± 1.3	8.1 ± 0.3
	10	6.1 ± 1.2	7.6 ± 1.7	8.2 ± 0.6
	100	6.6 ± 0.7	7.0 ± 1.7	8.5 ± 0.1
Gly-gly	0.5	6.4 ± 0.6	6.9 ± 0.1	7.2 ± 0.2
	1	6.6 ± 0.4	7.2 ± 0.4	7.6 ± 0.2
	10	6.4 ± 0.9	7.1 ± 0.4	8.1 ± 0.3
	100	6.7 ± 1.0	7.4 ± 0.3	8.3 ± 0.3
D-glucose	0.5	5.9 ± 1.2	6.4 ± 0.7	6.9 ± 0.6
	1	6.3 ± 2.6	6.9 ± 0.3	7.4 ± 0.4
	10	6.2 ± 1.9	6.7 ± 0.2	7.8 ± 0.3
	100	6.3 ± 2.4	6.9 ± 0.1	7.8 ± 0.4
Control		5.3 ± 3.2	5.6 ± 0.3	5.2 ± 0.3
MeOH		5.6 ± 2.3	5.4 ± 0.4	5.5 ± 0.7

Table - 3: Changes in germling length and biomass of *Ulva* spores cultured in the presence of three selected chemoattractants (n=4)

Chemoattractant	Conc. µg cm^{-2}	Germling	
		Length (mm)	Dry weight (mg)
Choline	0.5	43 ± 0.13	44 ± 0.09
	1	45 ± 0.11	48 ± 0.02
	10	53 ± 0.09	55 ± 0.15
	100	59 ± 0.09	59 ± 0.08
Gly-gly	0.5	40 ± 0.05	41 ± 0.04
	1	43 ± 0.06	45 ± 0.04
	10	52 ± 0.07	48 ± 0.14
	100	50 ± 0.05	49 ± 0.09
D-glucose	0.5	34 ± 0.10	40 ± 0.07
	1	31 ± 0.08	41 ± 0.10
	10	31 ± 0.05	44 ± 0.10
	100	38 ± 0.01	47 ± 0.13
Control		32 ± 0.13	38 ± 0.05
MeOH		30 ± 0.06	35 ± 0.05

***U. pertusa* spore culture:** In order to find out the growth promoting efficiency of chemoattractants, their impact on *Ulva* germlings was studied. Spore culture experiments (Kim and Lee, 1996) were conducted in Borosil beakers (100 ml) with different concentrations of chemoattractant coatings. The chemoattractant test solutions of choline, gly-gly and D-glucose were prepared as mentioned in earlier section and applied onto beaker bottom. Concentration of chemoattractant solutions were adjusted to give desired coatings with 0.5~100 µg cm^{-2}. Experimental beakers were oven dried at 40°C for 12 hr. Coated beakers were filled with PES medium (50 ml) and inoculated with 1 x 10^5 ml^{-1} of *Ulva* spores. Ampicillin solution was also added to each culture beaker. Experimental beakers were incubated in dark at 20°C. After spore attachment (12 hr), culture medium was discarded and fresh PES medium was added. To control the growth of microalgae, GeO_2 (100 mg l^{-1}; 20 µl) was added. Experiments were conducted in growth chamber under fluorescent lamps (55 µE m^{-2} s^{-1}) for 9 days with 12 : 12 LD cycle. During the experimental period thrice (72, 144 and 216 hr) DO content was estimated using tabletop DO meter (YSI 550A, USA). Length and biomass (dry weight) of *Ulva* germlings were estimated at the end of the experiments. Four replicates were used and the results were expressed as mean ±SD.

Preparation of chemoattractant coatings and field studies: Test panel studies were conducted at Ayajin harbor. For this a soluble matrix type of coating formulation was made with wood rosin, vinyl resin, MIBK, xylene and chemoattractant candidates (Sidharthan *et al*., 2004). Chemoattractant coating recipe was prepared by using high dispersion mechanical stirrer (Table 1).

The surfaces of the PVC panels (10 x 10 cm) were roughened with sand paper (#1000) to get uniform surface. Different concentrations of chemoattractant test coatings were sprayed on panels and kept at room temperature for 48 hr to dry. Thereafter, initial weight of each panel was recorded. All the experimental panels were randomly fixed on PVC pipe lines with the help of nylon ties. The panel setup was exposed to Ayajin harbor waters for 90 day. After removed from harbor waters, a nylon quadrat (10 x 10 cm) with 25 squares was used to examine the algal and animal fouling coverage on each panel. Each small square was equal to 4% of whole quadrat area. Algal and animal biomass were quantified and expressed as mean ±SD (D3623 - 78a: ASTM, 1998).

Statistical analyses were performed by using SPSS (Version 10.0). Significant differences were analyzed using one-way ANOVA followed by Tukey's test. Differences were considered to be significant at $*p < 0.05$ and $**p < 0.01$.

Results and Discussion

Different densities of spores inoculated and spores successfully germinated after 30 day incubation are shown in Fig. 1. When inoculated in high densities, *Ulva* spore germination (%) was found to be increased. In order to know the growth rate, *Ulva* germlings were cultured in PES medium for 77 days (Fig. 2). *Ulva* germling growth was less up to 30 day, later the length was gradually increased and after 60 day it reached a maximum of < 7.5 ± 0.8 cm.

Spores attached and successfully germinated on test coatings made with different concentrations of choline are shown in Fig. 3. Significant increase in spore attachment was observed on 1-100 µg cm^{-2} concentration of choline coatings. Up to 10 µg cm^{-2}, gly-gly coatings showed an increase in *Ulva* spore attachment (Fig. 4). At low concentration of 1 µg cm^{-2} of gly-gly, spore attachment was significantly increased (125.6 ± 29.7%; ANOVA, $p<0.05$). D-glucose coatings >1 µg cm^{-2} showed increased spore attachment when compared to control (Fig. 5). A maximum number of spore attachment was observed at 100 µg cm^{-2} levels (257.2 ± 52.9; ANOVA, $p<0.05$). Poly-L-lysine coatings 1-10 µg cm^{-2} attracted relatively more numbers of spores (Fig. 6). But concentrations ≥100 µg cm^{-2} of coatings, 40% decline in spore attachment was observed (ANOVA, $p<0.01$).

In all the four chemoattractants tested, *Ulva* spore attachment increased when compared to respective control. The maximum spore attachment (257.2 ± 52.9%) was recorded from D-glucose coating (100 µg cm^{-2}) with 150% over control (Fig. 5). At high concentrations (100 µg cm^{-2}) of gly-gly and poly –L-lysine, spore attachment was found to be decreased (Fig. 6). However, a reverse trend with increased spore attachment was estimated for choline and D-glucose.

Spore culture experiments were conducted in beakers with three selected chemoattractants (choline, gly-gly and D-glucose) and the changes in germling length, biomass and oxygen evolution of *U. pertusa* were recorded (Table 2 and 3). In which all the coatings were found to enhance the attachment and subsequent germination of spores. On choline coatings with 100 µg cm^{-2}, a maximum increase in growth of *U. pertusa* with 59 ± 0.9 mm was observed (Table 3). Overall performance of length and biomass of germlings estimated on choline coatings were high. In relation to this an increased DO evolution rate was observed (Table 2). Gly-gly coatings exhibited more or less similar effects on *Ulva* germling growth. Relatively less growth of germling was recorded for D-glucose coatings. However, all the coatings were found to increase the growth of *U. pertusa* germlings during increased culture duration. Correspondingly DO levels estimated were also found to be increased (Table 2).

Proportion of algae and animal fouling biomass recorded from test panels are shown in Fig. 7. The total fouling biomass recorded from panels coated with gly-gly was comparatively high (algal: 28.4 g dry wt. and animal: 14.6 g dry wt.). Very less fouling algal biomass was observed on poly-L-lysine coatings (17.0 g dry wt.). Algal biomass (predominantly *U. pertusa*) was high in coatings made with gly-gly whereas animal biomass was high in D-glucose coatings. Comparatively, all the chemoattractant coatings exhibited an increased algal fouling biomass.

Industrial pollution, sediment loading due to deforestation, extensive farming and coastal constructions are seriously damaging

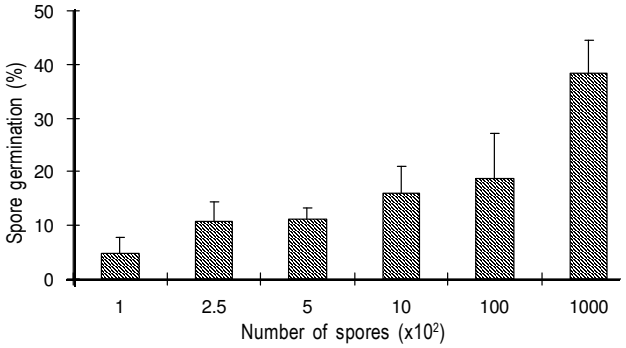

Fig. 1: Inoculation of different densities of *Ulva* spores and percentage of successful germination after 30 day incubation in PES medium

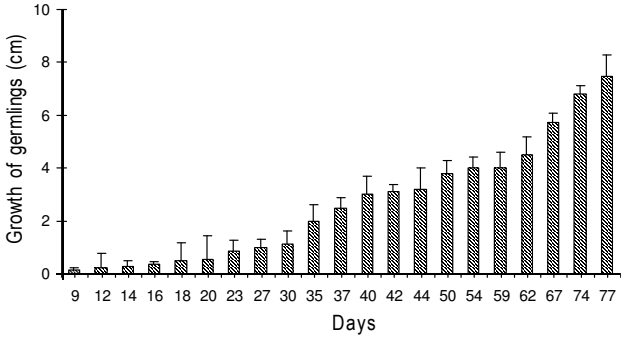

Fig. 2: Growth of *U. pertusa* germlings cultured in PES medium for 77 days

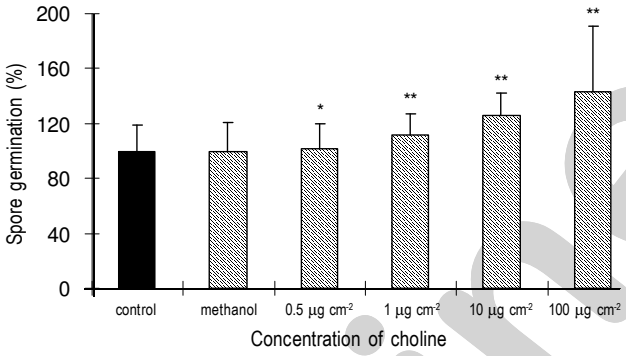

Fig. 3: Attachment and initial germination of *U. pertusa* spores on different concentrations of choline coatings (*p<0.05, **p<0.01)

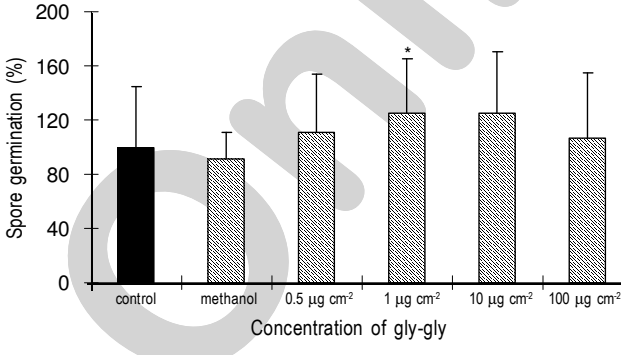

Fig. 4: Attachment and initial germination of *U. pertusa* spores on different concentrations of gly-gly coatings (*p<0.05, **p<0.01)

coral reefs. Besides these terrestrial influences, potential effects of toxic AF paints containing organotins, heavy metals and cyanides along with newly introduced AF biocides (sea-nine 211, Irgarol 1051, zinc pyrithione) on coral reef communities have recently attracted much attention (Owen *et al*., 2001; Negri *et al*., 2002; Jones, 2007). Several other natural disturbances also cause significant damage to coral reefs. The devastating algal whitening occurred in Korea and Japan (Tokuda *et al*., 1994); coral bleaching (Brown, 1997) and over grazing by herbivores (Watanabe and Harrold, 1991; Agateuma *et al*., 1997) are recognized as major threats to algal biodiversity. In recent years, artificial reefs are deployed in conditions described above, to increase the marine productivity. However, to attract propagule and juvenile stages of marine living forms application of suitable chemical coating (chemical stimuli) is one of the successful strategies.

The fouling alga *Ulva pertusa* is a very abundant species occurring along the Korean coast. This Chlorophyceae member exhibits a life cycle of isomorphic alternation of generations with life stages of similar morphologies. Reproductive activities (both gametogenesis and sporogenesis) occur near the margins of matured *Ulva* thallus, with the fertile portions turning slightly brown. In the diploid sporophytic thallus, specialized cells called sporocytes differentiate and undergo meiosis to produce haploid spores. These tetraflagellated spores swim away, and they find a suitable substrate to settle. Subsequently, they lose their flagella, and divide mitotically and develop as filaments then thallus. The life cycle of *Ulva* was well documented (Nordby, 1974; Bold and Wynne, 1978). Initial stages of spore adhesion, settlement and germination in Ulvaceae were well described (Callow *et al*., 1997; Callow and Callow, 2000).

There are two stages involved in the spore settlement, one is surface selection (chemical/physical cues) by spores and the other is by gravity. In general spores settle by themselves once surface contact is established. Spore adhesion takes place after surface selection. It comprises both primary attachment (just adhering on the surface) and in the secondary process spores discard their flagella, lift up their adhesive vesicles and finally secrete glycoproteins on the surface which result in permanent attachment.

Roughness and surface topography are important for settlement of many benthic organisms (Kohler *et al*., 1999; Lapointe and Bourget, 1999; Bers and Wahl, 2004). It has long been known that rough surfaces are preferred by *Ulva* spores/gametes to smooth surfaces (< 0.5 mm) (Christie and Shaw, 1968; Luther, 1976; Fletcher and Callow, 1992; Chaudhury *et al*., 2006).

Bacteria alter the topography of the surface and as well as change the physico-chemical properties of the substratum by altering its wettability, charge of by exposing different surface domains associated with extracellular polysaccharides (Holmstrom and Kjelleberg, 1994). Microbial biofilms were reported to enhance *Enteromorpha* (*Ulva*) spore settlement (Dillon *et al*., 1989). In other experimental studies, bacterial biofilm induced *Ulva* spore attachment was demonstrated (Joint *et al*., 2000; Tait *et al*., 2005). In contrary,

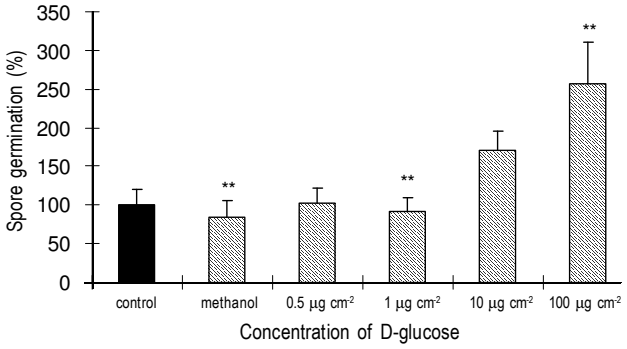

Fig. 5: Attachment and initial germination of *U. pertusa* spores on different concentrations of D-glucose coatings (*p<0.05, **p<0.01)

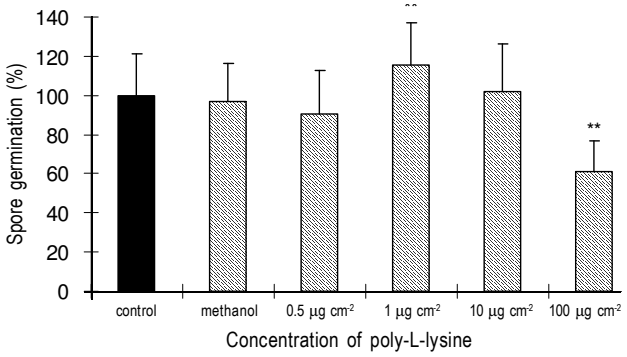

Fig. 6: Attachment and initial germination of *U. pertusa* spores on different concentrations of poly-L-lysine coatings (*p<0.05, **p<0.01)

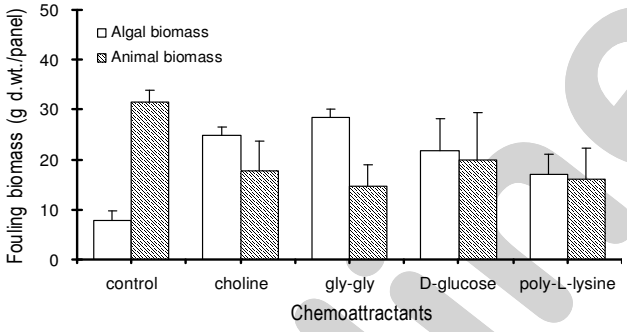

Fig. 7: Proportion of fouling biomass on different chemoattractant coatings (exposed to Ayajin harbor waters for 90 days)

Holmstrom *et al*. (1996) showed that a number of marine bacterial strains inhibited the settlement of algal cells, including spores of *Ulva* and/or invertebrate larvae.

Settlement is the stage, whereby the surface is located and surface contact is established. In many organisms, this stage is regulated by perception of a range of chemotactic signals as well as physical and physico-chemical interactions with the substratum. Algal chemotaxis to different chemical stimuli might involve different mechanisms of reception and signal transduction (Govorunova and Sineshchekov, 2003). Chemotactic responses mediated by secretion of pheromones by algal gametes are well known (Maier, 1995) but little is known regarding the existence or nature of diffusible settlement cues in the algae. Brown and green algal spores can detect and respond to a variety of inorganic and organic nutrients, thereby ensuring settlement in a habitat nutritionally favorable for growth (Maggs and Callow, 2002).

It was observed that very high numbers of spores adhered to silicone elastomeric coatings as they acted as chemoattractants (Callow and Callow, 1998). Experiments employing cover slips coated with a range of saturated fatty acids showed increased spores adhesion (Callow and Callow, 2000). Similar chemoattractant property of extracts of *Ecklonia cava* against *Ulva* spores was also reported (Sidharthan *et al*., 2007). *Ulva* spore attachment and subsequent germination were significantly increased in experimental coatings of choline and D-glucose (Fig. 3, 5).

Monosaccharides such as glucose and fructose are rich in seawater and various carbohydrates released from specific algal species considered as waterborne cues (Krug and Manzi, 1999). The role of carbohydrates as waterborne cue for larval and spore settlement was reported in earlier studies (Callow *et al*., 1981; Krug and Zimmer, 2000). Similarly, in the present study also a maximum of 150% increase in spore attachment was estimated from D-glucose coatings. It is comparable with the performance of polylysine coatings (200 spores cm^{-2}) reported by Santelices and Aedo (1999). Several other studies have revealed chemotaxis towards sugars in microalgae (Taylor and Panasenko, 1984; Cooksey and Cooksey, 1986; Ermilova *et al*., 1993).

Biofilms incubated in sugars and lectin compounds exhibited increased spore attachment (Michael and Smith, 1995). Quorum sensing enables cell to cell communication of bacterial population via specific diffusible chemical signals (Wheeler *et al*., 2006). Algae can directly respond to bacterial quorum sensing signals (Joint *et al*., 2002) to locate suitable substrata for settlement. Increased settlement of *Ulva* spores on *Vibrio angullarum* biofilms was correlated to presence of AHLs (acyl-homoserine lactones) (Tait *et al*., 2005). Wheeler *et al*. (2006) demonstrated the chemokinetic responses of *Ulva* spores induced by AHLs. Unlike chemotaxis, the swimming speed of *Ulva* spores was greatly reduced by AHLs signals thereby spores accumulated rapidly in particular area. Thus, chemical cues play an important role in benthic ecosystems, community structure and also in regulating the marine biodiversity.

Non-toxic substances that enhance the adhesion of seaweed spores are both ecologically and economically important. The roles of polylysine in enhancing the recruitment of seaweed propagules are consistent with known uses of this substrate as a cell surface ligand (Jacobson and Branton, 1977; Santelices and Aedo, 1999). The positive charge conferred by polylysine to the coated surface promotes the adherence of negatively charged cells. This capacity has been shown in wide variety of cells and organells (Jacobson and Branto, 1977) and now it is being utilized for seaweed propagules.

Application of spore adhesion-promoting substances may reduce nursery time and improve recruitment during low fertility seasons (Santelices and Aedo, 1999). Chemically active substances, used in the present study appeared as promising chemoattractants to enhance recruitment of selected algal species on artificial surfaces for both aquaculture and sea ranching purposes.

Acknowledgments

This work was supported by the Korea Research Foundation Grant by t0he Government of Korea (Regional Innovation Center Promotion Fund, MOCIE - RIC060605). Authors are thankful to Mr. Lim Chi Young, for his help during the field investigations.

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