Korean J. Environ. Biol. 17(3) : 315~319(1999)

Bioassay of Spores Attachment for the Weedy Green Alga *Ulva fasciata* on Agar and Agarose Surfaces

Hyun-Woung Shin

(Department Aquatic Resource Sciences, College of Natural Sciences, Soonchunhyang University, Asan City 336-600, Korea)

Abstract - Spore attachment of *Ulva fasciata* on agar and agaroses coating films showed various surfaces properties according to concentrations of agar or agaroses or via methanol. The highest number of spore attachment occurred in Sigma agarose coating film. Spore attachment on Bacto agar and other agaroses coating films showed the (2~36 times less than Sigma agarose coated film. Comparison of spore attachment on 2.5% and 5% SeaKem agarose and Bacto agar coating films differed in two concentrations while coating of 2.5% and 5% with NuSieve and Sigma agaroses did not differed. Spore attachment of coating made of 5% and 5% via 4% MeOH with Bacto agar and NuSieve agarose only differed. Overall, these results indicated agar and agarose coating films differed in spore attachment. Results of this work will be useful baseline for bioassay of antifouling activity of fouling organisms. [Agar, Agarose, Biofouling, Spore, *Ulva fasciata*].

INTRODUCTION

Development of biofouling on a surface immersed in water is a well-known phenomenon of ecological succession in marine environments (Evans 1981). The process begins with an organic film followed by accretion of microbia, soft and hard foulers including bacteria, fungi, diatoms, multicellular algae, and invertebrates. Rapid colonization occurs not only on inshore rock surfaces, pilings, piers, and aquatic vegetations but also on offshore man-made structures with a costly problem affecting structures such as ship-hulls, heat-exchanges, pilings among others (Shin 1995). The search for effective means to control fouling accumulation on man-made surfaces in aquatic environments began with the first attempts to explore and exploit the sea. Since the 1970's organometallic compounds such as tributyl-tin (TBT) have becoms the industry standard because they produce economical, highly effective and long-lasting antifouling (AF) coatings. Use of these compounds has become a problem as heavy metals (Copper & Zinc) have been accumulated in sediments and the water column (Callow 1990). Unfortunately, these highly effective materials are also extremely toxic and environmentally refractive, which has led to serious problems of environmental pollution associated with their application, use and disposal.

Recently, several programs have examined ways to develop novel AF agents that are biologically friendly to the marine organisms and environment (Zimmerman *et al.* 1993). Performance goals for novel AFs agents include: 1) maintenance of AF properties and constant flux rates, 2) a coating life time of 5 to 7 years, 3) effectiveness of the non-toxic AF agents to both target and non-target organisms, 4) the optimal dose AFs effectiveness should be less than 10 $\mu g/cm^2/day$, 5) low commercial cost for reagents and 6) effectiveness over the range of tropical to polar water temperatures. To demonstrate these essential novel AF features, it is necessary to test effectiveness of AF agents with bioassays from diverse marine organisms. Traditionally, newly develop-

ed AF agents have been examined primarily with bacteria or invertebrates (Todd *et al.* 1993). As foulers, species of the benthic, cosmopolitan green macroalgae *Ulva* and *Enteromorpha* have been overlooked as bioassay organisms despite their ubiquity in diverse marine environments and our knowledge of how to harvest cells for settlement (Shin 1996).

A method that has been used to assay microbial attachment is the Agar Media Diffusion Method. This method of primary screening for antibiotics detects bioactivity of specific compounds that can be utilized in production of specific molecules. It has been used broadly in the screening of microorganisms for antibiotic production in a wide range of fields (Johnson & Curl 1972; Waterman 1990), in assaying antimicrobial activity of chemicals (Collins & Lyne 1970; Ho & Ko 1980). And strain selection for drug resistance (Betina 1983; Jayasuriya *et al.* 1989; Sam 1993). The technique was used in the discovery of the principal antibiotics against the Gram-positive bacteria (Zahner *et al.* 1982; Berdy 1989) and to some extent, against the Gram-negative bacteria pathogens and pathogenic fungi (Ho & Ko 1980). This approach is based on the prior demonstration that agar media are biological material and active agents diffuse through the polysaccharide matrix.

It is the objective of this work to assess potential utility of this technique by using algae to detect bioactivity of specific non-toxic anti-fouling agents. This involves first assessing any effects of the agar or agarose as well as the solvent, methanol, that is commonly used to dissolve AF agents with phenolic acid components (Haslbeck *et al.* 1996) on settlement. This report includes: 1) a comparison of *Ulva fasciata* spore attachment on agar and agarose films at various concentrations, and 2) a comparison of attachment to specific concentrations of agar or agarose with methanol. These baseline determinations will allow further development of an assay for anti-fouling effectiveness with specific test organisms including the algae.

MATERIALS AND METHODS

1. Spore collection, density and statistical analysis

Twenty thalli of *U. fasciata* were collected randomly at Black Point from south coast of O'ahu Island, Hawaii. Whole thallis were transported to the laboratory within 30 minutes and cleaned in 0.2 μm filtered seawater to minimize epiphytes on thalli. To prepare the thalli for spore release, the material was blotted, separated, and individual plants were placed in dry petri dishes under 50 μmol $m^{-2}s^{-1}$ of fluorescence lights at room temperature (19.5°C). Twelve hours later the thalli were barely covered with filtered seawater (approximately 30 ml) which triggered the spore release.

To determine the density of spores, 0.5 ml of the spore suspension was added to 0.5 ml of 2% formalin and counted in a counting chamber under a 60 × 24 mm coverslip using a random field methods (APHA 1976). The number of spores in the innoculum was 1.2×10^{10}/ml ($\pm 8.9 \times 10^{2}$/ml).

The glass slides were immersed with 10% HCl for 24 hours and then rinsed within the distilled water for another day. The concentration of agar and agarose was diluted with distilled water (5% or 2.5% w/v). The 5% of agar and agarose were mixed with 4% methanol (MeOH). One ml of each agar or agarose concentration was spread on acid-cleaned glass slides and allowed to solidify in a horizontal position for 2 hours. All of the glass slides and media were autoclaved for 15 min. at 160°C. Constituents of experimental films are listed in Table 1. The most popular commercial agar and agarose brands, NuSieve, SeaKem, Bacto agar, Sigma products were selected. Four replicates were used, and all experiments were repeated at least two times.

2. Statistical analysis

The patterns of attachment by *U. fasciata* spore were analyzed with the use of t-tests which was compared with dosage-responses of 5% and 2.5% agarose concentrations, 5% agarose via 4% MeOH agarose.

RESULTS

1. Settlement on acid-cleaned glass slide as control

The mean of spore attachment on acid-cleaned glass slides was 60 spores/mm^2 (SD ± 9.3, n=4). Less spore attached on acid-cleaned glass slide than 2.5% Bacto agar (mean=148, SD ± 15, n=4), and 2.5%, 5% and 5% Sigma agarose with 4% MeOH (mean=1824, SD ± 56.2, n=4; mean=1833, SD ± 65, n=4, mean=1757, SD ± 60.3, n=4).

2. Bacto agar

Spore settlement on films of 2.5 and 5% of Bacto agar differed (mean=148, SD±15, n=4; mean=39, SD±9.4, n=4; t=0.001) (Table 2). Attachment of coating made of 5% Bacto agar and 5% with 4% MeOH with Bacto agar (mean=165, SD ±14.2, n=4) also differed significantly (t=0.001); however, attachment on the 5% Bacto agar was less than on the acid-cleaned slide.

3. NuSieve agarose

Spore attachment on the two concentrations, 2.5% and 5%, of NuSieve agarose did not differ significantly (mean=57, SD±28, n=4; mean=48.3. SD±20.1, n=4; t=0.204) (Table 2). Attachement on agarose films of 5% NuSieve and 5% NuSieve with 4% MeOH (mean=106.8, SD±14.5, n=4) differed significantly (t=0.001); the NuSieve films with MeOH were more attractive to the spores. The mean attachment on 2.5 and 5% NuSieve agarose films was lower than the control while the mean spore attachment on 5% NuSieve with MeOH films was higher than on the acid-cleaned slides.

4. SeaKem agarose

Spore attachment SeaKem agarose films differed significantly between 2.5% and 5% concentrations (mean=86.5, SD±11.8, n=4; mean=118, SD±12.1, n=4; t=0.02) (Table 2); the 5% agarose films were more attractive to the spores. A comparison of attachment on 5% SeaKem agarose and 5% SeaKem agarose with 4% MeOH (mean=119, SD±25, n=4) showed no significant difference (t=0.113). The mean spore attachment to 2.5 %, 5% SeaKem agarose and 5% SeaKem agarose with 4% MeOH agarose films were greater than on the acid-cleaned slides.

5. Sigma agarose

Spore attachment on 2.5% and 5% Sigram agarose films was not significantly different (mean=1824, SD±56.2, n=4; mean=1833, SD±65, n=4; t=0.45) (Table 2). A comparison of settlement on 5% Sigma agarose and the same concentration with 4% MeOH (mean=1757.3, SD±60.3, n=4) showed no significant difference (t=0.07). The attachment response of *U. fasciata* spores to two concentrations of Sigma agarose showed the spores preferred 5% over the 2.5% agarose films. The mean spore attachment to 2.5%, 5% Sigma agarose and 5% agarose with 4% MeOH agarose films was greater than the control slides. Spore attachment on both concentrations of Sigma agarose (2.5% and 5%) showed the highest number of spore attachment on coated film 12 times to 36 times higher than other coating films and on the acid-cleaned slides.

Table 1. Physical characterizations of agar and agarose

Name	Gelling point (°C)	Moisture (%)	Melting point (°C)	Co.
NuSieve GTC	≤35	10	≤65	MidWest Sci.
SeaKem LE	≤35	10	≤65	MidWest Sci.
Agarose	≤36	8.4	≤65	Sigma Co.
Bacto-Agar	?	?	?	Difoc Co.

Table 2. The results of comparisons between treatments (t-test)

Pair Comparison	t-test value
5% NuSieve & 2.5% NuSieve	0.204
5% NuSieve & 4% MeOH NuSieve	0.001*
5% SeaKem & 2.5 % SeaKem	0.020*
5% SeaKem & 4% MeOH SeaKem	0.113
5% Sigma agarose & 2.5% Sigma agarose	0.450
5% Sigma agarose & 4% MeOH Sigma agarose	0.067
5% Bactoagar & 2.5% Bactoagar	0.001*
5% Bactoagar & 4% MeOH Bactoagar	0.001*

* indicates the differed significantly (0.05).

DISCUSSION

This study shows that the agar and agarose tested here show differential responses by the attaching stage of *U. fasciata*. The agarose coating film was more stable than Bacto agar coating film as a substrate for antifouling assays with *U. fasciata* spores. The objective in using agar or agarose coated films to detect AF bioactivities is that the AF compounds diffuse through the agar or agarose coated film and is delivered to the surface at a specific release rate (this "flux" is commonly expressed in the unites of microorganisms per square centimeter of surface area per day). The constancy of the release rates are significant for efficient use of the AF agent throughout the life time of the coating.

The discovery of antifouling agents depends on the screening methodology used in crude extracts assay. Also, it is important to ensure that the sample taken is representative of the crude extract. Differential solubility can be a problem, particularly when the extract is not water soluble, in which case MeOH may need to be used.

The presence of large amounts of substances that can interfere with the assay, either dominating spectrometric measurement or masking the biological effects of smaller amount of an active principle, must be considered and overcome. Application of the Agar Diffusion Method must be based on knowledge of both differential diffusion that each AF compound as well as chemical reactions between the agar/agarose substrate and the AF agent.

The practical definition of agarose is a subset of agar molecules that possess the lowest charge content and therefore greatest gelling ability. According to Duckworth and Yaphe (1971), complete agar consist of three polysaccharides. These are neutral agarose, charged pyruvated agarose with little sulphated galactan. Commercial preparations of agarose contain different numbers of charged groups. According to the analytical values, Agarose from Sigma Co. contains a lot of charged groups and has higher gel strength than agarose SeaKem, NuSieve and Bacto agar which may be the reason why they exhibited such potential neutrality merits for bioassays with algal spores. SeaKem may also be useful for fouling studies based on the results which did not differ between 5% SeaKem and 4% MeOH with 5% SeaKem. In general, the results of spore attachment on films with 4% MeOH coating suggest that dissolving AF agents dissolved in MeOH does not influence attachment by the algal spores. However, depending upon the type of coating under evaluation, the release rate may be constant or not constant. It goes without saying that constant release rates are preferable in that they yield efficient use of the AF agent throughout the life time of the coating.

The Agar Diffusion Method can generate the resemble physical mechanism for testing the new AF agents. After incubation period, inhibition or enhancement of spore attachment of *U. fasciata* can be determined with microscopic observation. This study will be a useful baseline for bioassays of fouling using spores of *U. fasciata*.

ACKNOWLEDGMENT

The author gratefully recognizes the support of the Research Institute for Basic Sciences which assistance made this work possible grant #9810028 from Soonchunhyang University.

REFERENCES

Evan LV (1981) Marine algae fouling: A review with particular reference to shipfouling. *Bot. Mar.* **24** : 167-171.

Berdy J (1989) The discovery of new bioactive microbial metabolites: screening and identification. pp. 3-24. *In* Bioactive Metabolites from microorganisms: Progress in Industrial Microbiology Vol. 27 (Bushell ME & U Grafe *eds*). Elsevier. Amsterdam.

Betina V (1983) The chemistry and biology of antibiotics. Elsevier, Bratislava.

Callow M (1990) Ship fouling: Problems and solution. *Chem. Ind.* **5** : 123-127.

Collins GH & PM Lyne (1970) Microbiological methods. Butterworth & Co., Ltd. London.

Duckworth M & W Yaphe (1971) The structure of agar. Part I. Fraction of a complex mixture of polysaccharides. *Carbohyd. Res.* **16** : 189-197.

Haslbeck EG, CJ Kavanagh, HW Shin, WC Banta, P Song & GI Loeb (1996) Minimum effective release rate of antifoulants (2): Measurement of the effect of TBT and Zosteric acid on hard fouling. *Biofouling* **10** : 175-186.

Ho WC & Ko WH (1980) Agarose medium for bioassay of antimicrobial substances. *Phytopathology* **70** : 764-766.

Jayasuriya H, JD McChesney, SM Swanson & JM Pezzuto (1989) Antimicrobial and cytotoxic activity of rottlerin-type compounds from *Hypericum drummondii*. *J. Nat. Prod.* **52** : 325-332.

Johnson LF & EA Curl (1972) Methods for research on the ecology of soil-borne plant pathogens. Burgess, Minneapolis, MN.

Sam TW (1993) Toxicity testing using the brine shrimp: *Artemia salina*. pp. 441-456. *In*: Bioactive natural products: determination, and structural determination. (Colegate SM & RJ Molyneux eds). CRC press, Ann Arbor Michigan.

Shin HW (1995) Biofouling by marine weedy algae. *Kor. J. Phycol.* **10** : 97-108.

Shin HW (1996) Ecophysiological studies of reproductive motile cells for the Hawaiian macrophyte, *Ulva fasciata* (Chlorophyta). Ph. D. dissertation University of Hawaii. 162 pp.

Todd JS, RC Zimmerman, P Crew & RS Alberte (1993) The antifouling activity of natural and synthetic phenolic acid sulphate esters. *Phytochem.* **34** : 401-404.

Waterman PG (1990) Searching for bioactive compounds: various strategies. *J. Nat. Prod.* **53** : 13-21.

Zahner H, H Darutx & W Weber (1982) Novel approaches to metabolites screening. pp. 154-178. In: Bioactive Microbioal products: search and discovery (BuLock JD, LJ Nisbet.& DJ Winstanley *eds*) Academic Press, London.

Agar와 Agarose 코팅필름을 이용한 갈파래의 포자 부착 검색

신 현 웅

(순천향대학교 자연과학대 수산자원학과)

적 요 - 갈파래의 포자가 부착한 Agar와 Agarose 코팅필름은 agar, agarose 또는 메탄놀의 농도에 따라 다양한 표면의 성질을 나타냈다. Sigma agarose 코팅필름에서 가장 많은 수의 포자 부착이 일어났다. Bacto agar과 그외 agarose으로 코팅한 필름에 부착한 포자는 Sigma agarose으로 코팅한 필름보다 12~36배나 적었다. 2.5%와 5%의 SeaKem agarose와 Bacto agar 코팅필름의 비교에서 두 개의 농도 차이가 있는 반면에, 2.5%와 5%의 NuSieve와 Sigma agarose 코팅필름의 비교에서는 차이가 없었다. 5%의 Bacto agar와 4%의 메탄놀을 포함한 5% Bacto agar 비교, 5% NuSieve와 4%의 메탄놀을 포함한 5% NuSieve agarose 비교에서만 차이를 보였다. 본연구의 전반적인 결과는 agar와 agarose로 코팅한 필름에서 포자 부착의 차이가 있음을 알 수 있었다. 본연구 결과는 착생생물에 대한 반착생을 검색할 수 있는 기초가 되어질 것이다.