JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2009. Vol. **3**(2), p. 649-654

Improved Recoverability of Bacterial Strains from Soft Coral, *Lobophytum* sp. for Antagonistic Activity

C. Chellaram* and J.K.P. Edward

*Department of Biomedical Engineering, Vel Tech Multi Tech
Dr. R.R. Dr. S.R. Engineering College, Avadi. Chennai - 600 062, India.
Suganthi Devadason Marine Research Institute, Tuticorin - 628 001, India.

(Received: 02 March 2009; accepted: 13 April 2009)

Bacteria associated with the soft coral, *Lobophytum* sp. was cultured in a varied combination of low to high nutrient media. Each of the media was supplemented with sodium pyruvate. This study was aimed to find out whether the addition of sodium pyruvate to all solid media, could significantly increase the recovery of microbes. Microorganisms on maltose amended seawater agar were highly recovered in the supplemented media than on non-supplemented media. These findings suggest that the addition of sodium pyruvate to solid growth and isolation media may improve recoverability of microorganisms from soft coral. The results also indicated that the higher percentage of the antagonistic bacteria was found on supplemented media when compared to that of the non-supplemented media and hence the highest percentage of antagonistic bacteria were found to be exhibited by the supplemented media of MA+SE media.

Key words: Soft corals, Different media, Bacterial recoverability, Antagonistic activity.

Interactions between marine bacteria and their host organisms are known to play a significant role in many marine ecosystems, but historically this association has received little attention. Numerous investigators have studied the interactions between corals and microbes (Chellaram *et al*., 2006 and Gnanambal *et al*., 2005). These studies have shown that there is a dynamic microbiota living on the surface and possibly within the tissues of corals and in the surrounding reef waters. Analyses of microbial communities have been hindered by inability to cultivate most of the organisms within a sample. There is a wide consensus among microbial ecologists that the majority of bacteria in complex natural communities do not form colonies on the rich media traditionally used for enumerating and isolating bacterial species, even though they may be viable (Zobell, 1946). Estimate of bacterial recoverability from environmental samples ranges from only 0.01 to 12.5% of existing community (Amann *et al*., 1995; Sobecky *et al*., 1998 and Ward *et al*., 1990).

The simple act of taking microorganisms from their natural environment and placing them onto laboratory media exposes the organisms to a wide variety of environmental stresses and

* To whom all correspondence should be addressed.
Tel.: +91-9944040538;
E-mail: dr.chellaram@gmail.com.

subjects them to unnatural growth conditions. These injured cells are commonly unable to produce colonies on media used for their enumeration. Therefore, recovery of microorganisms from environmental sample is likely reduced as a result of potential stress and injury. Numerous methods have been employed in attempts to increase the number of bacteria retrievable from a sample, with varying but still minimal success. In an effort to minimize injury and stress, scientists have advocated the exogenous addition of various supplements, most often catalase and sodium pyruvate to culture media.

The soft coral, *Lobophytum* sp are found in abundance in Tuticorin coastal waters. But works pertaining to the associated bacteria and their recovery from these corals are not carried out *hitherto*. Thus an attempt has been made to use different types of media and media supplementation to determine if recovery of the natural microbial community associated to the soft coral could be increased by the supplementation and the use of different types of media. The efficacy of the media supplements was determined in terms of enhancing the antibacterial activity of the isolated strains by employing appropriate bioassays.

MATERIAL AND METHODS

The bacteria associated with coral surface were collected by swabbing a small area (approximately 1 cm^2) of the external surface of soft coral, *Lobophytum* sp (Coelenterata: Octocoralia: Alcyonacea) from Tuticorin coastal waters. After serially dilution (10^2, 10^3, 10^4) a combination of low to high nutrient media (50:50, Maltose amended sea water agar, Oligotrophic agar , Carbon mix agar, Free Lunch Medium, Marine Agar media and Seawater Extract media) and the media additions (Sodium pyruvate) was employed in an attempt to recover the largest number of representative microorganisms (Table. 1). 100µl from each of the dilutions in triplicates were poured onto different media containing supplement and controls (without Sodium pyruvate) and spread with a sterile glass rod. Inoculated plates were stored inverted in sealed sleeves in the dark at room temperature (approx. 20-25°C).

Colony forming units (CFUs) were counted on 4, 8, 10 and 12 days of the experiment following growth and inoculation. However, day 10 counts were used for all analyses as they yielded the most representative counts for all experiments. All recovered (amended and unamended) bacterial strains were screened for production of antibacterial substances, using double agar overlay method (Dopazo *et al*., 1988).

Double agar overlay method was used for the assay of isolated bacterial strains against the 3 human pathogens (*Klebsiella pneumoniae*, *Staphylococcus aureus* and *Shigella dysentriae*) and 3 fish pathogens (*Proteus mirabilis*, *Serratia marcescens* and *Aeromonas hydrophila)*. Colonies of isolated bacteria were developed on ZMA plates by spotting 18 h old culture and incubating at room temperature for 32 h. About 10 µL of the culture was suspended in 8 ml of soft Tryptone Soya Agar (TSA) with soft agar was poured immediately over the macro-colonies of the antagonistic marine bacteria on the ZMA plates. The percentage of antagonistic activity was made between two recovered strains.

RESULTS

The numbers of culturable bacteria per 100µl obtained on low to high nutrient of the control and supplemented media isolated from *Lobophytum* sp. are presented in Table 2. The CFU counts for each medium used, with the corresponding differences from control values are given in Fig 1. There was a significant variation between the control and the sodium pyruvate supplemented media used for the recoverability of the microbial colonies. More bacterial colonies were formed on supplemented media than control (Un-supplemented) media like, Oligotrophic medium ($4.25\pm0.1X10^4$), C-Mix Medium (4.5 ± 0.26), Marine Agar (7.55 ± 0.087) and MA+SE medium ($7.9\pm.33X10^4$) showed moderate CFU counts, while supplement added media showed higher CFU counts of, 6.3 ± 0.26, 6.5 ± 0.18, 8.3 ± 0.155 and $8.9\pm0.63X10^4$ respectively.

Among sodium pyruvate supplemented isolates from sample, 50:50 and FL medium were able to recuperate the highest representatives of $1\pm0.087X10^5$ and $3\pm0.2X10^5$ respectively in comparison with control media. It was noted that

Table 1. Media recipes

Medium	Reference	Composition
50:50	This study	0.6ml trace metal solution,[a] 0.6ml PO_4^- solution,[b] 500ml filtered seawater, 500ml distilled water, 10g agar.
Maltose Amended seawater (MsH_20)	This study	2.5 g maltose, 0.1.5ml trace metal solution,[a] 1.5ml PO_4^- solution,[b] 1 L filtered seawater, 10 g agar
Oligotrophic Media (OLIGO)	Santavy and Colwell,1990	0.5g tryptone, 0.1 g sodium glycerophosphate, 0.05 g yeast extract, 1 L filtered seawater, 12 g agar.
Carbon Mix (C-mix)	This study	0.2 g maltose, 0.2 g mannitol, 0.2 g glucose, 0.2 g soluble starch, 0.2 g galactose, 0.1 g peptone, 0.1 g tryptone, 0.1 g yeast extract, 1ml trace metal solution,[a] 1ml PO_4É solution,[b] 1 L filtered seawater, 10g agar.
Free Lunch Medium (FLM)	Currin *et al*.,1990	23.4g NaCl, 0.75g HCL, 7g $MgSO_4$, 0.2g $CaCL_2$ 0.015 g KH_2PO_4, 1 g mannitol, 1 g yeast extract, 1 g peptone, 1ml trace metal solution,[a] 1L distilled water, 10g agar.
Marine Agar 2216 (MA)	Difco purchase	55.3 g Marine Agar 2216, 1L distilled water.
Seawater + Soft coral Extract Media (sH_2O + SE)	This study 10g agar,	1.5ml trace metal dolution,[a] PO_4É solution,[b] , 1 L filtered seawater, 5-10% filter sterilized soft coral extract prior to pouring plates.

[a] 2.86g H_3BO_3, 1.81 g $MnCl_2$. $4H_2O$,1.36g Fe EDTA, 0.08g $CuSO_4$-$5H_2O$, 0.049g $Co(NO_3)_2$-6H2O. 0.39g NaMoO-$2H_2O$, 0.22gg ZnSO4-$7H_2O$, 1000 ml distilled H_2O.; [b] 5.0g NaH_2PO4-H_2O, 1000 ml distilled H_2O.

Table 2. Cultivable cells/100µl inoculum on different medium

DifferentMedia	Control CFU (Mean of 3 values ± S. D)	Sodium pyruvateCFU (Mean of 3 values ± S. D)	Statistical significance	
			t value	*p* value
50:50	$0.47\pm0.043\times10^4$	$1.5\pm0.15\times10^4$	-11.3714	0.000171**
MsH_2O	$0.65\pm0.1\times10^4$	$1.1\pm0.22\times10^4$	-3.250	0.015682*
OLIGO	$4.25\pm0.1\times10^4$	$6.3\pm0.26\times10^4$	-12.5536	0.000116**
C-MI×	$4.5\pm0.26\times10^4$	$6.5\pm0.18\times10^4$	-10.9545	0.000197**
FLM	$3\pm0.65\times10^4$	$4.2\pm0.218\times10^4$	-3.0317	0.019357*
MA	$7.55\pm0.087\times10^4$	$8.3\pm0.155\times10^4$	-7.3	0.0009**
MA+SE	$7.9\pm0.33\times10^4$	$8.9\pm0.63\times10^4$	-2.25	0.035*

All values are mean ± SD of triplicates; ** - P<0.01 (Highly significant); * - P<0.05 (Significant); NS – Non-significant

Table 3. Improved recoverability of producer strains on different media with supplement

Different	Control			Sodium pyruvate		
Media	Total strains	Producer strains	%	Total strains	Producer strains	%
50:50	47	11	18.18	150	31	19.77
MsH_2O	65	11	18.09	135	24	18.8
OLIGO	425	61	15.04	630	93	16.2
C-MIX	450	75	15.07	650	101	15.62
FLM	300	38	17.09	420	82	18.53
MA	755	117	15.69	830	137	18.5
MA+SE	790	133	20.85	890	175	22.19

DISCUSSION

Microorganisms from environmental samples exposed to significant environmental changes and stresses in the transition from natural environment to laboratory media may become sub-lethally injured. In general addition of pyruvate to media improves the recovery of injured bacteria through its action in degrading peroxides and promoting cell recovery (McDonald *et al*., 1983). Martin *et al*. (1976) demonstrated that the presence of either catalase or sodium pyruvate in various non-optimal media permitted the increased enumeration of injured and uninjured *Staphylococcus aureus* cells, often to levels above those obtained using the un supplemented growth media. There are earlier reports to indicate that exogenous addition of various supplements, most often catalase or sodium pyruvate can improve the detection of microbes in stress (Kreig and Hoffman, 1986).

In the present study, sodium pyruvate supplemented MA+SE medium was found to have the highest bacterial representatives. Bacterial strains isolated from *Lobpyhtum* sp, using the sodium pyruvate supplemented MA+SE medium have the highest CFU counts, $8.9 \pm 0.1X10^4$ as compared to other medium. The supplemented media had the highest capacity to recover the bacterial strains than un-supplemented media. Similar works have focused on the fact that addition of sodium pyruvate to laboratory media resulted in the recovery of stressed bacteria by way of degrading toxic H_2O_2 that builds in bacterial cells. Calabrese and Bissnnette (1990) and Olson *et al*. (2000) demonstrated that sodium pyruvate additions as well as combinations of Sodium pyruvate was effective in recovering sub-lethally injured cells and increasing the detection of total heterotrophic bacteria from acid mine water and marine sponges respectively. The present study is in agreement with their study and the results indicate that the application of different approaches to the cultivation of microorganisms from marine samples increases the percentage of microbes recoverable from the samples. The function of secondary metabolites in nature is a controversy raging for decades. Secondary metabolite production has also been hypothesized as "elbow space' to microbial species, which coexist in the same environment (Zahner *et al*., 1982). Long and Azam, (2001) studied the antagonistic interaction among the marine pelagic bacteria. The antagonistic bacteria isolated from surface of the soft coral *Lobophytum* sp. was able to inhibit the test organisms used for the experiments. It has been documented that bacteria associated with the soft coral, *Dendronephthya* sp. are suggested to produce bioactive compounds against Gram negative and Gram positive bacteria. Chellaram, *et al*. (2005) observed that the presence of antagonistic bacteria on the surface of the gorgonid is to inhibit both human and fish pathogens. In the present study, the highest percentage (221.19%) of antibiotic producing strains was isolated with supplemented medium of MA+SE. A better recovery and antagonistic results of bacterial strains from the surface of these soft corals suggests that these organisms represent as ecological niche, which harbors largely uncharacterized organisms attached to the surface may yield a vast array of new compounds with novel activity.

ACKNOWLEDGMENTS

CC thanks sincerely the Director, Suganthi Devadason Marine Research Institute, Tuticorin, for the financial and laboratory support and Chairman and Director, Vel Tech Multi Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Chennai, for their unremitting encouragement.

REFERENCES

1. Amann, R. I., Ludwig, W., Schleifer, K. H. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 1995; **59**: 143-169.
2. Calabrese, J. P., Bissonnette, G. K. Improved detection of acid mine water stressed coliforms bacteria on media containing catalase and sodium pyruvate. *Can. J. Microbiol.* 1990; **36**: 544-550.
3. Chellaram C., Gnanambal, K. M. E., Edward, J. K. P. Isolation and screening of mucus associated bacteria of the gastropod, *Drupa margariticola* for antagonistic activity. *J. Mar. Biol. Ass. India*. 2005; **47**(2): 154-159.
4. Dopazo, C. P., Lemos, M. L., Lodeiros, C.,

Bolinches, J., Barja, J. L., Toranzo, A. E. Inhibitory activity of antibiotic producing marine bacteria against the fish pathogens. 1988; *J. Appl. Bacteriol.* **65**: 97-101.

5. Gnanambal, K. M. E., Chellaram, C., Jamila Patterson. Isolation of antagonistic marine bacteria from the surface of the gorgonian corals at Tuticorin, Southeast coast of India. *Indian J. Mar. Sci*. 2005; **34**(3): 316-319.
6. Kreig, N. R and Hoffman, P. S. Microaerophily and oxygen toxicity. *Annu Rev. Microbiol,* 1986; **40**: 107-130.
7. Long, R. A., Azam, F. Antagonistic interatctions among marine pelagic bacteria. *Appl. Environ. Microbiol*. 2001; **67**: 4975-4983.
8. Martin, S. E., Flowers, R. S., Ordal, Z. J. Catalase: Its effects on microbial enumeration. *Appl. Environ. Microbiol.* 1976; **32**(5): 731-734.
9. McDonald, L. C., Hackney, C. R., Ray, B. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. *Appl. Environ. Microbiol.* 1983; **45**(2): 360-365.
10. Olson, J. B., Lord, C., Mc Carthy, P. J. Improved recoverability of microbial colonies from marine sponge samples. *Microbial Ecol*. 2000; **40**: 139-147.
11. Sobecky, P. A., Mincer, T. J., Chang, M.C., Toukdarian, A., Helinski, D. R. Isolation of broad-host-range replicons from marine sediment bacteria., *Appl. Environ. Microbiol.* 1998; **64**: 2822-2830.
12. Ward, D. M., Weller, R., Bateson, M. M. 16s rRNA sequences reveal numerous uncultured microorganisms in a natural community., *Nature,* 1990; **345**: 63-65.
13. Zahner, H., Bachmann, E., Hutter, R., Nuesch, J. Sideramine, eisenhaltige Wachstumsfaktoren aus Mikroorganismen. *Pathol*. *Microbiol*. 1962; **25**: 708-736.