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Full Length Research Paper

Enhanced cultivability of antagonistic bacterial strains from soft coral *Sinularia* sp., Gulf of Mannar, Southeastern India

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Studies to increase the recoverability of antagonistic bacterial strains carried out from soft coral species *Sinularia* 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 ($2.1\pm0.1X10^4$), followed by Free Lunch Medium ($0.98\pm0.07X10^4$) and OLIGO medium ($1.61\pm0.4X10^4$) which were highly recovered in the supplemented media than in 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. Hence, the highest percentage of antagonistic bacteria was found to be exhibited by the supplemented media of MA+SE media, which are isolated from *Sinularia* sp. was (24.89%).

Key words: Improved recoverability, antagonistic, sodium pyruvate, *Sinularia* sp.

INTRODUCTION

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. Surface associated bacteria represent distinct populations of aquatic bacteria. Most studies addressing the cultureability of marine bacteria have focused on seawater populations, but it remains possible that surface associated bacteria have a greater capacity for growth on agar media than planktonic forms. The bacteria associated with macrophyte surface can be distinct from surface rounding seawater populations (Bolinches et al., 1988).

Numerous investigators have studied the interactions between corals and microbes (Paul et al., 1986; Chellaram and Patterson, 2009; 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. 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). Analyses of microbial communities have been hindered by our inability to cultivate most of the organisms within a sample. 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; 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

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Table 1. Media recipes.

Medium	Reference	Composition
50:50	This study	0.6 ml trace metal solution,[a] 0.6 ml PO_4^- solution,[b] 500 ml filtered seawater, 500 ml distilled water, 10 g agar.
Maltose Amended seawater (MsH_20)	This study	2.5 g maltose, 0.1.5 ml trace metal solution,[a] 1.5 ml PO_4^- solution,[b] 1 L filtered seawater, 10 g agar
Oligotrophic Media (OLIGO)	Santavy, 1995	0.5 g 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_4^- solution,[b] 1 L filtered seawater, 10g agar.
Free Lunch Medium (FLM)	Santavy, 1995	23.4 g NaCl, 0.75 g HCL, 7 g $MgSO_4$, 0.2 g $CaCL_2$ 0.015 g KH_2PO_4, 1 g mannitol, 1 g yeast extract, 1 g peptone, 1 ml trace metal solution,[a] 1 L distilled water, 10 g agar.
Marine Agar 2216 (MA)	Difco purchase	55.3 g Marine Agar 2216, 1 L distilled water.
Seawater + Soft coral Extract Media (sH_2O + SE)	This study	1.5 ml trace metal dolution,[a] PO_4^- solution,[b] , 1 L filtered seawater, 10 g agar, 5-10% filter sterilized soft coral extract prior to pouring plates.

[a] 2.86 g H_3BO_3, 1.81 g $MnCl_2$.$4H_2O$,1.36 g Fe EDTA, 0.08 g $CuSO_4$-$5H_2O$, 0.049 g $Co(NO_3)_2$-$6H_2O$. 0.39 g NaMoO-$2H_2O$, 0.22 g $ZnSO_4$-$7H_2O$, 1000 ml distilled H_2O. [b] 5.0 g NaH_2PO4-H_2O, 1000 ml distilled H_2O.

wide variety of environmental stresses and 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.

The observation that large percent (often >99%) of the bacteria in marine samples do not form colonies on the agar media, traditionally used for the isolation of marine bacteria (e.g. Bacto Marine Agar 2216. Difco Laboratories, Detroit) suggests that traditional media formulations may be inappropriate for the growth of most marine bacteria. Although no single medium can be expected to support the growth of all bacteria in a mixed population, it should be possible to improve our ability to culture marine bacteria by developing media formulations that better reflect the types and quantities of nutrients present in the environment sampled. 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 corals, *Sinularia* sp are found in abundance in Tuticorin coastal waters, Southeastern India. But works pertaining to the associated bacteria and their recovery from the 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.

MATERIALS AND METHODS

Sample collection

The bacteria associated with coral surface were collected by swabbing a small area (approximately 1 cm^2) of the external surface of soft coral, *Sinularia* sp (Coelenterata: Octocoralia: Alcyonacea) from Tuticorin coastal waters. Swabs were suspended in 99 ml autoclaved, filtered seawater (FSW) and vortex-mixed for 30 s. The buds were removed from the flask and the resulting bacterial suspension was vigorously hand shaken and serially diluted (10^2, 10^3 and 10^4).

Media and supplement used

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 micro-organisms (Table 1). All the media were prepared immediately prior to sample collection and stored in sealed sleeves in the dark to minimize photo-oxidation and free radical formation. 100 µl 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.

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

Different media	Control	Sodium pyruvate	Statistical significance	
	CFU (Mean of 3 values ± S. D)	CFU (Mean of 3 values ± S. D)	t value	p value
50:50	$0.5\pm0.05X10^4$	$1.9\pm0.2X10^4$	-11.762	0.000149**
50:50	$0.3\pm0.07X10^5$	$1.2\pm0.05X10^5$	-18.122	2.7265E-05**
50:50	$0\ X10^6$	$0.13\pm0.02X10^6$	-11.258	0.000177**
MsH_2O	$0.6\pm0.035\ X10^4$	$2.1\pm0.218X10^4$	-11.773	0.000149**
MsH_2O	$0.2\pm0.026X10^5$	$1.4\pm0.05X10^5$	-36.742	1.63799E-06**
MsH_2O	$0X10^6$	$0.3\pm0.066X10^6$	-7.856	0.000709**
OLIGO	$6.5\pm0.436\ X10^4$	$7.1\pm0.46X10^4$	-1.643	0.087847*
OLIGO	$3.1\pm0.17\ 3X10^5$	$3.9\pm0.13X10^5$	-6.358	0.001568**
OLIGO	$1.1\pm0.17X10^6$	$1.7\pm0.2X10^6$	-3.928	0.008566**
C-MIX	$4.1\pm0.26\ X10^4$	$6.15\pm0.23X10^4$	-10.145	0.000266**
C-MIX	$1.9\pm0.50X10^5$	$2.8\pm0.13X10^5$	-11.023	0.000193**
C-MIX	$0.4\pm0.55\ X10^6$	$1.1\pm05X10^6$	-16.202	4.24534E-5**
FLM	$3.1\pm0.095\ X10^4$	$5.1\pm0.13X10^4$	-21.240	1.453E-05**
FLM	$1.3\pm0.05X10^5$	$3\pm0.18X10^5$	-15.740	4.76E-05**
FLM	$0.35\pm0.03\ X10^6$	$1.0\pm0.05X10^6$	-19.310	2.1206E-05**
MA	$8.2\pm0.33X10^4$	$9.5\pm0.25X10^4$	-5.460	0.002733**
MA	$3.1\pm0.23X10^5$	$5.5\pm0.43X10^5$	-6.720	0.001279**
MA	$0.5\pm0.087\ X10^6$	$1.8\pm0.1X10^6$	-17.021	3.49E-05**
MA+SE	$8.3\pm0.458X10^4$	$9.9\pm0.09X10^4$	-5.942	0.0020**
MA+SE	$4.4\pm0.018X10^5$	$5.0\pm0.43X10^5$	-2.216	0.0455*
MA+SE	$1.3\pm0.132X10^6$	$2.1\pm0.1X10^6$	-8.356	0.000561**

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

20-25°C).

Bacterial counting

Colony forming units (CFUs) were counted using colony counter 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. Day 12 counts were not utilized as significantly more clumped was present, limiting the number of triplicate counts available. All recovered (amended and unamended) bacterial strains were screened for production of antibacterial substances, using double agar overlay method (Dopazo et al., 1988).

Antagonistic activity

Double agar overlay method was used for the assay of isolated bacterial strains against the 3 human pathogens [*Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus aureus* (ATCC 29737) and *Shigella dysentriae* (ATCC 13313)] and 3 fish pathogens [*Proteus mirabilis* (MTCC 1429), *Serratia marcescens* (MTCC 97) and *Aeromonas hydrophila* (ATCC 7966)]. The test strains were obtained from Vellore Christian Medical College (CMC). 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 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 cultureable bacteria per 100 µl obtained on low to high nutrient of the control and supplemented media isolated from *Sinularia* sp are presented in Table 2. The CFU counts for each medium used with the corresponding differences from control values for coral are given in Figure 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 in both samples. The Oligotrophic agar ($6.5\pm0.436\ X10^4$), C-mix medium ($4.1\pm0.26\ X10^4$), Marine agar ($8.2\pm0.33\ X10^4$) and MA+SE agar ($8.3\pm\ 0.548X10^4$) showed moderate CFU counts, whereas sodium pyruvate added media showed higher CFU counts of $7.1\pm0.46X10^4$, $6.15\pm0.23X10^4$, $9.5\pm0.25X10^4$ and $9.9\pm0.09X10^4$ respectively as against controls. It was observed in the present study that there was a significant difference between the supplemented and unsupplemented media in microbial colony recoverability.

The addition of sodium pyruvate increased counts in all the media used in the present study. Among sodium pyruvate supplemented isolates from soft coral, C-MIX and Marine agar were able to recover the maximum numbers of the representatives ($6.15\pm0.23X10^4$ and

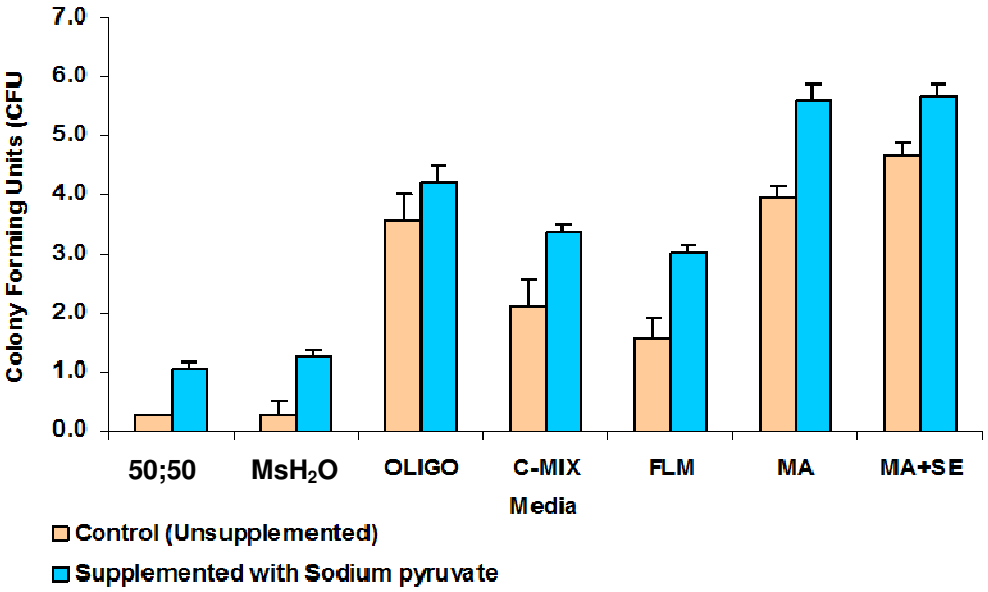

Figure 1. Microbial recoverability from *Sinularia* sp., using different media combination and supplementation.

9.5±0.25X10^4 respectively) in comparison with control media. The control plates of 50:50 (10^5) media had no colonies, however, some microbial recoverability was observed on the supplemented medium of the same media. It was noted that there was a highly significant difference between the control and the supplemented media combination used in recovering the microbial strains as evidenced by t and p values.

All media supplemented with sodium pyruvate has highest percentage of antagonistic activity against 3 human and 3 fish pathogens than unsupplemented media (Tables 3). The highest percentage of antibiotic producing strains was isolated from *Sinularia* sp. of supplemented medium (MA+SE, 24.89%). Among the 7 media, sodium pyruvate amended media of the MA+SE, MA and C-MIX recovered the highest percentage of potent producer strains than unsupplemented media as given in Figure 2.

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 sodium 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 in various non-optimal media permitted the increased enumeration of injured and uninjured *S. aureus* cells, often to levels above those obtained using unsupplemented 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; Chang et al., 1993).

In the present study, sodium pyruvate supplemented MA+SE medium was found to have the highest bacterial representatives. Bacterial strains isolated from *Sinularia* sp, using the sodium pyruvate supplemented MA+SE medium have the highest CFU counts, 9.9±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 (Martin et al., 1976). Chang et al. (1993) also established the positive effect of sodium pyruvate on seawater-injured *E. coli* when it was added as a supplement to nutrient agar, a solid medium used for cultivating a wide variety of microorganisms and containing peptone and beef extract. Calabrese and Bissnnette (1990) and Olson et al. (2000) demonstrated that sodium pyruvate additions as well as combinations of Sodium pyruvate and catalase were 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 research 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

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

Different media	Control			Sodium pyruvate		
	Total strains	Producer strains	%	Total strains	Producer strains	%
50:50	50	10		190	34	
50:50	30	4	17.5	120	21	18.57
50:50	0	0		13	5	
MsH_2O	89	15		210	38	
MsH_2O	21	4	17.27	140	29	19.47
MsH_2O	0	0		30	7	
OLIGO	650	93		710	141	
OLIGO	310	61	17.28	390	60	18.36
OLIGO	110	31		170	36	
C-MIX	410	62		615	86	
C-MIX	190	34	16.70	280	64	18.7
C-MIX	40	11		110	38	
FLM	310	50		510	84	
FLM	130	29	18.30	300	55	19.1
FLM	35	8		100	34	
MA	820	152		930	177	
MA	310	55	18.47	435	73	19.47
MA	50	11		121	39	
MA+SE	830	174		965	223	
MA+SE	440	69	19.2	460	121	24.89
MA+SE	130	26		190	58	

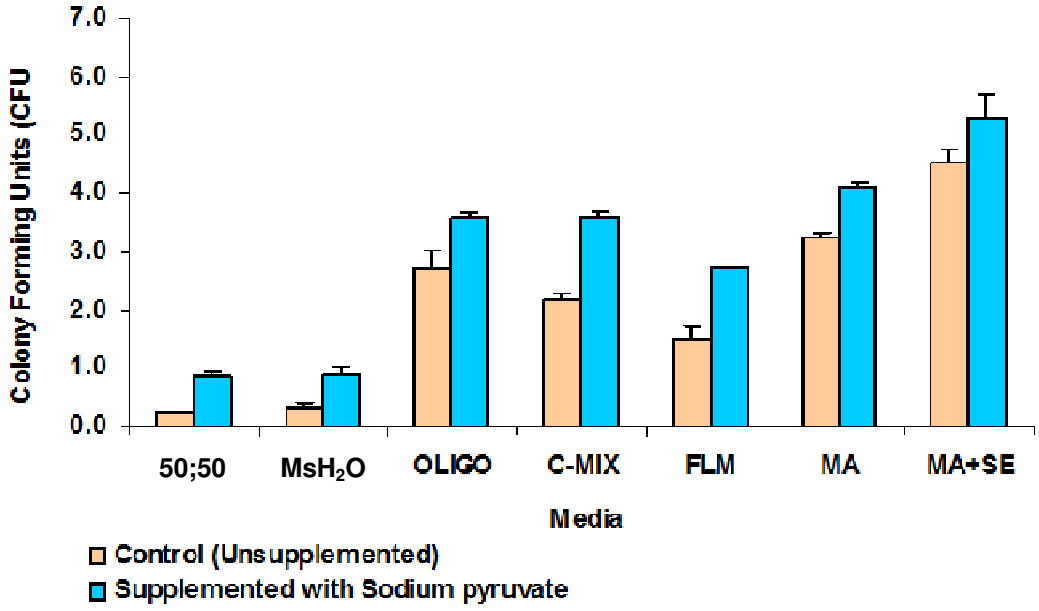

Figure 2. Antagonistic activity of bacterial isolates of *Sinuaria* sp., using different media combination and supplementation.

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. They also observed that microbes are homogeneously distributed in sea water; however there is a changing perception that microbes are distributed heterogeneously. Bacterial species richness is also variable at the millimeter scale and the variability increases in response to increase in the concentration of particulate organic matter in sea water.

The antagonistic bacteria isolated from surface of the soft coral, *Sinularia* 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 gorgonian is to inhibit both human and fish pathogens. In this study, the highest percentage (24.8%) of antibiotic producing strains was isolated from *Sinularia* sp. with supplemented medium of MA+SE. A better recovery and antagonistic results of bacterial strains from the surface of these soft coral suggests that these organisms represented as ecological niche, which harbors largely uncharacterized organisms attached to the surface may yield a vast array of new compounds with novel activity.

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