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Improved Recoverability of Microbial Colonies from Marine Sponge Samples

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ABSTRACT

The growth of microorganisms from marine sponge samples was studied on various low to high nutrient solid media using media supplements. The supplements utilized were catalase, sodium pyruvate, and a combination of the two. Medium composition was found to influence the growth response on the supplemented media. Microorganisms on low nutrient media responded more favorably to the media additions than on high nutrient media. Thirty-five percent of the supplemented media demonstrated colony forming unit (CFU) recoveries that were 50% or greater than those of the unamended control plates. Twenty-one percent showed recoveries of more than 100% of the control values, with sodium pyruvate additions providing for the greatest overall increase in recovery, whether alone or in conjunction with catalase. These findings suggest that addition of catalase or sodium pyruvate to solid growth and isolation media may improve recoverability of microorganisms from natural samples.

Introduction

Analyses of microbial communities have been hindered by our inability to cultivate most of the organisms within a sample. Estimates of bacterial recoverability from environmental samples range from 0.01% to 12.5% of the existing community [1, 25, 28]. Some environmental recoverability estimates are derived from comparisons of direct counts of bacteria (using stains such as acridine orange or DAPI) and counts of cultivated colonies (e.g., [6, 12]). Unfortunately, this method of approximating the number of bacteria in a given sample is not effective for examining microbial communities associated with a living host. The stains detect nucleic acids and do not differentiate between those of the bacteria and of the host organism [9]. This becomes especially problematic when potentially dealing with large numbers of intracellular bacteria (within host cells). Numerous methods have been employed in attempts to increase the

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number of bacteria retrievable from a sample, with varying but still minimal success. This growth restriction has severely limited our understanding of microbial diversity and function. Although a variety of molecular methods have been developed to assist in defining community composition, these techniques are not yet capable of providing the information that can be obtained from examinations of pure cultures. Information pertaining to growth preferences, nutrient utilization, and biochemical characterization can best be gained from isolation and study of individual species.

A number of studies have investigated the effects of various environmental parameters (e.g., starvation, heating, freezing, chlorine, pH) on the recoverability of microorganisms. Stresses such as these often render a portion of the surviving cells sublethally injured or debilitated. 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 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 samples is likely reduced as a result of potential stress and injury. In an effort to minimize injury and stress and thus improve detection of these microorganisms, workers have advocated the exogenous addition of various supplements, most often catalase or sodium pyruvate, to culture media. Most of these studies focused on the recovery of specific organisms rather than improved overall recovery from an environmental sample. Microorganisms examined include: *Escherichia coli* as an enteric indicator species [4]; numerous microaerophilic organisms such as *Camplyobacter fetus* [10], *Treponema pallidum* [26], *Clostridium perfringens* [7, 8], and *Spirillum volutans* [19]; and *Beggiatoa leptomitiformis* [3].

Increases in the aerotolerance of various microaerophiles were noted with the addition of certain supplements to liquid or solid media, with the common link between the supplements being their ability to quench toxic forms of oxygen [14]. The most commonly utilized and successful supplements include catalase, sodium pyruvate, and superoxide dismutase. These agents act to destroy free radicals and peroxide in the media, thereby allowing the organisms to grow at higher O_2 tensions. Martin et al. [16] demonstrated that the presence of either catalase or sodium pyruvate in various nonoptimal media permitted the increased enumeration of injured and uninjured *Staphylococcus aureus* cells, often to levels above those obtained using the unsupplemented, recommended growth medium. For *Beggiatoa* spp. cultures, the addition of catalase to culture medium increased the period of viability from 1 week to 2 months [3]. If, as McLeod and Gordon [18] hypothesized, one of the targets of sublethal injury to stressed cells is catalase, then the exogenous addition of catalase or another oxygen radical scavenger into the culture medium may prevent the accumulation of hydrogen peroxide (H_2O_2) during metabolism and permit the growth and replication of the organisms.

Oxygen toxicity of culture media resulting from photooxidation due to illumination has been found to inhibit the growth of even aerobic or facultative organisms [11]. Harmon and Kautter [7, 8] found that aerobic storage of media, especially those designed for the cultivation of anaerobes, caused formation of inhibitory levels of H_2O_2, but that the inhibition could be reversed by the addition of a H_2O_2-degrading agent prior to use. In addition to the toxic reactive oxygen species formed through oxidation processes, accumulation of H_2O_2 was found to be a universal phenomenon associated with cellular injury following sublethal stress [16]. Recovery of *E. coli* cells whose membranes were damaged by freezing or heating was enhanced by compounds that destroyed H_2O_2, including pyruvate and catalase [17]. Single-stranded breaks in bacterial DNA have also been attributed to high levels of H_2O_2 [27], suggesting that toxicity of reactive oxygen species, in particular H_2O_2, may have very serious detrimental effects on the physical and genetic stability and recoverability of many microorganisms.

Since it is thought that the culturability of naturally occurring microbial communities is negatively affected by the stresses associated with removing the sample from its natural environment and plating onto solid culture media, the objective of this study was to determine if recovery of the natural microbial community could be increased by the use of medium supplements. We demonstrate that enhanced recoverability was noted with the addition of catalase, sodium pyruvate, or a combination of the two to the growth media utilized for the cultivation of marine microorganisms associated with a deep-water sponge. Significant differences in colony forming unit (CFU) counts were noted between control (unamended) media and supplemented media. Superoxide dismutase additions were also examined in early studies but were discontinued when there were no significant differences in CFU counts between unamended and supplemented media (data not shown). Composition of the growth medium and its influence on the recovery of natural microbial communities were also examined.

Table 1. Medium recipes

Medium name	Reference	Formula
Marine Agar 2216 (**MA**)	Difco purchase	55.3 g Marine Agar 2216, 1 L distilled water
Free Lunch (**FL**)	[5]	23.4 g NaCl, 0.75 g KCl, 7.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.015 g KH_2PO_4, 1.0 g mannitol, 1.0 g yeast extract, 1.0 g peptone, 1 ml trace metal sol'n,[a] 1 L distilled water, 10 g agar
Carbon Mix (**C-mix**)	This study	0.1 g maltose, 0.1 g mannitol, 0.1 g glucose, 0.1 g soluble starch, 0.1 g galactose, 0.1 g peptone, 0.1 g tryptone, 0.1 g yeast extract, 1.0 ml trace metal sol'n,[a] 1.0 ml PO_4^- sol'n,[b] 1 L filtered seawater, 10 g agar
Maltose amended seawater (**MsH_2O**)	This study	2.0 g maltose, 1.0 ml trace metal sol'n,[a] 1.0 ml PO_4^- sol'n,[b] 1 L filtered seawater, 10 g agar
Oligotrophic Media (**OLIGO**)	[23]	0.5 g tryptone, 0.1 g sodium glycerophosphate, 0.05 g yeast extract, 1 L filtered seawater, 12 g Noble agar
Seawater + Sponge Extract Media (**sH_2O + SE**)	HBOI use	1.0 ml trace metal sol'n,[a] 1.0 ml PO_4^- sol'n,[b] 1 L filtered seawater, 10 g agar, 3–4% filter sterilized sponge extract prior to pouring plates
60:40	This study	0.6 ml trace metal sol'n,[a] 0.6 ml PO_4^- sol'n,[b] 600 ml filtered seawater, 400 ml distilled water, 10 g agar
Gram Positive Media (**GP**)	Modified from [24]	10.0 g tryptose, 5.0 g NaCl, 3.0 g beef extract, 2.5 ml phenylethanol, 1 L distilled water, 15.0 g agar

[a] 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 1.36 g FeEDTA, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.049 g $Co(NO_3)_2 \cdot 6H_2O$, 0.39 g $NaMoO_4 \cdot 2H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 1000 ml distilled H_2O

[b] 5.0 g $NaH_2PO_4 \cdot H_2O$, 1000 ml distilled H_2O

Methods

Sample Acquisition

Samples of two undescribed sponge species of the genus *Discodermia* were obtained from various sites in the Bahamas at depths exceeding 400 feet using HBOI's Johnson-Sea-Link I submersible from November 11 to November 25, 1998. Sponges were collected by suction and placed into Plexiglas buckets mounted on the submersible work platform. Sponges were aseptically removed from the buckets on return to the surface and small portions were subsampled for analysis. Collection sites were Cat Island (experiment 1), Eleuthera (expt. 2), Egg Island (expt. 3), and Black Rock (expt. 4), Bahamas. The sponge samples were identified through examination of spicule preparations and morphological features prior to experimentation.

Media and Growth Conditions

See Table 1 for a list of the media used for cultivation of marine microorganisms. A combination of low to high nutrient media and the various media additions shown in Table 2 were employed in an attempt to recover the largest number of microorganisms. All media were prepared immediately prior to the expedition and stored in sealed sleeves in the dark to minimize photo-oxidation and free radical formation. Inoculated plates were stored inverted in sealed sleeves in the dark at room temperature (approx. 20–25°C). Plates were removed and colony forming units (CFUs) counted using a Gallenkamp colony counter (Leicester, UK) on days 5, 10, 15, and 20 following inoculation. Day 15 counts were used for all analyses as they yielded the most representative counts for all experiments. Day 20 counts were not utilized as significantly more fungal contamination was present, limiting the number of replicate counts available.

Growth Studies (Experiments 1 and 2)

Small sections of the sponges were gently rinsed in sterile natural seawater, cut into smaller pieces, and homogenized at low speed with an ethanol sterilized VirTis high-speed homogenizer (VirTis Co., Inc., Gardiner, NY). Resulting supernatants were serially diluted in sterile natural seawater. Control (unsupplemented), catalase, sodium pyruvate (SP), and SP + catalase supplemented plates were used for each medium tested. Triplicate plates of each treatment were inoculated for two dilutions per experiment with 50 µl of the selected sponge supernatant dilutions using the spread plate technique. All plates were allowed to dry overnight in the dark prior to placing into sleeves for storage.

Sponge Extract Addition Studies (Experiments 3 and 4)

Small sections of the sponges were gently rinsed in sterile seawater and flushed repeatedly with sterile seawater using a tuberculin syringe. Host extract used as a media supplement was prepared by homogenizing pieces of the host sponge, prefiltering the suspension through a glass fiber filter, and filter sterilizing the extract using a 0.2 µm filter and sterile collection bottle. Final media concentra-

Table 2. Media additions

Addition	Reference	Concentration
Catalase (**Cat**)	[4, 19]	100 units/ml filter sterilized and added to molten media (45°C) following autoclaving
Sodium pyruvate (**SP**)	[4, 16]	1% wt/vol added to media prior to autoclaving
Sponge extract (**SE**)	HBOI use	3–4% vol/vol filter sterilized and added to molten media (45°C) OR 50 µl of extract prepared on site from exact host and plated with inoculum
Combinations of above	This study	Maintain suggested conc. for each addition

tions were typically 3–4% (v/v). Sponge-specific host extract was prepared in the field by removing and homogenizing a small piece of the host sponge. The resulting suspension was prefiltered using a 0.45 µm syringe-tip filter followed by filter sterilization with a 0.2 µm syringe-tip filter. Each plate for the host extract amended experiments received 50 µl of the sponge extract concurrently with 50 µl of inoculum. Again, control, catalase, SP, and SP + catalase supplemented plates were used for each medium tested. Two plates were inoculated per treatment for two dilutions.

Statistical Analyses

CFU counts from replicate plates (triplicate or duplicate as noted for the dilution that provided between 30 and 300 colonies per plate) were analyzed using analysis of variance (ANOVA). In experiments 1 and 2, the main effects were medium and presence or absence of each supplement; in experiments 3 and 4, the main effects were medium, presence or absence of each supplement, and the presence or absence of host extract. Because of low sample sizes, only interactions between supplements were considered. Significant ANOVAs ($p < 0.05$) were followed by means comparisons to identify which conditions produced higher recoverability. Means comparisons were carried out using the Ryan–Einot–Gabriel–Welsch multiple range test [22], which controls the experiment-wise error rate ($\alpha = 0.05$) rather than the per-comparison error rate. The SAS general linear model (GLM) procedure [22] was used for all statistics.

Results

Growth Studies Experiments 1 and 2

Experiments 1 and 2 tested the effect of media additions on recoverability of microbial colonies from various low to high nutrient media. The CFU counts for each medium used in experiments 1 and 2 are shown in Fig. 1A and 1C, respectively, with the corresponding percent differences from control values shown in Figs. 1B and 1D. In both experiments, there was a significant effect of the conditions for microbial recoverability.

The media used had a significant effect in both experiments, although the supplements used significantly affected recoverability only in experiment 1 (see Table 3). For experiment 1, means comparison showed no obvious pattern of significant differences in CFUs in the combinations of media and supplement. Experiment 2 means comparisons showed no obvious pattern of significance for media. In both experiments, the 60:40 medium supplemented with SP and catalase showed higher CFU counts. There was a trend for FL medium (experiment 2) and catalase-supplemented Gram positive selective medium (GP; experiment 1) to produce lower recoverability, and for SP supplemented media to produce higher CFU counts in experiment 1. The addition of catalase decreased counts in most media in experiment 1, although means comparisons indicated this was significant only for GP (data not shown). Adding both catalase and SP tended to recover counts to near SP-only levels, except in GP medium.

Of the media tested, GP yielded the smallest colonies based on visual examination. This presumptive Gram-positive selective medium also demonstrated less variation in colonial morphology than the other media studied, likely indicative of a smaller community of Gram-positive microorganisms than Gram-negative microbes associated with marine deep-water sponges. Gram stains of crude *Discodermia* spp. sponge suspensions have shown that the associated microbial community largely consists of Gram negative species (~90%; unpublished results). All growth on plates was counted, including initial fungal growth. Counts were discontinued after the initial presence of fungi was observed as plates were quickly overrun by sporulating fungi. Extremely variable cell recoveries were noted for the individual experiments. Actual calculations of cultivable cells/ml inoculum are shown in Table 4 for each experiment.

Host Extract Growth Experiments: Experiments 3 and 4

Experiments 3 and 4 tested not only the effect of various media and medium additions on microbial recoverability, but also the effect of adding host extract to the plates. The

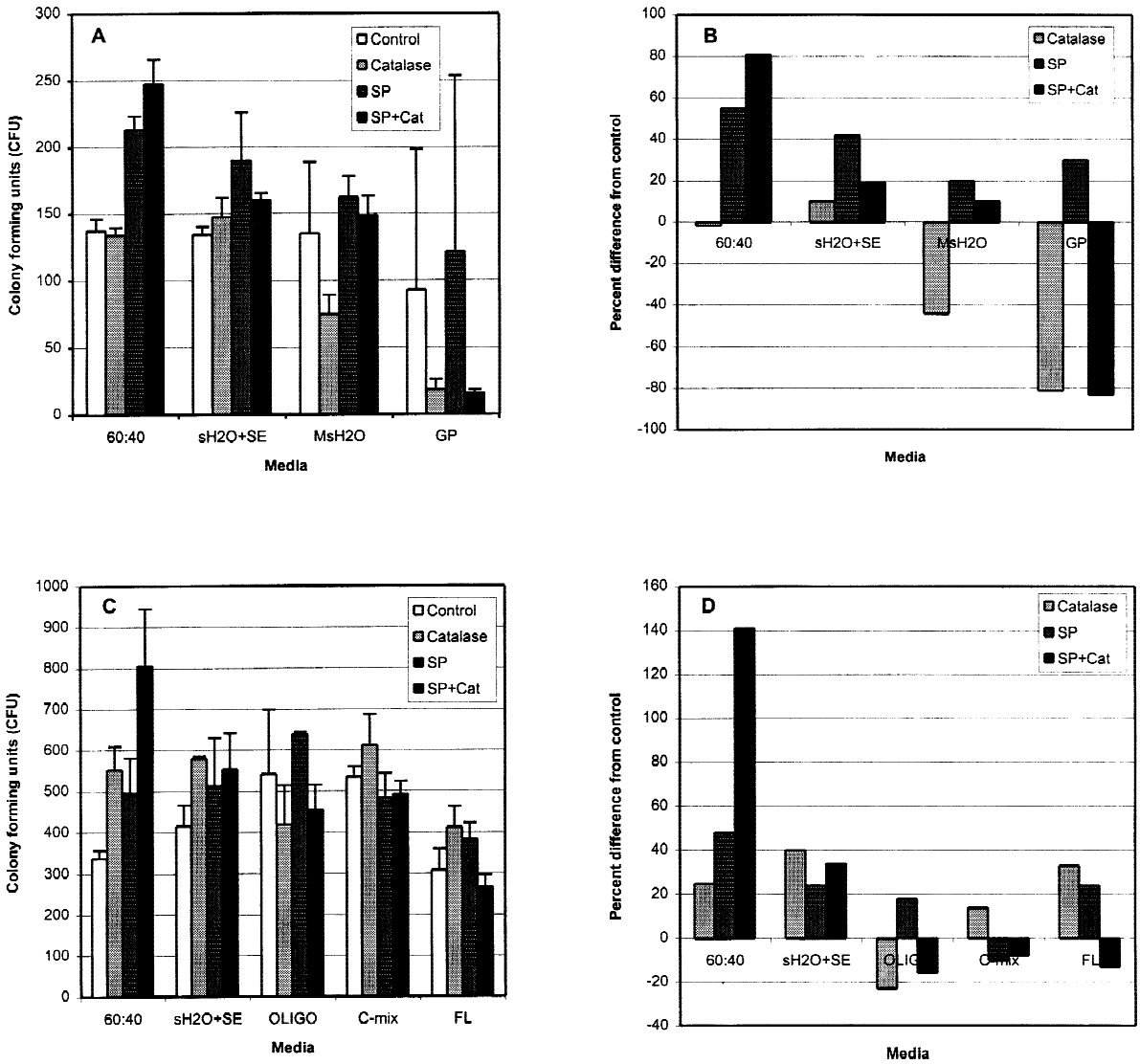

Fig. 1. Microbial recoverability from deep-water marine sponge samples using various media and media additions (Cat = catalase, SP = sodium pyruvate). (A and B) Experiment 1 data; (C and D) experiment 2 data. All CFU counts are from 15 days after inoculation. A and C show means from triplicate plates with the corresponding standard deviation. B and D are average percent differences from control plate counts, including negative differences. Note the variation in *y* axis values between all figures.

total CFU counts for experiment 3 are shown in Fig. 2A with the corresponding percent difference from control in Fig. 2B, followed by experiment 4 in the same format in Figs. 2C and 2D.

The first experiment adding host extract (expt. 3) at the time of inoculation showed a significant effect of media and supplement, but no effect of the addition of host extract (Table 3). The second experiment with host extract (expt. 4) showed no significant effects of any growth conditions (see Table 3 for ANOVA results). This is, in part, due to the low sample sizes (two replicates) and the overall low recoverability seen in this particular experiment.

As with experiments 1 and 2, there were no clear patterns in how the medium affected recoverability. In experiment 3, the 60:40 medium tended to produce lower counts than other media. This medium also demonstrated a trend for higher recoverabilities when host extract was used (especially when supplemented with SP); the nonsignificance of extract in the overall experiment may have been due to the lack of an effect in the other two media. Only the SP supplement had a significant effect on microbial recoverability in experiment 3.

For both experiments 3 and 4, control CFU counts were generally lower than those of supplemented plates. Note the

Table 3. ANOVA results

	Expt 1	Expt 2	Expt 3	Expt 4
F value	6.77	3.78	5.20	2.20
p value	0.0001	0.0022	0.0005	ns
Medium	0.0001	0.001	0.0002	ns
Extract	—	—	ns	ns
Catalase	ns	ns	ns	ns
SP	0.0062	ns	0.0173	0.0212
SP + Catalase	ns	ns	ns	ns

ns = nonsignificant, *p* value greater than 0.05

differences in the *y* axis values between experiments 3 and 4 (Fig. 2). The CFU control plate counts are an order of magnitude lower in experiment 4 than in experiment 3. The high variability between the duplicate plate counts potentially masked differences between the variables that might have otherwise been significant. In experiment 4, 60:40 medium yielded CFU counts for all additions, with and without host extract, that were at least 50% greater than CFU counts from control plates. SP additions provided the greatest recoverabilities on 60:40, the single low nutrient medium, while having dramatically less effect on the remaining higher nutrient media.

Discussion

The differences in collection site locations, environmental conditions, microbial associations of individual host sponges, and experimental treatments may explain the large variability noted in the recoverabilities of microorganisms from the host organisms. Direct counts of the sponge-associated microbial community were very difficult and highly unreliable as a result of the presence of intact sponge cells and cell debris. Because most counting methods rely on nucleic acid presence for cell detection, the large amount of eukaryotic DNA/RNA in the samples made this process unsuitable. Even with the variability in CFU counts, the trends remained relatively consistent for individual media throughout the four experiments. Post-hoc analysis (ANOVA with experiment as main effect; followed by means comparison) of the 60:40 medium indicated that the experiments were significantly different (data not shown). SP additions were found to promote the best recoverability on 60:40 media in all experiments, whereas catalase additions yielded mixed results, being nonsignificantly beneficial for recoverability in two experiments and slightly detrimental in the remaining two experiments.

The medium additions employed appeared to affect microbial recoverability differently according to the composition of the medium. Generally, low nutrient media were more positively affected by medium additions than were high nutrient media. This may suggest that the organisms recovered on low nutrient media were more affected by culturing stress than their high nutrient tolerant counterparts, or that they were utilizing the medium additions as supplemental carbon sources. Sodium pyruvate additions provided approximately 9 mg C/plate, whereas the C addition of catalase was negligible (<50 μg protein/plate). If the recovered organisms were using the medium additions as a C source, then the growth on high nutrient media (e.g., MA, C-mix, FL) should not be affected to the same extent as that on the low nutrient media. The results obtained suggest that these additions were not being utilized solely as a carbon source. Alternately, the organisms recovered from amended media may be more representative of the diversity of the microaerophilic community, as the O_2 concentration of waters of similar depths as the collection sites is lower than that of

Table 4. Total cultivable cells/ml inoculum on each medium

Media	Control	Catalase	SP	SP + Cat
Experiment 1				
60:40	2.74×10^4	2.68×10^4	4.26×10^4	4.96×10^4
sH_2O + SE	2.68×10^4	2.94×10^4	3.80×10^4	3.20×10^4
MsH_2O	2.70×10^4	1.50×10^4	3.24×10^4	2.98×10^4
GP	1.86×10^4	3.6×10^3	2.42×10^4	3.2×10^3
Experiment 2				
60:40	6.68×10^6	8.38×10^6	9.90×10^6	1.61×10^7
OLIGO	1.08×10^7	8.38×10^6	1.28×10^7	9.12×10^6
C-mix	1.07×10^7	1.22×10^7	9.68×10^6	9.84×10^6
sH_2O + SE	8.30×10^6	1.16×10^7	1.03×10^7	1.11×10^7
FL	6.12×10^6	8.22×10^6	7.66×10^6	5.34×10^6
Experiment 3				
60:40 C	1.22×10^4	1.16×10^4	2.50×10^4	2.02×10^4
60:40 C + SE	4.88×10^4	4.72×10^4	1.15×10^5	1.07×10^5
C-mix C	1.02×10^5	1.53×10^5	1.14×10^5	1.16×10^5
C-mix C + SE	5.00×10^4	8.14×10^4	7.68×10^4	1.37×10^5
MA C	5.66×10^4	4.80×10^4	6.40×10^4	8.32×10^4
MA C + SE	5.52×10^4	7.32×10^4	7.82×10^4	9.94×10^4
Experiment 4				
60:40 C	2.50×10^3	6.30×10^3	1.38×10^4	1.14×10^4
60:40 C + SE	3.60×10^3	7.10×10^3	1.19×10^4	6.10×10^3
C-mix C	8.40×10^3	1.14×10^4	8.00×10^3	9.10×10^3
C-mix C + SE	1.02×10^4	1.59×10^4	1.28×10^4	1.10×10^4
MA C	5.60×10^3	8.00×10^3	6.90×10^3	1.44×10^4
MA C + SE	6.00×10^3	7.70×10^3	1.99×10^4	8.90×10^3

All values are calculated from means. Triplicate plate counts were used for experiments 1 and 2 with duplicate plate counts for experiments 3 and 4. "C"; designates plates without host extract addition, "C + SE" indicates addition of 50 μl of host extract with the inoculum.

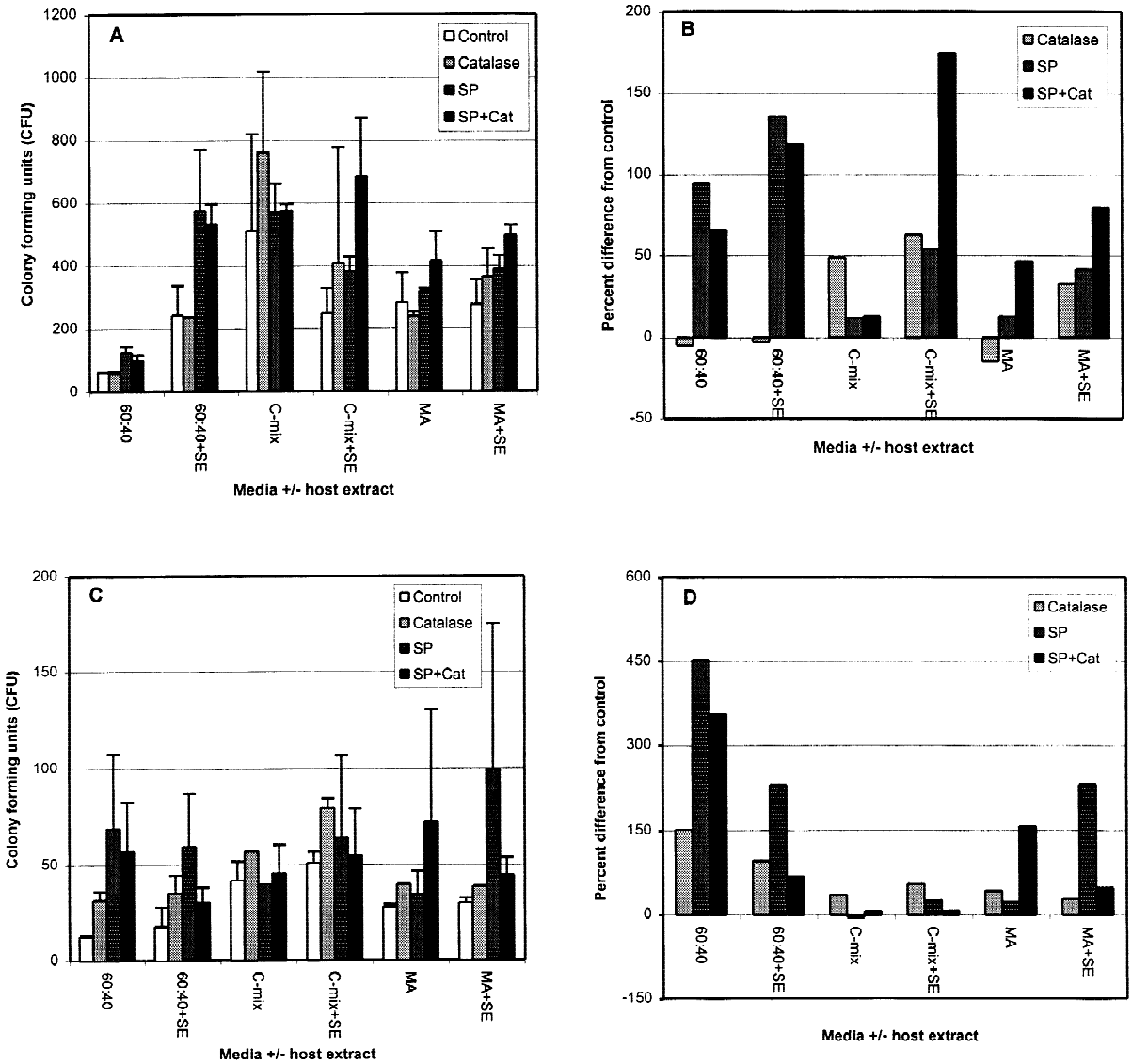

Fig. 2. Microbial recoverability using host-extract amended media and various medium additions (Cat = catalase, SP = sodium pyruvate) with deep-sea sponge samples. A and B are from experiment 3 data; C and D are from experiment 4 data. A and C show means from duplicate plates with the corresponding standard deviation. B and D are average percent differences from control plate counts, including negative differences. Note the variation in *y* axis values between all figures.

well-oxygenated surface waters, and these medium additions are known to increase aerotolerance [14]. Bacterial and yeast colonies were significantly larger on high nutrient media, while the corresponding colonial morphology visually appeared to be dramatically less diverse, especially on MA and FL. The identifications and genetic affiliations of the cultured organisms are still unknown at this time, and therefore, true microbial diversity comparisons of the recovered microorganisms on the various media cannot be made. Nonetheless, a recent study comparing partial 16S rRNA gene sequences from various microbial isolates and clones obtained from environmental DNA extractions of *Discodermia* spp. sponges demonstrated that a wide diversity of microorganisms were associated with the sponges [15].

Sodium pyruvate (SP) amended media appeared to provide the most consistently increased microbial recoveries for each sponge sample (there was a significant effect of SP addition in two of the four ANOVAs). SP additions increased recoveries over control plate counts for all media but C-mix, which demonstrated slightly reduced recoveries in two of the three experiments in which it was used. Catalase additions varied in their efficacy between samples, but never

showed a significant difference by ANOVA. Predominantly high nutrient media (>1 g C/L) appeared to be positively affected by catalase additions, whereas low nutrient media showed markedly less effect, with catalase amended plates often yielding fewer colonies than unamended control plates. Interestingly, the combination of SP and catalase additions yielded extremely varied results, following neither of the trends noted for individual additions and producing no additive effect of the individual treatments. None of the experiments showed a significant effect of the SP + catalase interaction, although the resulting CFU counts were extremely varied and may have masked trends in individual media.

Regardless of the medium supplement used, additions increased microbial recovery from deep-water marine environmental samples. Thirty-five percent of the amended media demonstrated CFU recoveries that were 50% or greater of those of the unamended controls. Twenty-one percent showed recoveries of over 100% of the control values, of which 10% were from catalase additions, 40% from SP additions, and 50% from SP + Cat additions. Over half of these increased recoveries were from inoculated 60:40 plates, suggesting that oxygen toxicity and stress may play a large role in regulating growth of species on low nutrient, oligotrophic media.

The addition of supplementary filter-sterilized host sponge extract to the various media tested whether sponge extract additions promoted or reduced microbial recoverability. It has been hypothesized that the host organism provides some essential nutrient to the associated microorganisms to promote the association and their growth [20, 29]. Alternately, some sponge-derived secondary metabolites have been shown to possess potent anti-microbial activity that may act to minimize microbial colonization and proliferation [2, 13, 21]. These experiments yielded variable results for the various media and for the different sponge samples. Host extract appeared to promote growth and recoverability for one sponge sample using 60:40 medium while demonstrating little effect on recoverability for the second sample, at least indicating that it was not detrimental to the growth of the culturable organisms within the associated community. There was no appreciable difference as a result of extract additions for C-mix and MA media. Until the isolates recovered from these studies are identified, the true effect of host extract addition on microbial recoverability and diversity cannot be determined, but initial results would suggest that an extract of the host sponge does not significantly affect the recoverability of the microbial associates.

This study was a preliminary survey of the potential effects of medium additions for enhancing microbial recoverability from deep-water marine sponges. Because of time, space, and the constraints of working aboard a research vessel, only a certain number of media, medium additions, and replicates could be tested. Attempts were made to maximize the impact of the experiments, but obviously future studies and additional replications are warranted. Continued experimentation will be comparable only in terms of significance noted for increased or decreased microbial recoverability for individual additions, as the environmental factors present at the collection sites will vary dramatically. Medium compositions and additions for future experimentation will be determined from the results obtained in this study.

Results from this study suggest that improved recovery of marine sponge-associated microorganisms can be achieved by using a wide variety of low to high nutrient media in conjunction with medium additions that increase resistance to harmful reactive oxygen species. Microorganisms from environmental samples are exposed to significant environmental changes and stresses in the transition from natural environment to laboratory media and may become sublethally injured or debilitated. Calabrese and Bissonnette [4] demonstrated that sodium pyruvate additions, as well as combinations of SP and catalase, were effective in recovering sublethally injured cells and increasing the detection of total heterotrophic bacteria from acid mine water. Their study and our results indicate that the application of different approaches to the cultivation of microorganisms from environmental samples increases the percentage of microbes recoverable from the sample. Continued research into preferred growth conditions and methods to alleviate stress associated with laboratory cultivation will, it is hoped, allow scientists to culture more of the microbial community in future studies, thereby gaining a better understanding of the true breadth of microbial diversity.

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