The Utilization of Rum Slops by Marine Bacteria. I. Isolation of Efficient Strains[1]

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ABSTRACT

Bacterial strains have been isolated from selected marine communities, and their ability to utilize and/or detoxify the crude slops has been assessed. These bacterial strains were isolated from 1) marine mud, 2) decaying sea grass, *Thalassia testudinum*, 3) the surfaces of the roots of the mangrove *Rhizophora mangle* and 4) gravel sediment. The isolates were grown on solid modified sea water media containing slops.

A total of twenty isolates were obtained from the slops media. Colony shape and color of these strains were determined. The slops isolates were grown in liquid culture media in the presence of slops. Eight of the original isolates grew in this media, showing little tendency to form aggregates. The growth of these strains was examined in the presence of variable concentrations of the slops. The results of this study indicate that the growth inhibitors in the slops can be partially detoxified by these bacterial strains, and the remaining material utilized for population growth.

INTRODUCTION

Marine bacteria are a basic and elemental part of the food chain that fosters the production of marine organisms. However, it does appear on the basis of previous work[3] that the crude slops are toxic in general to marine microorganisms. Thus the discharge of this material in crude form in the ocean might not be considered an efficient means of disposal. The object of this work was to examine a number of bacterial strains obtained from selected marine communities with respect to their ability to utilize and/or detoxify the crude slops. Such a preparation of marine micro-organisms might then be employed to convert the slops into proteinaceous material for use as food or in fact render it harmless by detoxification, thus alleviating some of the problems regarding its disposal.

PROCEDURE

PREPARATION OF SLOPS MEDIA

Crude slops were centrifuged (27,000 × g, 4° C, 1 h) and then filtered through millipore filters (14- and 7-micron pore size). The resulting solution was then autoclaved, and when it had cooled to room temperature (22° to 24° C) the density of the solution was determined. This slops

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[3] Tosteson, T. R. et al., Effect of the mosto on the growth of marine micro-organisms, Rum Pilot Plant, Agr. Exp. Stn. Univ. P.R., Report RPP 1-73, October 1973.

stock solution was then diluted with millipore filtered sea water to concentrations ranging from 0.5 to 10 mg/ml. The solid growth media employed in these studies was made by the addition of agar (1.25%) to a slops solution of a concentration of 1 mg/ml.

Crude clarified slops solution was dialyzed against distilled water. The dialyzed preparation was then lyophilized and the resulting powder dissolved in filtered sea water at a concentration of 1 mg/ml. The preparation was converted into a solid growth media by the addition of agar as described above.

PREPARATION OF BACTERIAL SAMPLES

The bacteria employed in these studies were isolated from four sources: 1) marine mud; 2) decaying sea grass, *Thalassia testudinum*; 3) the surfaces of the roots of the mangrove, *Rhizophora mangle*; and 4) gravel sediment. Samples of *T. testudinum* and mangrove roots were brought to the laboratory and carefully rinsed with sterile sea water. A sterile wire loop was rubbed on the surfaces of the samples and the resulting material carefully streaked on the agar plates containing the solid slops growth media. In the case of the mangrove samples, scrapings were made from segments of roots constantly immersed in sea water and colonized by organisms, as well as from segments of the root within the splash zone, without obvious colonization by macro-organisms.

The samples of gravel sediment and marine mud were added to sterile sea water and the mixture agitated. Aliquots of these mixtures were streaked on the solid slops growth media as described above.

During the course of the procedure carried out to isolate bacterial strains that grow on the solid slops media, duplicate samples of the material obtained from the sea grass *T. testudinum*, the mangrove roots and the marine sediments were plated on a nutrient agar media that did not contain the slops. All of the agar plates prepared in the manner described in this section of the Report were incubated at laboratory temperature (22° to 24° C). After 24 to 28 h of growth, those colonies that exhibited the most prolific growth in the slops media were replated on similar media. Successive replating and consequent purification of these colonies were carried out, resulting in the purified colony, the morphology and appearance of which were noted.

The purified isolates from both the dialyzed and crude slops media were inoculated into 20 ml aliquots of sterile liquid slops media in Dulong shake flasks. These cultures were incubated overnight at a temperature of 26° to 29° C. The culture flasks were agitated during this time. Following this, the suspension was centrifuged (1,500 × g, 18° C, 10 min), the supernatant solution discarded and the cells resuspended in sterile sea water. The densities of suspensions were determined with a colorim-

eter (wave length, 660 nm). In the case of each inoculum, dilutions were made to bring these stock cultures of bacteria to a uniform optical density (0.39). From these stocks each suspension of the isolated bacterial strains was mixed with various concentrations of the sterile liquid slops media, to an initial optical density of 0.075. The final concentration of slops employed in these suspensions ranged from 0.4 to 8.08 mg/ml. In general each of the eight selected strains isolated from the solid slops media was subsequently tested at seven different concentrations of liquid slops media. Control suspensions of the slops isolated bacteria were incubated in sterile sea water, in parallel with the slops media experiments.

The suspensions were incubated as described above. The growth of the bacterial populations in each case was assessed using the colorimeter. Two growth periods were followed in these experiments: a relatively short period of 3 to 7 h following the initiation of the incubation, and a period of 24 to 30 h of growth. Thus in the case of each suspension the optical density was determined after 3 to 7 h of growth and subsequently after 24 to 30 h of growth.

The results of these experiments are expressed in terms of the change in the optical density (OD) as a function of time, with respect to the short incubation period and the longer one. The efficiency of the use of the slops substrate is expressed in terms of the change in the OD divided by the particular concentration of slops originally present in that suspension at the initiation of its growth. The purpose of obtaining data for both the short and long periods of incubation was to determine when most of the growth in a given suspension took place.

RESULTS AND DISCUSSIONS

Twenty isolates were obtained from the selected source materials. All of those that were isolated onto crude slops agar were able to grow on the dialyzed slops agar, and vice versa. In general the best growth was always found with the crude slops agar. The solid media made from the slops was much more selective than the nutrient agar.

Strict quantification of the differences in the number and type of bacterial colonies growing on the nutrient agar as compared to that found on the slops agar was not attempted. Certain qualitative differences were, however, immediately obvious. No pigmented forms ever appeared on the slops plates, while these types of bacteria were rather common on the nutrient agar plates. Nutrient agar plates streaked with material from the sea grass *Thalassia* and those made from the upper segments of the mangrove root were well covered with pigmented and white colonies. Plates made of the material from the mangrove root segment that was constantly immersed in the sea, and well colonized by macro-organisms, showed no pigmented forms. In all cases, the corresponding slops plates

TABLE 1.—*Colony description of the 20 bacterial strains isolated on slops media*

Strain code name	Source	Colony color	Colony shape	Cell aggregation
CRRT 1	Thalassia	Cream	Large, round, darkened center, irregular edges	+
CRRT 2	Thalassia	White, sheen	Small, round	0
CRRT 3	Thalassia	White	Small, swirls	0
CRRT 4	Thalassia	Cream	Small, round, darkened center, irregular edges	+
CRMR 1	Mangrove immersed	White, opaque	Large, amorphous	0
CRMRS 1	Mangrove exposed	White, translucent	Round, irregular edges	+
CRMRS 2	Mangrove exposed	White	Streaks	0
CRMRS 3	Mangrove exposed	Off-white, yellow tinge	Round, irregular edges	+
CRSED 1	Gravel sediment	White to translucent	Elongate, irregular edges	+
DIALRT 1	Thalassia	White, translucent	Swirls, wisps	+
DIALRT 2	Thalassia	White, opaque	Round	0
DIALMR 1	Mangrove immersed	White, sheen	Wispy	0
DIALMRS 1	Mangrove exposed	White, sheen	Round, irregular edges	+
DIALMRS 2	Mangrove exposed	White	Round	+
DIALSED 1	Gravel sediment	White, sheen	Round, irregular edges	+
CRMUD 1	Marine Mud	White, translucent	Round, small	0
CRMUD 2	Marine Mud	White	Round, large	0
CRMUD 3	Marine Mud	Clear, sheen	Round, large	+
DIALMUD 1[1]	Marine Mud	White	Round, irregular edges	0
DIALMUD 2[1]	Marine Mud	White	Round, thin	0

[1] Test not completed.

TABLE 2.—*Effect of the slops concentration in the growth of selected bacterial strains*

Crude slops concentration	ΔOD—hours								
	3.0	3.5	5.0	6.5	23	24	25	27	SUE*
Mg/ml									
						CRRT 2			
0.00				−0.042				−0.05	
0.808				+0.264				+0.293	0.36
1.62				+0.505				+0.495	0.306
4.04				+0.638				+1.878	0.465
6.06				−0.02				+0.15	
8.08				0.00				0.00	
						CRRT 3			
0.00	0.00						−0.018		
0.404	+0.08						+0.07		0.173
0.808	+0.147						+0.176		0.217
1.62	+0.220						+0.550		0.339
4.04	−0.02						0.00		
6.06	−0.033						+0.018		
8.08	+0.01						+0.04		
						CRMR 1			
0.00			0.00					−0.016	
0.404			+0.138					−0.156	0.386
0.808			+0.246					−0.274	0.339
1.62			+0.308					−0.497	0.307
4.04			0.00					−0.05	
6.06			0.00					−0.04	
8.08			0.00					−0.03	

				CRMRS 2				
0.00	0.00				−0.023			
0.404	+0.087				+0.101		0.25	
0.808	+0.146				+0.240		0.297	
1.62	+0.208				+0.583		0.360	
4.04	0.00				0.00			
6.06	0.00				−0.025			
8.08	0.00				−0.02			
				DIALRT 2				
0.00			−0.01	−0.019				
0.404			+0.12	+0.107			0.265	
0.808			+0.257	+0.267			0.330	
1.62			+0.533	+0.593			0.366	
4.04			−0.006	+0.015				
6.06			+0.015	+0.01				
8.08			+0.02	+0.01				
				DIALMR 1				
0.00		−0.012		−0.014				
0.404		+0.082		+0.089			0.220	
0.808		+0.113		+0.272			0.336	
1.62		+0.154		+0.61			0.376	
4.04		+0.04		+0.99			0.245	
6.06		+0.01		+0.01				
8.08		0.00		0.00				
				CRMUD 1				
0.00			−0.031		−0.036			
0.404			+0.145		+0.097	0.799	0.240	
0.808			+0.324		+0.287	0.401	0.355	
1.62			+0.462		+0.682	0.285	0.421	
4.04			+0.015		0.000			
6.06			−0.020		+0.01			
8.08			0.000		0.000			

TABLE 2—*Continued*

Crude slops concentration	ΔOD—hours									
	3.0	3.5	5.0	6.5	23	24	25	27	SUE*	
						CRMUD 2				
0.00			−0.018			−0.036				
0.404			+0.088			+0.083			0.218	0.205
0.808			+0.202			+0.302			0.250	0.374
1.62			+0.320			+0.845			0.197	0.522
4.04			−0.020			+0.790				0.195
6.06			−0.010			+0.03				
8.08			0.000			0.000				

on which the selected materials were streaked, were more sparsely populated with non-pigmented forms, some of which showed good growth. Table 1 gives a listing and description of the colonies of 20 strains of bacteria isolated on the slops media.

Preliminary experimentation with the isolates revealed that when they were grown in liquid culture, in many cases the cells tended to form aggregates. The formation of clumps of cells in suspension greatly hinders the turbidimetric assessment of the growth of the bacterial population. Thus eight of the isolates, those that showed little or no tendency to aggregate during their growth in liquid culture, were selected for the experiments designed to assess the effect of the various concentrations of the slops on their growth in liquid slops. These eight strains are indicated in table 1. The growth of isolates that showed significant aggregation will be assessed by determining the total protein nitrogen at the beginning and end of their experimental growth periods.

Table 2 shows the results of the study of the growth of the selected eight strains in liquid media of varying slops concentration. The table gives the change in the optical density (Δ OD) for the short growth period (3 to 7 h) and that found over the total growth period (24 to 30 h). The substrate utilization efficiency (SUE) is equal to the Δ OD divided by the concentration of slops employed. This index has been calculated in each case based on the change in OD found in the longer growth period. In general, the substrate utilization efficiency (SUE) increased to an optimum with increasing slops concentration. Many of the cultures showed optimum growth and their highest SUE at a slops concentration of 1.62 mg/ml. The following tabulation summarizes the values of SUE for those strains showing an optimum value for this index at this concentration of slops.

Strain	*Optimum SUE*[4]
CRMUD 2	0.522
CRMUD 1	0.421
DIALMR 1	0.376
DIALRT 2	0.366
CRMRS 2	0.360
CRRT 3	0.339
CRMR 1	0.307

At concentrations of slops greater than that which gives maximum utilization (the highest value of SUE), growth tends to be inhibited. However, three of the isolated strains were able to grow at concentrations of slops that were as high as 4.04 mg/ml. One of these strains (CRRT 2)

[4] In each case above, the optimum SUE was determined at the end of the long incubation period for cultures growing on crude slops at a concentration of 1.62 mg/ml.

showed its best growth and highest efficiency at this concentration. The following tabulation summarizes utilization efficiency of those strains that grow at this concentration of slops (4.04 mg/ml). There was no significant growth found with any of the strains at slops concentrations of 6.06 mg/ml or higher.

Strain	*Optimum SUE*[5]
CRRT 2	*0.465*
DIALMR 1	*0.245*
CRMUD 2	*0.195*

Comparison of the amount of growth that took place in the short incubation period to that of the total growth period, at the various concentrations of slops tested, suggests that there are inhibitors in the slops medium that can at least be partially overcome by the bacteria if the concentration of the slops is not too high, and a sufficient incubation period is employed. The optimal SUE concentration represents the threshold concentration of inhibitory factors. At this point, the concentration of utilizable components is as high as it can be, with the inhibitors not quite in sufficient concentration to affect the growth. At concentrations below the SUE optimum, growth is limited by the amount of substrate available. It is of interest to note that at high concentrations of the slops the inhibitory factors are generally not bacteriolytic, but bacteriostatic. This is evidenced by the fact that at concentrations of 6.06 mg/ml and higher there is not a significant decrease in the OD.

Strain CRRT 2 appears to be the most promising strain of marine bacteria tested so far, with respect to the utilization of the slops.

RESUMEN

Bacterias de comunidades marinas seleccionadas se aislaron de: a) lodo marino; b) la yerba de mar, *Thalassia testudinum* en estado de descomposición; c) las superficies de raíces del mangle *Rhizophora mangle*; y d) sedimento de grava.

Veinte bacterias aisladas se obtuvieron cuando estas cepas bacterianas se cultivaron en un medio de cultivo sólido que contenía agua de mar y mosto.

Ocho de las bacterias originalmente aisladas crecen en un medio de cultivo líquido que contiene mosto en concentraciones variables.

Los resultados de este trabajo indican que los inhibidores de crecimiento encontrados en el mosto pueden ser parcialmente detoxificados por estas cepas bacterianas, y el material restante puede ser utilizado para la multiplicación de poblaciones.

[5] In each case above, the optimum SUE was determined at the end of the long incubation period for cultures growing in crude slops at a concentration of 4.04 mg/ml.