In vitro inhibition of *Rhodotorula minuta* by a variant of the marine bacterium, *Pseudomonas piscicida*[1]

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KURZFASSUNG: **In-vitro-Inhibierung von *Rhodotorula minuta* durch eine Variante des marinen Bakteriums *Pseudomonas piscicida*.** Die Wirkung verschieden behandelter zellfreier Filtrate einer Variante des marinen Bakteriums *Pseudomonas piscicida*, die antibiotische Wirksamkeit gegen die im marinen Milieu vorkommende Hefe *Rhodotorula minuta* besitzt, wurde untersucht. Maximale Filtrat-Aktivität wurde von 4 Tage alten Bakterienkulturen (Pepton-Flüssigkeits-Schüttelkulturen) bei Verwendung von Hefezellen als Testorganismen erhalten, welche die logarithmische Wuchsphase noch nicht erreicht hatten. Die antibiotische Wirksamkeit der Filtrate ging bei pH 4 verloren. Höhere pH-Werte sowie Temperaturen bis zu 100° C hatten keine Wirkung auf die Aktivität des hindernden Prinzips. Bei Dialyse der Bakterienfiltrate gegen Peptonbouillon beziehungsweise gegen ein zellfreies Filtrat einer anderen Pseudomonaskultur zeigte sich, daß dialysiertes Material wie Dialysierrückstand aktiv waren. Verminderte Aktivität trat in definierten Medien auf. Wurde das Filtrat zwei „natürlichen" marinen Adsorbantien „carbonate rock leching" und getrockneten Amphipoden ausgesetzt, so war die antibiotische Aktivität reduziert. Es werden Beweise für die in-situ-Wirkung der Pseudomonaden-Antihefen-Aktivität auf die Biologie von Populationen Hefen und Bakterien in Mikrohabitaten vorgelegt.

INTRODUCTION

The occurrence of marine bacteria with antiyeast activity was reported initially from this laboratory in 1962 (BUCK, MEYERS & KAMP 1962), followed by more detailed studies (BUCK et al. 1963) of the inhibitory spectrum of one marine isolate of *Pseudomonas* characterized by noteworthy specific antiyeast properties. More recently (BUCK & MEYERS 1965), further data was presented on the general significance of this activity in marine environments, especially in regard to yeast-bacteria associations of possible relevance to yeast bionomics in estuarine habitats. In view of the significant inhibition of species of *Rhodotorula*, especially *R. minuta*, by the aforementioned

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pseudomonad, the *in vitro* effect of variously-treated cell-free bacterial filtrates were tested on this common, marine-occurring yeast.

METHODS AND MATERIALS

The bacterium used is a variant of *Pseudomonas piscicida* BEIN ATCC 15057 (BUCK, MEYERS & LEIFSON 1963) and is designated in this paper as AEB (amphipod extract bacterium). This variant differs almost solely by its unique antiyeast properties, for both organisms exhibit noteworthy ichthyotoxicity and have other physiological properties in common (BUCK & MEYERS 1966). AEB was isolated from an intertidal tube-dwelling amphipod (crustacean) community developing on wood panels in Biscayne Bay, Florida. The bacterium is maintained routinely in our laboratory at 20° C on an artifical sea water, designated ASW, (LYMAN & FLEMING 1940) agar medium containing 1.0 % Bacto-peptone. Unless designated otherwise, all growth media for the various assays were prepared with ASW. *Rhodotorula minuta* FY-75, a marine isolate, was obtained from the culture collection of the Department of Microbiology, University of Miami. This test yeast was maintained on an ASW agar medium of 1 % glucose and 0.1 % Bacto-yeast extract (G/Y). Transfers were made monthly and stored at 20° C.

For the assay tests, AEB was cultured at 25° C in 1 % peptone broth with various concentrations of the resultant filtrate subsequently tested for antiyeast activity. A maximal concentration of 20 % was used, based on the volume of the yeast growth medium. Cultures of the bacterium at 0, 1, 2, 4, 7, 10 and 21 days of growth were checked for possible relationship between age of cells and inhibitory properties. *R. minuta* cells at various stages of development similarly were exposed to AEB cultures.

Following centrifugation, subsequent filtrates of AEB were adjusted to pH 4.0, 6.0, 8.0 and 10.0, and assayed immediately as well as after storage at 4° C for 2 and 7 days at these pH values. The initial pH of the bacterial cultures was 8.0 to 8.2. Filtrates also were exposed for 30 minutes to temperatures of 3°, 20°, 30°, 45°, 60°, 80° and 100° C in water baths.

Determinations of antiyeast activity were made in broth cultures, with growth of the yeast measured turbidimetrically in specially prepared test vessels. AEB cells were removed from peptone cultures by centrifugation at 10,000 rpm. After appropriate treatment, described below, cell-free preparations, as well as all additions to flasks of *R. minuta*, were filter-sterilized through UF porosity sintered glass filters (Morton flasks).

Test culture vessels were fashioned by fusing 5" × 1/2" Pyrex culture tubes approximately 5/8" above the base of 125 ml Erlenmeyer flasks and tilted slightly downward. The yeast growth medium was G/Y broth, with 20 to 25 ml dispensed per flask and sterilized for 15 minutes at 121° C. The various filter-sterilized materials were added aseptically to the culture vessels which were placed on a reciprocating shaker (100 strokes/min) at 25° C. Periodically, the flasks were removed, the contents tipped into the side-arm and optical densities of yeast cultures measured with a Bausch and Lomb "Spectronic 20" colorimeter at 600 mμ.

Cytological examinations of *R. minuta* cells were made, including site, occurrence and frequency of budding as seen in wet mount preparations and general stainability of the cells from Gram stains and application of 1 % aqueous methylene blue. Chance nuclear stains (CHANCE 1952) were used for detection of chromatinic aberrations.

The dialysis characteristics of the active fraction(s) produced by AEB were examined. Initially, cell-free cultures were dialyzed against equal volumes of ASW, using cellulose tubing of 48 Å porosity. The dialysis test was run at 4° C for 30 hours, with both dialyzate (within the tube) and dialyzant (outside the tube) checked for activity. To provide approximate molecular equivalents both within and outside of the dialysis tube, the culture also was dialyzed against both 1 % peptone broth and cell-free material from a 48-hour culture of *P. piscicida* ATCC 15057. All fractions were assayed with suitable controls.

Filtrates from AEB cultures were exposed to several adsorbants, i. e., anion (Dowex 2-X8) and cation (Dowex 50W-X8) exchange resins, powdered cellulose (Whatman), powdered charcoal (Darco G-60; Atlas Powder Co.) and silica gel (100–200 mesh; Curtin and Co.). Several dried and powdered "natural" marine adsorbants, including algae *(Ulva* and *Centroceras),* amphipod animals and tubes, HCl leachings from high carbonate-containing marine coral rocks and $CaCO_3$ (Baker) also were tested for inhibition. The latter were used since both AEB and *P. piscicida* precipitate $CaCO_3$ in sea water media (GREENFIELD 1963) which might act as a natural adsorbant. Approximately 10 to 15 % (by weight) of adsorbant was added to 20 ml of cell-free peptone cultures. The adsorbant and broth were mixed and stored at 4° C for 24 hours, followed by centrifugation with subsequent filter sterilization of the filtrate. Twenty milliliters of natural sea water was added as an elutant to the same adsorbant, stored and centrifuged as above, and assayed. Several "extracts" from the amphipod community also were tested. These included "drippings" (as panels were removed from the water), "squeezings" (pressed exudate) and homogenates (from an amphipod slurry in sea water).

RESULTS AND DISCUSSION

The effects of various concentrations of culture filtrates of AEB on the growth of *R. minuta* are plotted in Figure 1. Below 10 % concentration of filtrate antiyeast activity was absent. A slight stimulatory effect may be observed in the peptone broth control. While 10 and 20 % filtrate inhibited yeast growth with equal effectiveness, other studies using *Cryptococcus neoformans* as a test yeast indicated that the higher concentration was required for inhibition. Based on these observations, a level of 20 % AEB filtrate was employed routinely in the present study. Since maximal activity was obtained from four-day AEB cultures, all assays were run with filtrates from 72-96-hour cultures. Those older or younger than 96 hours, especially cultures at 7 to 21 days, were less active. Nevertheless, activity was present in cultures as young as 24 hours.

Possible relationship between age of *R. minuta* cells and degree of inhibition was examined (Fig. 2). Cells taken prior to the logarithmic stage were more susceptible to AEB filtrates than those harvested later in the multiplication cycle. However, as noted by the low turbidimetric readings, all stages of cells tested exhibited poor growth. For

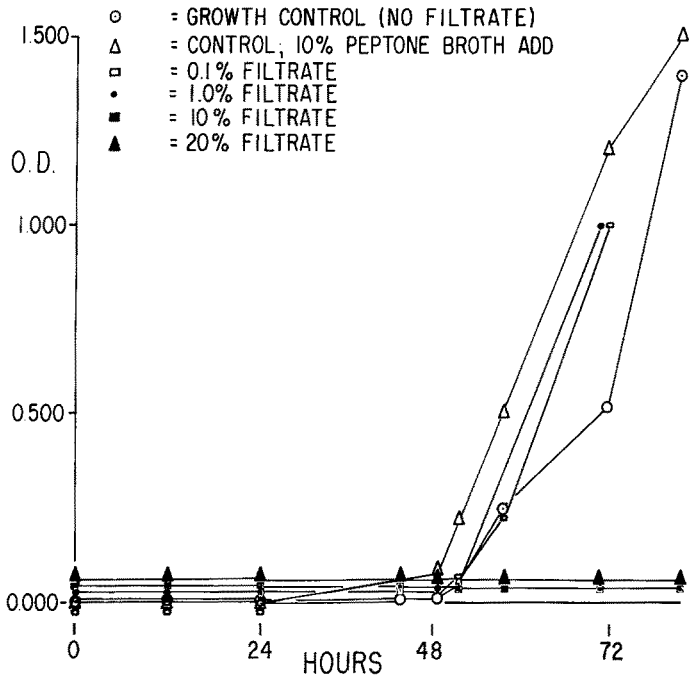

Fig. 1: Effect of various concentrations (final) of AEB filtrate on the growth of *Rhodotorula minuta*

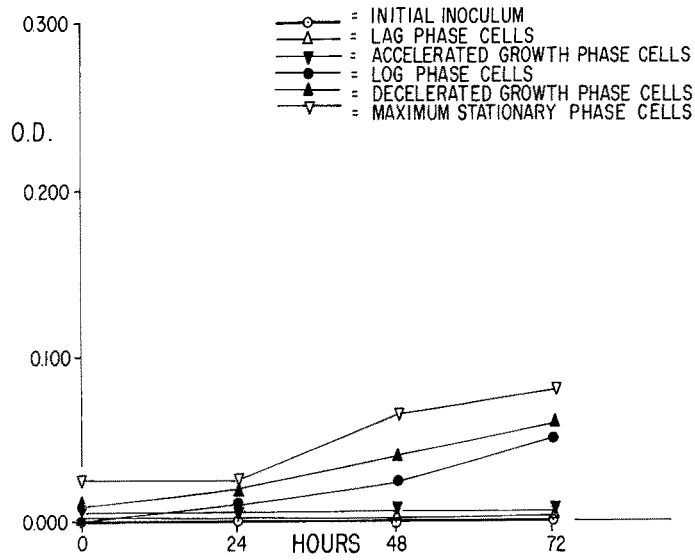

Fig. 2: Effect of age of *Rhodotorula minuta* cells on their inhibition by AEB filtrate

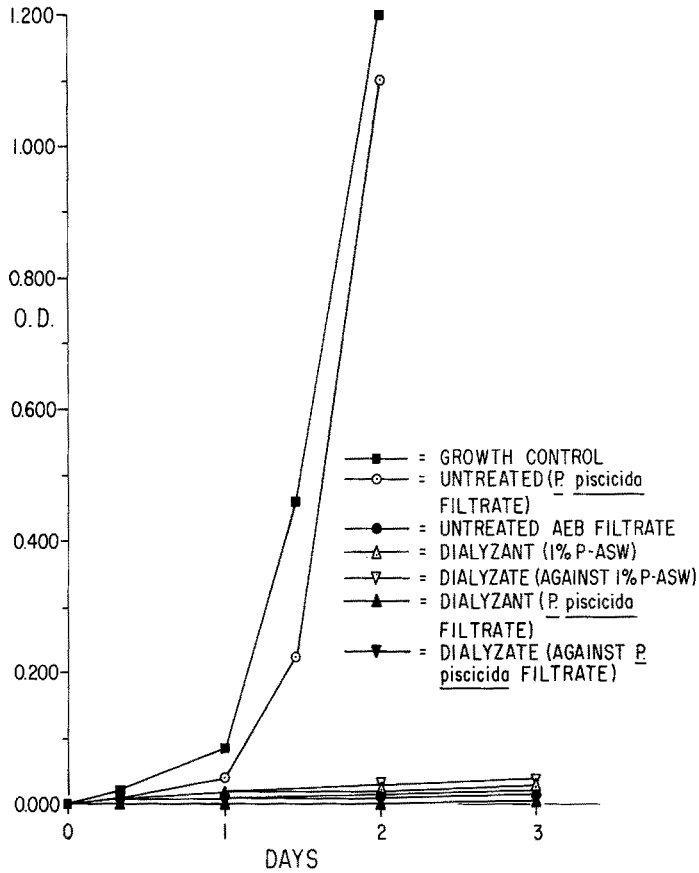

Fig. 3: Inhibition of *Rhodotorula minuta* by various dialysis fractions

convenience, yeast inocula were taken from 24-30-hour (middle-late log phase) shaken cultures.

Activity of AEB broth cultures was completely lost at pH 4.0. Adjustment of the pH to 6.0, 8.0 and 10.0 had little effect on antiyeast properties. Filtrates with adjusted pH retained activity on storage. Inhibition at the four pH values was in the order 10>8>6>4. Cell-free filtrate was unaffected by exposure for 30 minutes to temperatures of 3, 20, 30, 45, 60, 80 and 100° C. Earlier we had noted that heat treatment to 50° C for one hour did not reduce the inhibitory activity of the dialyzed fraction or the filtrates, whereas heating to 60° C for one hour resulted in reduction or loss of activity. Exposure to 100° C for one hour always produced complete loss of inhibition. Filtrates and dialyzed portions have retained activity as long as 60 days when stored at 4° C.

The ammoniacal nature of the fish toxin produced by *P. piscicida* has been demonstrated (Meyers & Greenfield 1963). Both *P. piscicida* and AEB actively deaminate proteinaceous substrates with production of copious amounts of NH_3. To eliminate the

possible role of ammonia as the antiyeast agent, peptone filtrates from cultures of AEB, adjusted to pH 9.6 and unadjusted (pH 8.1), were autoclaved for 20 minutes, readjusted to 8.1 and assayed. Material at the higher pH was negative to Nessler's reagent, indicating the absence of NH_3. This removal of NH_3 from AEB cultures by a combination of alkaline pH and heat had no effect on activity.

Initially, the antiyeast fraction present in AEB cultures was found to be nondialyzable against ASW. However, additional studies indicated that all fractions were active when filtrate was dialyzed against peptone broth or cell-free *P. piscicida* cultures (Fig. 3). These results suggest a stability of the inhibitory material in the presence of molecules normally present in peptone broth or metabolic end products of pseudomonad growth.

Both AEB and *P. piscicida* exhibit excellent growth in several nutritionally defined media in artificial sea water. The more complex of these (B-1) contained sodium pyruvate (150 mg %), L-glutamic acid (150 mg %), L-arginine (50 mg %), L-glycine (50 mg %) and adenylic acid (diphosphate; 50 mg %); pH 7.3. AEB also showed equally good growth in a medium composed of L-arginine (75 mg %) and either adenylic acid (25 mg %) or inorganic phosphate (10 mg %), added as mixed M/15 Na_2HPO_4-KH_2PO_4 buffer. Filtrates of AEB from the complete B-1 medium inhibited *R. minuta* even after a 21-day incubation period. Antiyeast activity was reduced when the bacterium was grown in an arginine and inorganic phosphate medium and was lost completely in an ASW medium containing arginine and adenylic acid. However, as noted, growth of AEB in both of these media was comparable to that present in peptone or B-1 media. Possibly this reduction in antiyeast activity in simple media may be due to the decrease in the total substrate concentration present.

Cells of *R. minuta* in contact with AEB filtrate (10 %) do not appear to remove appreciable amounts of the active material. Yeast cells, maintained for 72 hours in shake flasks of AEB filtrate, were removed by centrifugation, and the culture medium filter-sterilized and reinoculated with fresh *R. minuta* cells (0.1 ml of a 24-hour culture), comparable to the original inoculum. This procedure was repeated five times until the total volume of the filtrate was sufficiently reduced through evaporation to negate further runs. While the inhibitory capacity of the AEB filtrate was reduced somewhat, considerable antiyeast activity still was maintained throughout the test. It appears that either a minimum of material is required for inhibition of *R. minuta* or the process is merely a surface phenomenon and the substance(s) can be removed readily by washing of the yeast cells. Similarly, while a minimum of 20 % by volume of AEB filtrate is required for effective inhibition, it is entirely possible that the quantity of active material in culture fluids is small and might be active at a microlevel upon suitable concentration.

The effect of AEB filtrates on *R. minuta* is static, since inhibited cells develop normally when transferred to fresh, filtrate-free media. The principal inhibitory effect is that of interference with bud formation and/or bud separation from the parent cell. In affected cells, buds are slow to develop and are small when formed. Two to three buds per parent cell may occur rather than the single bud found in cells of normal cultures. The Gram reaction of affected cells is not altered and the gross permeability of the yeasts to stains such as methylene blue does not change markedly. However, it is

noted that inhibited cells stain uniformly while normal cells tend to stain non-homogeneously. Discrete changes in the chromatin material of the former cells are suggested.

In another test, the effect of full strength AEB filtrate (48-hour culture) on *R. minuta* cells over an extended period was examined. The test was maintained at 5° C to prevent initial yeast growth. The effect of various periods of incubation at this temperature was checked at 5 minutes, one hour, 24 hours, 72 hours and 8 to 21 days, by removal of the yeast from the filtrate by centrifugation with subsequent resuspension of the cells in filtrate-free G/Y broth at room temperature. Appropriate controls were run including flasks of yeast cells in G/Y broth alone at the same temperature as the test. No permanent inhibitory effect was noted even for yeast cells maintained in the AEB filtrate at 5° C for as long as 21 days, although exposure to the filtrate for more than eight days does produce a slight growth lag. Subsequent development of the yeast is approximately similar in spite of the period of exposure to filtrate. It appears that yeast cells in an active stage of growth and metabolism are materially affected by the substance(s) present in the bacterial filtrate.

Our earlier efforts to characterize the physical and chemical properties of the inhibitory substance present in filtrates of AEB included evaporation in vacuo at 25, 45, and 60° C with collection of acid- and base-trapped volatile fractions and solvent extraction of cells and filtrates with methyl ether, acetone, methanol, and ethanol. In general, little or no antimicrobial activity was found in the various fractions obtained by these treatments.

In view of the known stimulatory and inhibitory effects of various organic acids on the growth of microorganisms, including yeasts (NIEMAN 1954, NOVAK et al. 1964), various compounds were selected for comparison with the activity of AEB. These materials, in final concentration of 0.1 and 0.001 %, included sodium and calcium acrylate, sodium caprylate, sodium glycolate and formic, propionic, valeric, trichloroacetic, malonic, undecylenic and stearic acids. Sodium caprylate inhibited *R. minuta* at 0.001 % concentration for 48 hours and 0.1 % formic acid was inhibitory even after 96 hours. The antimycotic activity of sodium caprylate against *Candida albicans* has been reported elsewhere (WATT, ADAMS & PAYNE 1962). The activity of 0.1 % propionic, valeric, trichloroacetic and malonic acids was similar to that of 0.1 % formic acid. Formic, propionic, valeric, trichloracetic and malonic acids were ineffective at the lower concentration.

Acrylate and glycolate, ecologically important in marine food chains (FOGG 1964, SIEBURTH 1960) were shown to be ineffective, as were the long chain saturated fatty acids, undecylenic and stearic. The cytological effects noted for organic acid-inhibited yeast cells (WATT, ADAMS & PAYNE 1962), namely, impairment of bud separation and nuclear alteration, have been observed in cells of *R minuta* inhibited by AEB.

The results of adsorbant-exposed cell-free AEB material on *R. minuta* cells are listed in Table 1. While none of the artificial products removed the active material, two of the natural substrates, dried amphipod animals and tubes and rock leachings decreased filtrate activity. It would be expected that the tubes, consisting of sand grains cemented together by mucopolysaccharide-like secretions with various inclusions, would possess some adsorptive capacity.

Filtrates exposed to the cation exchange resin and the subsequent eluant were both inhibitory, but the fraction treated with the resin was less inhibitory than the eluate. This partial adsorption on the cation exchange resin suggests that the inhibitor may be a weak small molecular weight (based on dialysis tests) base. Disc assay of a selected group of ammonia and amine-containing compounds (including ammonium chloride, methylamine, ethylamine, tetraethyl ammonium chloride, n-propyl amine, dimethyl

Table 1

Effect of various adsorbants on antiyeast properties of AEB filtrates

Adsorbant treatment	Inhibitory effect after treatment	Inhibitory effect of eluant
Ulva powder	+	0
Centrocerus powder	+	0
$CaCO_3$	+	0
Silica gel	+	0
Rock leachings	0	0
Anion resin	+	0
Cation resin	+	+
Amphipod	partial	0
Cellulose	+	0
Charcoal	+	+
Filtrate, untreated	+	
Control, no filtrate	0	

amine, triethylamine, t-butylamine and di-isopropyl amine), 10^{-1} to 10^{-5} dilutions against *R. minuta* and other test yeasts gave negative results. Natural amphipod material, in the form of drippings, squeezings and homogenates were, in general, non-inhibitory, although the homogenized sample slightly retarded the growth of *R. minuta*. Some ecological significance may be attached to the possible binding of active fractions by marine materials under natural conditions.

While chemical characterization of the active principal in culture filtrates of AEB has been inconclusive, nevertheless, the data presented provide additional pertinent information on the activity of this ecologically significant antiyeast bacterium. These data may be useful in further in vitro testing of other pseudomonads with comparable activity. For, as noted earlier (Buck, Meyers & Kamp 1962, Buck & Meyers 1965), antiyeast properties among pseudomonads of soil and clinical origin are not uncommon. Ten of 13 clinical isolates of *Pseudomonas* examined were active as was a species isolated from soil as well as *Pseudomonas aeruginosa*. These data suggest a wide range of possible associative inhibitory relationships may exist in various environments characterized by large concentrations of yeasts and bacteria. The increased activity of AEB at higher pH values, and the suggested involvement of natural marine materials in adsorption of inhibitory fractions are further evidence for in situ control of yeast development in marine environments. Our ecological data (Buck & Meyers 1965) on yeast/bacteria population dynamics in marine micro-habitats support these considerations.

SUMMARY

1. Maximal activity of cell-free filtrates of the antiyeast bacterium, *Pseudomonas piscicida*, against *Rhodotorula minuta* was obtained from four-day shaken peptone broth cultures. Yeast cells were taken prior to their logarithmic growth phase.
2. Antiyeast activity was lost when filtrates were adjusted to pH 4.0, while higher pH values, as well as temperatures up to 100° C for 30 minutes, had no effect on inhibition.
3. The principal inhibitory effect is that of interference with bud formation and/or bud separation from the parent cell. Yeast cells in an active stage of growth and metabolism are materially affected by the substance(s) present in the bacterial filtrate.
4. Both dialyzates and dialyzants were active when filtrates were dialyzed against peptone broth or another pseudomonad culture. Decreased activity was noted when the bacterium was grown in chemically defined media.
5. Exposure of filtrates to two "natural" marine adsorbants, carbonate rock leachings and dried amphipod material, resulted in lessened inhibitory properties.
6. Chemical characterization of the active principal in filtrates of the bacterium is inconclusive. While experimental evidence (cation exchange resin) suggests that the inhibitor may be a small molecular weight base, disc assay of a selected group of ammonia and amine-containing compounds gave negative results.
7. Ecological data indicate in situ action of the antiyeast material in discrete marine environments.

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