Cultural Estimation of Yeasts on Seaweeds

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Five per cent suspensions of freshly harvested seaweeds were used as an inoculum to develop a selective medium for epiphytic yeasts. Conditions for satisfactory yeast growth and visualization as red colonies on membrane filters were obtained by supplementing a basal glucose-Trypticase-yeast extract agar at pH 7.0 with 100 mg each of chloramphenicol and 2,3,5-triphenyl tetrazolium chloride per liter. Maximal counts were obtained by triturating the algae in prechilled (4 C) seawater with a blender for 2 to 5 min. Inhibitory phenolic materials released from phaeophytes during this process were removed with a modified Cholodny filtration. A preliminary survey indicated that yeasts were epiphytic on all nine species of seaweeds and that maximal populations occurred on the chlorophytes and rhodophytes especially during the periods of warmer water.

The yeasts are receiving attention as members of the marine microflora (14, 31). Bunt (3), van Uden and Castelo Branco (30), Suehiro (27), and Suehiro and Tomiyasu (28, 29) have demonstrated considerable yeast populations on decaying seaweeds. Although an association between phytoplankton and yeasts was suggested by Kriss and Novozhilova (10), Roth et al. (18) examined marine algae and grasses for the presence of yeasts and found no specific association even though the surrounding waters and sediments contained yeasts.

Sample preparation is a problem in examining littoral marine algae for epiphytic microorganisms. Decaying algae may fragment when shaken, but fresh algae require more rigorous treatment. Trituration in a blender without proper precautions (4) not only subjects heat-sensitive microorganisms to excessive heat, but also liberates protein-precipitating and inhibitory substances (19, 20). Media commonly used for the detection and enumeration of marine yeasts (1, 13, 31) have their limitations. Fungi rapidly overgrow these plates before maximal yeast development. Light-colored yeast colonies are sometimes indistinguishable from glistening detritus and plant materials on the surface of the white membrane filter. The low pH of many media may inhibit acid-sensitive yeasts growing in alkaline seawater. Antibiotics appear to be used (1) at concentrations up to 50 times greater than required for bacterial inhibition. The indiscriminate use of antibiotics to suppress bacteria may inhibit some yeasts (16, 25). It became apparent that a procedure for the preparation of seaweeds and a satisfactory medium for the cultivation of yeasts had to be developed before the association of yeasts with seaweeds could be studied further. This paper reports the results of such an attempt.

MATERIALS AND METHODS

The basal medium found satisfactory for mycological work and referred to as "C" medium (22) consisted of 0.1% yeast extract (BBL), 1.0% Trypticase (BBL), 3.0% glucose, and 1.5% agar (Difco) prepared with an artificial seawater (ASW; Balderston et al., in preparation). To prepare an ASW with a salinity of approximately 30%, the following procedure is used. NaCl (~300 g), Na2SO4 (~50 g), and KCl (~10 g) are dried at 125 C for at least 4 hr and cooled in a desiccator. For solution A, NaCl (dried), 205.1 g; KCl (dried), 5.8 g; MgCl2·6H2O, 92.8 g; and CaCl2·2H2O, 13.0 g are dissolved in 7,000 ml of distilled water at room temperature. For solution B, 34.4 g of Na2SO4 (dry) is dissolved in 2,580 ml of distilled water at room temperature. For solution C, 16.8 g of NaHCO3, and 0.014 g of Na2HPO4 are dissolved in 1,000 ml of distilled water and filter-sterilized with a 0.2-μm membrane filter. To prepare a liter of medium C or ASW, 730 ml of solution A is used to dissolve the ingredients in one flask while 260 ml of solution B is put in a second flask. After these flask are autoclaved, 10 ml of solution C is added to flask B which is combined with flask A to avoid phosphate and trace-element precipitation during autoclaving. The pH of the unadjusted medium after autoclave sterilization was between 6.0 and 6.1. The pH was adjusted as required with either sterile 10% lactic acid or with 1 N NaOH. Filter-sterilized antibiotic solutions and 2,3,5-triphenyl tetrazolium chloride were added to sterile and cooled (45 C) media before pouring.
The seaweeds used for natural yeast populations and the survey species were collected at Camp Varnum, R.I., some 7 km south of the Narragansett Marine Laboratory. Approximately 5% seaweed (wet weight) was suspended in sterile ASW. The yeasts were released either by shaking with glass beads for 10 min or by blending with prechilled (4°C) ASW for 2 to 5 min. For cultivation, volumes of the above suspension equivalent to 0.1 to 25 ml in a minimum volume of 10 ml of ASW were filtered directly on sterile membrane filters which were placed on the agar media. Except where stated otherwise, 0.45-μm membrane filters (Millipore Corp.) were used.

To avoid cultivation on membranes which may have adsorbed inhibitory substances from the seaweeds, a modification of the Cholodny procedure (9) was used. The yeasts were kept in suspension above the membrane with a stirring blade while 10× the sample volume of sterile seawater was used to dilute out inhibitory materials. The volume was reduced to 5 ml and quantitatively transferred to a second membrane for cultivation. All membranes and plates were incubated at 18°C for 96 hr before counting and the isolation of representative yeast colonies. These isolates were maintained on malt extract-agar, studied with standard procedures, and identified according to Lodder (11).

Yeast cells for inocula were prepared by scraping growth from a 48- to 72-hr-old agar slant of C medium and making a suspension in ASW equal to a McFarland no. 1 turbidity (12). Serial decimal dilutions were used for direct microscopic counts in a hemocytometer as well as colony counts by the spread plate method (2).

RESULTS AND DISCUSSION

Because yeasts are generally more acid tolerant than bacteria, selective media for yeasts have traditionally been acidified (pH 4.5 to 5.5) to inhibit bacterial development. For some obscure reason, some investigators still acidify the yeast growth medium although sufficient antibiotics have been added to suppress bacterial growth completely (1). Morris (14) questions the use of acidic media to cultivate yeasts from alkaline seawater. To select the pH optimal for growth of yeasts epiphytic on algae, the pH of C medium was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0. Nine yeast cultures isolated from four seaweed species and seawater were used as inocula. The results are shown in Table 1.

The seaweed isolates, including Candida parapsilosis, showed good growth at all pH levels with a slightly higher count at pH 7.0. Only the C. parapsilosis isolate from seawater appeared to be inhibited at pH 8.0 and 9.0. This confirms the observation of Norkrans (15), who found that this species was inhibited above pH 8.1.

In the early part of this study, difficulty was experienced in distinguishing cream-colored yeasts from plant detritus on white membrane filters. Tetrazolium salts have been used for the vital staining of microorganisms (6). Rioux et al. (17) reported that 36 out of 42 yeast species took up 2,3,5-triphenyl tetrazolium chloride (TTC) at a concentration of 100 mg/liter which became reduced to formazan and pigmented the colonies red. In an attempt to make the cream-colored yeast colonies visible on white membrane filters by the addition of TTC to the medium, we found that this reduction was pH-dependent. The natural yeast population on a fresh specimen of the red alga Chondrus crispus was used to determine the effect of pH on yeast pigmentation by TTC. The results are given in Table 2. At pH 7.0, the yeasts were able to reduce 100 mg of TTC per liter and to pigment all of the colonies. Higher concentrations of TTC inhibited yeast growth and produced large rough colonies. Lower yeast counts were obtained at pH levels lower and higher than 7.0. TTC pigmentation was poor under acidic conditions. A satisfactory medium for pigmentation and growth appears to be one at pH 7.0 which contains 100 mg of TTC per liter. During a 15-month survey of yeasts occurring on nine species of seaweeds, no non-TTC-pigmented yeast colonies were encountered. An added feature of TTC is that it is inhibitory to some bacteria (6) and in our medium it probably supplements chloramphenicol in suppressing bacterial growth.

In the marine environment, bacteria are usually more numerous than yeasts. For the selective isolation of yeasts from marine materials, bacterial inhibitors such as chloramphenicol, chlortetracycline, and streptomycin are generally used in the culture medium either alone (1) or in combination (7). The concentrations and combinations of antibiotics used to suppress bacterial growth in media used for marine yeasts appear excessive. Richards and Elliott (16) have cautioned against the indiscriminate use of antibiotics and have shown that streptomycin at a concentration of 200 mg/liter inhibited 10 species of yeasts tested at pH 6.9 and 7.0. Ahearn et al. (1) have used chloramphenicol at concentrations between 500 mg/liter and 5,000 mg/liter. In addition to the prohibitory cost of chloramphenicol at such concentrations, it may also have an adverse effect on yeasts. It has been shown that chloramphenicol at a concentration of 500 mg/liter inhibited Rhodotorula glutinis (25), whereas 1,000 mg/liter caused an unbalanced cell wall synthesis (26). Our preliminary observations indicated that, although 50 mg of chloramphenicol per liter permitted bacteria to grow, 100
mg/liter completely suppressed growth. The effect of minimal concentrations of antibiotics alone and in combination on the natural bacterial and yeast populations of C. crispus is shown in Table 3. It was found that chloramphenicol alone completely inhibited bacterial growth but did not affect the yeast count. Penicillin and streptomycin alone or in combination and chlorotetracycline alone did not completely prevent bacterial growth. A satisfactory medium for the cultivation of yeasts appears to be C medium at pH 7.0 supplemented with 100 mg each of chloramphenicol and TTC per liter. Such a medium (CCT) was used in the remainder of the study.

A number of procedures were compared for their effectiveness in releasing yeasts attached to seaweed. C. crispus was suspended in sterile ASW and treated by manual shaking in prescription bottles with and without glass beads, trituration in "Eberbach" semi-micro-stainless-steel and Waring (Pyrex glass) blender cups, and ultrasonic vibration using a 120-w, 20-kc probe. Treatments were conducted for 2.5-, 5.0-, and 10-min periods. The results are given in Table 4. The Waring blender yielded yeast counts 20 to 50 times greater than the other methods. Trituration in the semi-micro-stainless-steel blender caused an apparent thermal damage to the yeast cells which may be due to the smaller capacity. Ultrasonic treatment was ineffective in releasing yeast cells. To prevent any thermal damage to the yeast cells during trituration, sterile prechilled (4 C) ASW was used to suspend the algae.

In brown algae, phenolic materials which account for up to 9% of the dry weight (8) are readily exuded from whole plants under living conditions (21) and cause a yellow discoloration in the suspending water (5). These phenolic materials are highly reactive and readily denature protein. A 1:1,000 dilution of a seaweed extract of Sargassum natans killed hydroids (22), whereas the median lethal dose of phenols from Ascophyllum nodosum for fish larvae was 0.32 mg/liter (24). In our study, 5% seaweed suspensions triturated in the blender released some 30 mg of phenolic material per liter. During the filtration
of phaeophyte extracts, about 64% of this phenolic material was adsorbed on the membrane filter. The intimate contact between the adsorbed phenolic materials and the filtered yeast cells apparently inhibited their growth and gave a low count. A modified Cholodny filtration (9) was used in an attempt to avoid inhibition caused by this adsorption. Fucus vesiculosus was used to prepare the phenol-containing algal extract which was used to test the procedure. A suspension of the yeast C. parapsilosis (isolated from F. vesiculosus) was added to the algal extract. Sufficient cells were used to overshadow any natural yeast flora present in the algal extract. The yeast-algal extract mixture was filtered through Millipore,

Sartorius, and Gelman filters of 0.2- and 0.45-μm pore sizes by using both the direct and Cholodny filtration techniques. The data are shown in Table 5. Similar results were obtained with both porosities of all three filter types. The mean values indicated that the phenolics adsorbing on to the membranes reduced the yeast counts by 85%. The Cholodny filtration (second filter) recovered some 88% of the yeasts. If 12% of the yeast population adhered to the first filter and inhibition was 85%, then one would expect that 2% of the population grew on the first filter. Such is the case. Greater recovery than the 88% obtained may be possible by the use of a flexible stirring blade which scrapes the membrane surface.

After both the CCT growth medium and the seaweed preparation procedures were developed, a preliminary survey was undertaken to enumerate the yeast populations epiphytic on seaweeds. Algal species selected for this survey were: F. vesiculosus (L.), A. nodosum Le Jol., Laminaria digitata (Huds.) Lamour., L. agardhii Kjellm., Ulva lactuca (L.), C. crispus Stackh., Rhodymenia palmata (L.) Grev., Polysiphonia lanosa (L.) Tandy, and P. harveyi Bail. These species were picked due to their dominance and availability throughout the year. Rocks were included in the survey to determine the population attaching to a nonliving surface. Results of the survey are shown in Table 6. All nine species of seaweeds tested harbored a yeast microflora. Maximum numbers occurred during the warm period of the year when the seawater temperature exceeded 20°C. The lower population of yeasts on phaeophytes may be due to a release of inhibitory phenolic materials. The Cholodny method increased the yeast count from the phaeophytes some threefold.

### Table 3. Effect of antibiotics on the suppression of bacterial growth and on the detection of yeasts from a fresh sample of the red alga Chondrus crispus

| Antibiotics | No. of microorganisms | Trial 1 | Trial 2 |
|-------------|-----------------------|---------|---------|
|             | Bacteria | Yeasts | Bacteria | Yeasts |
| None        | ~1,000    | 1.7    | ~10,000  | 2.1    |
| P           | ~100      | 1.9    | ~1,000   | 2.0    |
| S           | ~100      | 1.8    | ~100     | 2.0    |
| P + S       | 0.1       | 1.7    | 0.0      | 2.2    |
| Ct          | 0.1       | 1.8    | 0.2      | 1.8    |
| Cp          | 0         | 1.5    | 0        | 1.9    |
| P + S + Ct  | 0.1       | 1.6    | 0        | 2.0    |
| P + S + Cp  | 0         | 1.6    | 0        | 2.0    |

a Antibiotics were used to supplement the basal medium C at pH 7.0 which contained 100 mg of TTC per liter. Abbreviations: P = penicillin G potassium, 200 mg/liter; S = streptomycin sulfate, 100 mg/liter; Ct = chlorotetracycline HCl, 100 mg/liter; Cp = chloramphenicol, 100 mg/liter.

b × 10^6 per gram (dry weight) of algae.

### Table 4. Comparison of methods for the release of epiphytic yeasts from the red alga Chondrus crispus

| Method | Trial 1 | Trial 2 |
|--------|---------|---------|
|        | 2.5 min | 5.0 min | 10.0 min | 2.5 min | 5.0 min | 10.0 min |
| Hand shaking | 3.0 | 3.5 | 6.4 | 0.1 | 3.6 | 5.7 |
| Hand shaking with glass beads | 6.5 | 14.9 | 163 | 6.1 | 13.3 | 15.1 |
| Blending in "Eberbach" semi-micro stainless-steel blender | 4.1 | 1.0 | <0.03 | 5.04 | 0.2 | <0.03 |
| Blending in Waring (Pyrex glass) blender | 145.2 | 211.2 | 99.0 | 279.8 | 204.6 | 84.5 |
| Ultrasonic dispersion | 4.0 | 4.3 | 3.9 | 6.5 | 6.5 | 6.7 |

a Fresh seaweed (10 g) suspended in 200 ml of sterile seawater.

b Values × 10^6 per g (dry weight) of algae. Basal medium C at pH 7.0 was supplemented with 100 mg of chloramphenicol and TTC per liter, respectively.

c Excessive heating of blender.
TABLE 5. Inhibition of yeast (Candida parapsilosis) recovery by the extract of Fucus vesiculosus and its reversal by the modified Cholodny method

| Prepn | Filtration technique | Trial 1 | Trial 2 | Mean recovery | Per cent recovery |
|-------|----------------------|---------|---------|---------------|------------------|
|       |                      | Millipore | Sartorius | Gelman | Millipore | Sartorius | Gelman | Millipore | Sartorius | Gelman |
| Yeast suspension | Direct | 70 | 72 | 76 | 70 | 72 | 72 | 70 | 72 | 70 | 72 | 72 | 62.9 | 100.0 |
| Yeast suspension | Direct<sup>a</sup> | 10 | 11 | 11 | 14 | 17 | 12 | 5 | 7 | 6 | 8 | 4 | 7 | 9.3 | 15.0 |
| +%5 algal extract (w/v)<sup>b</sup> | Cholodny (first filter)<sup>c</sup> | 4 | 2 | 2 | 1 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1.2 | 2.0 |
| | Cholodny (second filter) | 65 | 58 | 60 | 62 | 67 | 60 | 41 | 47 | 52 | 49 | 53 | 50 | 55.3 | 88.0 |

<sup>a</sup> Sufficient Candida parapsilosis cells were added to overshadow any natural yeast flora present.
<sup>b</sup> Phenolic content expressed as Phloroglucinol was 36 mg/liter (trial 1) and 29 mg/liter (trial 2).
<sup>c</sup> Membrane filters absorb about 64% of the phenolic material in algal extract.

TABLE 6. A survey of yeasts epiphytic on seaweeds at Camp Varum Beach in Narragansett, R.I. (41°27'N, 71°26'E) during 1970

| Class     | Sample       | Filtration | No. of yeast cells<sup>a</sup> |
|-----------|--------------|------------|--------------------------------|
|           |              | 6 Apr. | 20 May | 20 June | 20 July | 17 Aug. | 15 Sept. |
| Chlorophycale | Ulva lactuca | Direct | 40 | 300 | 4 | 1,410 | 45,000 | 2,800 |
| Rhodophycale | Chondrus crispus | Direct | 290 | 140 | 40 | 1,020 | 42,000 | 1,700 |
| Rhodymenia palmata | Direct | 56 | 100 | 4 | 710 | 8,600 | 200 |
| Polysiphonia lanosa | Direct | 190 | 230 | 55 | 2,110 | 56,000 | 7,300 |
| P. harveyi | Direct | 400 | 290 | 40 | 1,450 | 72,000 | 1,900 |
| Phaeophycale | Fucus vesiculosus | Direct | 60 | 80 | 30 | 210 | 850 | 150 |
| Ascophyllum nodosum | Direct | 120 | 180 | 50 | 350 | 2,930 | 390 |
| Laminaria digitata | Direct | 140 | 140 | 10 | 270 | 730 | 110 |
| L. agardii | Direct | 180 | 210 | 20 | 390 | 2,120 | 230 |
| Rock | Direct | 20 | 10 | 1 | 60 | 90 | 90 |
| Seawater<sup>b</sup> | Direct | 20 | 10 | 1 | 70 | 130 | 130 |

<sup>a</sup> Values expressed as number of yeast cells per gram (dry weight) of algae. Incubation temperature, 18 C. Seawater temperatures: 6 April, 10 C; 20 May, 16 C; 19 June, 19 C; 20 July, 21 C; 17 August, 27 C; 15 September, 17.5 C.

<sup>b</sup> No. of cells/liter.

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