Possible Isolation of Halophilic, Cellulolytic Bacteria

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ABSTRACT

Isolation of halophilic, cellulolytic bacteria was attempted using inocula from the periphery of Sippewissett Salt Marsh, from a Red Sea saltern microbial mat sample, from wooden pier posts in the Eel Pond, and from a road salt storage facility at the Falmouth Department of Public Works. Aerobic and anaerobic liquid culture enrichments were performed using cellulosic substrates (filter paper, carboxymethylcellulose or Solka-floc) as the carbon source in 10% NaCl. Significant growth was detected only under aerobic conditions with carboxymethylcellulose (CMC) or Solka-floc. Colonies were screened for cellulolytic activity using CMC plates and congo red staining. Though no isolates tested positive using this technique, some liquid cultures of the same isolates grew in CMC, solka-floc, and/or cellobiose after 7-10 days in the presence of 15% NaCl. Further investigation using more sensitive assays for cellulolytic activity are necessary to determine if these isolates can use cellulose (or derivatives) as a sole carbon source.

INTRODUCTION

Hypersaline environments occur throughout the world in environments where seawater is isolated or trapped inland and then concentrated through evaporation. Although animal and plant diversity tends to decrease as salinity increases, some organisms, including many bacteria, can survive in and often require high salt. Such organisms are classified according to the range of salt concentrations they tolerate. A widely accepted definition for halotolerant and halophilic microbes is outlined below (Ventosa, 1989):

1) non-halophilic microorganisms--grow best in media containing less than 0.2M (~1%) salt.
2) halotolerant--can tolerate higher concentrations of salt
3) slight halophiles--grow best in media with 0.2-0.5 M (~1-3%) salt
4) moderate halophiles--grow best in media with 0.5-2.5 M (~3-15%) salt
5) borderline extreme halophiles--grow best in media with 1.5-4.0 M (~9-23%) salt
6) extreme halophiles--grow best in media with 2.5-5.2 M (~15-32%) salt

Proteins and other biological macromolecules in hypersaline environments must have special properties that prevent precipitation and allow function in high salts. It is, therefore, of interest to study the physiology and biochemistry of halophilic microorganisms. Cellulose is the most abundant polysaccharide in nature, as it is the primary structural component of plant cell walls. Many cellulolytic bacteria have been isolated and described from various terrestrial and marine habitats. Since the size and shape of cellulose prevent its entry and internal breakdown in such organisms, their cellulases are extracellular where they are associated with the outer membrane or secreted into the media.

A halophilic, cellulolytic bacteria would be an ideal model for studies on halophilic bacteria and enzymes. As an external enzyme, the cellulase must be functional in high salt environments and it would be relatively easy to purify. Furthermore, cellulolytic activity is well studied and easily assayed.

Only one halophilic, cellulolytic bacteria has been isolated and described in the literature (Simankova et. al., 1993). This study attempted to isolate such an organism from habitats in the Woods Hole area and from samples imported from the Red Sea saltern microbial mat. Woods Hole area samples included a road salt storage facility in Falmouth and marine habitats that contain plant matter and might produce entrapments of water which are subjected to evaporation.
MATERIALS AND METHODS:

Local samples were collected from the inocula source list in Figure 1. Red Sea saltern microbial mat sample was obtained from Yehuda Cohen. An overview of the isolation procedure is outlined in a flowchart in Figure 2. Media composition was adapted from Simkanova et al. (1993) and the Microbial Diversity Methanogen/Acetogen Handout (Breznek, 1994). Unless otherwise stated, cellulosic enrichment and isolation media contained the following: 1.02 g MgCl₂ x 2 H₂O, 0.04 g yeast extract, 2.38 g HEPES, 20.0 g NaCl, 200 ml distilled water, cellulosic compound, and reazurin (a few drops, added to anaerobic media only). After autoclaving, 0.2 ml of each of the following solutions were added to the media: SL-12 trace elements, vitamins, vitamin B12 (all described in Breznec, 1994) and 1 mg/ml filter sterilized cyclohexamide dissolved in 20% acetone. Media pH was adjusted to ~7. Anaerobic media received 3.6 ml 1 M NaHCO₃ and 2 ml 125 mM Na₂S. Anaerobic media was prepared in a Widdle flask with stirring and cooling under a nitrogen and carbon dioxide mixture and dispensed into sterile Balsch tubes. Aerobic media was prepared in an erlenmeyer flask and dispensed with a repeating syringe.

The initial filter paper and carboxymethylcellulose (CMC) enrichment media was made as described above, but initially without cellulosic compounds. Filter paper (Whatman No. 1) was cut into ~0.1 g pieces and autoclaved in appropriate tubes. Half of the media was delivered to these tubes (10 ml x 10 tubes for aerobic and anaerobic). To the remaining 100 ml was added 12.5 ml sterile, deoxygenated CMC solution (0.45 g in 50 ml water). This CMC media was dispensed in empty sterile tubes. Solka-floc (SF) was added (1.8 g in 200 ml media) to separate flasks of media before autoclaving. Problems with the Widdle Flask and anaerobic technique yielded pink media in many anaerobic tubes. Some were regassed and resealed or further reduced with additional hydrogen sulfide in attempt to maintain an anaerobic environment.

Samples were diluted (approximately 1 ml or 1 g) in 6 ml sterile deoxygenated seawater. 0.75 - 1.5 ml inoculum was then transferred with a sterile syringe into primary enrichment tubes. Tubes were incubated at room temperature (~24°C) on a platform shaker. SF tubes were vortexed (to resuspend insoluble SF) and examined a few times each day. When growth was observed as turbidity, cultures were transferred to new media using a 5-10% inoculum.

When secondary enrichments were turbid, they were streaked onto 10% NaCl nutrient agar plates with 1.5% glycerol added. Anaerobic enrichments were streaked and incubated in the glove box. Aerobic enrichments were incubated at room temperature. Isolated colonies grew only in aerobic conditions and were triplicate plated on CMC, SF, and nutrient agar plates containing 10% NaCl. (0.9 g SF or CMC in 200 ml media, 2.38 g HEPES, 3.0 g noble agar to CMC/4.0 g noble agar to SF. All other additions as with aerobic liquid media above). Secondary enrichments were also streaked on 15 and 30% NaCl nutrient agar plates to look for any generic extreme halophiles. Growth appearing on the CMC plates was transferred to new CMC plates and to liquid cultures containing 15 or 25% NaCl and CMC, SF, or Cellobiose (CB) to a final glucose concentration of ~30 mM and only 0.01% yeast extract. All liquid cultures were incubated at room temperature with shaking. CMC plates were then tested for CMC degradation using the congo red staining procedure (Wood and Kellogg, 1988).

RESULTS

After five or six days, most CMC and SF primary enrichments were turbid and microscopic examination showed numerous bacteria of various sizes and morphologies. Similarly, secondary enrichments grew rapidly.

Colony isolatates grew only under aerobic conditions, therefore anaerobic isolations were not pursued further.

CMC congo red screening showed no CMC degraders. However, some of the same isolates grew in 15% NaCl liquid media with only CMC, SF, or CB as a carbon source (Table 1). Significant growth (turbidity) was seen only in F.D.P.W. wood sample 15%/SF, Eel Pond pier wood 15%/CMC, and Red Sea mat, bottom, 25%/CB and 15%/SF (displayed in bold type). It should be noted that different colonies on the CMC plates may have originally come from the same inoculum source. The same CMC colony was not necessarily used to inoculate all 6 types of isolation media, and all colonies were not inoculated into all 6 media types. Therefore, different
INOCULA SOURCES
1) Sippewissett Salt Marsh--water and sediment from edge
2) Sippewissett Salt Marsh--water
3) Sippewissett Salt Marsh--wood and grass
4) Falmouth Department of Public Works (F.D.P.W.) salt pile I
5) F.D.P.W.--salt and sand pile
6) F.D.P.W.--wall area salt
7) F.D.P.W.--wood
8) Eel Pond--Pier wood
9) Eel Pond--seaweed and grass
10) Red Sea saltern microbial mat--top
11) Red Sea saltern microbial mat--center
12) Red Sea saltern microbial mat--bottom

ENRICHMENT MEDIA

• 10% NaCl
• trace elements
• vitamins
• 0.02% yeast extract
• cyclohexamide

**Aerobic**
Carboxymethylcellulose (CMC)
Solka-floc
Filter paper

**Anaerobic**
Carboxymethylcellulose
Solka-floc
Filter paper
**FLOWCHART**

Inoculated enrichment media with 1:6 dilutions of all 12 sources → Incubated on shaker at room temperature

Growth (turbidity) in aerobic and anaerobic CMC and SF tubes within 5 days → Transfered to fresh media

All 2° tubes with growth streaked on 10% NaCl nutrient agar plates. Anaerobic plates streaked and incubated in glove box → Also streak 30% NaCl n.a. to look for generic extreme halophiles

Colonies on n.a. plates transferred with toothpick to set of CMC, SF, and NA plates (all 10% NaCl) → No growth on 30% plates

Colonies on CMC used to inoculate liquid media with 15% or 25% NaCl and CMC, SF, or Cellobiose as carbon source → Incubated at room temperature on shaker

CMC plate assayed for cellulolytic activity with congo red screening procedure → No positives

7-9 days later, growth seen in some of the liquid cultures
cell morphologies in different types of media within the same inocula category may have come from different CMC colonies.

Growth in filter paper enrichments was very slow. No obvious degradation of filter paper occurred under these conditions, though some enrichments showed visible clumping at tears in the paper.

No colonies were observed after 10 days on the 25% NaCl nutrient agar plates.

**DISCUSSION**

Primary and secondary enrichments yielded rapid, abundant growth leading to the misconception that cellulolytic halophile isolation would be quick and easy. There are a few explanations for the difficulty experienced in isolation, as compared to apparent ease in enrichment. First of all, growth in the enrichment was probably due mainly to carryover of substrates in the inoculum. Organisms were not forced to grow on cellulose and therefore grew quicker.

Secondly, the enrichment media contained a small percentage of yeast extract. Concerned that the yeast extract might be promoting growth of non-cellulolytic organisms, I reduced the amount in the isolation liquid cultures to 0.01%. This increased the selection for cellulolytic organisms and forced use of cellulosic substrates.

Cellulolytic activity on CMC was not observed when screened with the congo red assay. However, before screening, the same colonies were used to inoculate the liquid isolation media and many of these tubes eventually showed growth. The congo red assay may not be sensitive enough for such slow growing (slow cellulose degrading?) bacteria. Another more sensitive assay and reinoculation of cellulosic-substrate-only plates could confirm whether the obtained isolations can indeed use cellulose as a sole carbon source.

Cellulolytic bacteria can be slow growers when grown only on cellulose. The time limitations in this course did not allow the intended investigation of the desired isolate. Perhaps a future Microbial Diversity student could attempt isolation using more selective conditions initially (no yeast extract, little inoculum) and could start earlier to allow more time for growth. If a pure culture is obtained, it would be interesting to characterize the bacteria in terms of degree of salt tolerance, optimal temperature, preferred cellulose substrates, etc. If extracellular cellulase could be purified, it could be assayed for activity in the presence of varying salt concentrations to determine if salt is required for activity, an interesting characteristic of other halophilic enzymes.

**BIBLIOGRAPHY**


<table>
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<tr>
<th>Sample</th>
<th>15%/CMC</th>
<th>25%/CMC</th>
<th>15%/CB</th>
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<td>rods(C2)</td>
<td>rods &amp; cocci(C2)</td>
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<td>rod clumps(C14)</td>
<td>cocci(C14)</td>
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<td>small mot. rods(A4)</td>
<td>small motile rods(A4)</td>
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( ) = CMC colony source # bold = Significant growth / turbidity. Other samples required several field searches to find cells. — = not done  0 = no growth

chns. = chains