Survey of α-Chitin and β-Chitin Utilizing Bacteria and Archaea in Selected Marine Habitats Using Both Culturing and Molecular Techniques

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Introduction

Chitin is polysaccharide that is the main constituent of arthropod shells, some mollusk shells and the cells walls of fungi (Shimoda et al., 1996). Chitin is the second most abundant organic compound on the earth (behind only cellulose), yet remains an almost unutilized biomass resource. There are three polymorphic crystal structures of chitin are known, α, β and γ. α-Chitin is the most tightly compacted, most crystalline form where the chains are arranged in an anti-parallel form (Lee et al., 1996). It can be readily obtained from the shells of crabs, lobsters and shrimps. β-Chitin, found in the pen of squid, has the monoclinic form where the chains are parallel (Tokura et al., 1990).

Chitin is known as a potentially useful biopolymer produced in huge amounts in the natural environment. Most chitin studies are based on the relatively easily obtained α-chitin whose main chains are arranged in an anti-parallel manner with strong intermolecular hydrogen bonding. In contrast, β-chitin has much weaker intermolecular hydrogen bonding (as a result of the parallel arrangement of the main chains) and this property is thought to possibly have important ramifications in the bioavailability of β-chitin as an energy source for chitin-degrading microorganisms (Svitil et al., 1997). The ability to utilize chitin as a carbon source is not thought to be essential for heterotrophic bacteria living in most oceanic environments because many other carbon sources are available, but a number of Bacteria and Archaea are known to facultatively degrade chitin (Kirchman and White, 1999) and that is an essential first step in the degradation of organic material in nature (Cottrell et al., 1999). With rare exception, chitin is always found cross-linked to other structural components, such as proteins and glucans (Gooday, 1990).

Objectives

- Culture and isolate microorganisms utilizing α-chitin (from the shells of lobsters and unbleached chitin from chemical grade chitin) and β-chitin (from squid pens) as the sole Carbon and Nitrogen source
- Grow and visualize biofilms formed on lobster shells and squid pens

- Extract genomic DNA, amplify via PCR and sequence 16S rRNA genes using Bacterial and Archaeal primers

- Compare differences in the chitin-degrading microbial community structure between proximate but dissimilar sampling environments using traditional ecological indices of diversity, evenness and richness

Materials and Methods

Media was prepared using 1 liter of distilled water and the pH was adjusted to 7.7 using 1M NaOH and/or HCl and then autoclaved.

ASW (Artificial Seawater)

Stocks

(1)	Extra salts	per liter
	$NaNO_3$	30.0 g
	Na_2HPO_4	1.2 g
	K_2HPO_4	1.0 g

(2)	Vitamin solution	per liter
	Biotin	0.0002 g
	Calcium pantothenate	0.02 g
	Cyanocobalamin	0.004 g
	Folic acid	0.0004 g
	Inositol	1.0 g
	Nicotinic acid	0.02 g
	Thiamine HCl	0.1 g
	Thymine	0.6 g
	(can be stored frozen at -20°C)	

Medium	per liter
"Ultramarine Synthetica" sea salts	33.6 g
Extra salts stock solution (1)	3.75 ml
Vitamin stock solution (2)	2.5 ml
Soil extract	25.0 ml
Tricine	0.50 g

Whole lobster shells were obtained from the Falmouth Fish Market. Shells were separated from the body of the lobster, broken into pieces and pulverized using a mortar and pestle. A total of three grams of lobster shell chitin ("lobster") was extracted and

ground from four different individuals. Shell chitin was dried overnight in a 37°C oven. Three grams of β chitin ("squid") were isolated from more than 120 squid obtained from a local Falmouth bait and tackle store. Individual squid were dissected and the gladius (squid pen) was excised. The squid pens were cut into small pieces, dried overnight in a 37°C oven and pulverized using a mortar and pestle. Dry, powdered, unbleached chitin ("powdered") was obtained from Sigma Chemical Company.

Plates were poured and partially cooled until the agar reached a semi-solid consistency. Because chitin does not dissolve in water, 0.2 grams of chitin (overall chiti ı concentration of 10 grams of chitin per liter of media) was spread on top of the plate and the majority was held on top of the plates by surface tension. The resulting plates therefore contained the chitin in a thin layer on the top of the plate, accessible to the inoculated microbes for use as a carbon source.

Samples were obtained from four areas:

1. Salt Pond (seawater)
2. Eel Pond (seawater)
3. "Surfside Beach" (seawater)
4. "Surfside Beach" sand (slurry prepared from sand sample)

One set of media plates was inoculated with 100 µl of either sample seawater (or a slurry prepared from the Surfside Beach sand sample) and a second set of plates were inoculated with a 1:100 dilution of the same samples in sterile seawater.

Results

Plates were inspected for growth every day after inoculation. In general, no obvious growth was detected before 5 full days of growth. After five days, colonies were formed only on actual particles of chitin and growth typically did not occur on parts of the surface of the plate without chitin. Irregularly shaped "clouds" of microorganisms formed on the chitin and appeared yellowish or translucent white by unaided visual inspection. Under a 40x power microscope, the densely populated colonies were obviously composed of several different cell types and no monotypic colonies were isolated, even after restreaking colonies onto new plates in several successive attempts.

Growth patterns were essentially the same for directly plated samples and 1:100 dilution samples. Given longer than five days to grow, diluted samples grew in the same irregularly shaped "clouds" directly on the chitin as undiluted samples.

Samples inoculated onto powdered chitin plates did not show growth after 10 days. The majority of powdered chitin did not stay on the surface of the plates and broke the surface tension and sank to the bottom of the plates, out of contact with inoculated microorganisms.

Day 5 Results

Surfside Beach Sand

α chitin

lobster: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms demonstrated motility. This sample had the most extensive growth of white and yellow "clouds" of microorganisms among lobster chitin plates.

powdered: no growth

β chitin

squid: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms did not demonstrate motility. This sample had the most extensive growth of white and yellow "clouds" of microorganisms among squid chitin plates.

Surfside Beach Seawater

α chitin

lobster: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms demonstrated motility.

powdered: no growth

β chitin

squid: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms did not demonstrate motility.

Eel Pond Seawater

α chitin

lobster: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds

of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms demonstrated motility.
powdered: no growth

β chitin

squid: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth on the chitin itself.

Salt Pond Seawater

α chitin

lobster: Growth of microorganisms resulted in the yellowing of most of the chitin on the plate. Close inspection revealed irregularly shaped clouds of microorganism growth (both rods and cocci) on the chitin itself. Most microorganisms demonstrated motility.

powdered: no growth

β chitin

squid: Generally the same as squid chitin plates from Eel Pond and the two Surfside Beach samples with the exception that there were colonies growing on the plate and not in contact with chitin. There were 10 colonies of this type on the non-diluted plate and it is possible that they were agar degraders, although no depression in the agar was visible. No similar colonies appeared on the diluted plate. Microscopic inspection revealed the presence of motile rods in these colonies.

Microscopic inspection of white and yellow "clouds" of microorganisms revealed that they are composed of numerous morphotypes. The community consisted of rods and cocci of varying sizes. Some were motile, some were not and the percentage of motile organisms differed between squid pen degraders and lobster shell degraders. As a general rule, there were a higher percentage of microorganisms demonstrating motility growing on lobster chitin than on squid chitin.

Subsequent attempts to streak and isolate types of microorganisms were unsuccessful. Microscopic inspection of streak plates all revealed a consortium of morphotypes. No differences in the number of types were obvious between the original inoculation plates and streak plates.

Molecular Investigations

Due to time constraints and the relatively slow growth of chitin degrading microorganisms in this study, the successful isolation of particular strains of microorganisms was not accomplished after repeated attempts. The original goal of RFLP analysis and sequencing of particular strains of chitin degrading microorganisms was not

reached. The original plan to compare differences in the chitin-degrading microbial community structure between proximate but dissimilar sampling environments using traditional ecological indices of diversity, evenness and richness was therefore not possible. No conclusions about the role of Archaeal microorganisms in the degradation of α and β chitin can be reached.

References

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