Mierobial Diversity Personal Project

Isolation of Cellulolytic Bacterium using Turnip as Cellulose Source (Substrate)

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Introduction

Interested in cellulose decomposition and in the microorganisms that produce the relevant enzyme systems has been stimulated by a desire for a greater understanding of one of the most important processes in nature. This, in turn, has been prompted by the need to optimize exploitation of the potential of cellulose as a source of energy and chemical feedstocks and to improve the efficiencies of digestion of fodder by ruminants.

Cellulose accounts, on average, for 50% of the dry weight of plant biomass, it also accounts for about 50% of the dry weight of secondary source of biomass, such as the surpluses, wastes of agricultural, forest, industrial and domestic origin. Cellulose is a homopolymer consisting of glucose units joined by β-1,4 bonds. The disaccharide cellobiose is regarded as the repeating unit in cellulose in as much as each glucose units is rotated by 180^0 relative to its neighbor.

Cellulolytic microorganisms are ubiquitous in nature, and representative species are found in many different generation and in a great variety of environments such as soils, swamps, seawater sediments, cotton bales, animal gut ect. Many enrichments have been done for cellulolytic bacteria and many different types of cellulolytic bacteria have been isolated and pure cultured. All of these, enrichments have used microcrystalline cellulose, carboxymethyl cellulose (CMC) or other forms of purified cellulose as carbon sources. These celluloses are quite different from the cellulose found in many vegetables or other natural sources. In that natural, cellulose is more hydrated than purified cellulose. As turnips contain a high concentration of hydrated cellulose, an enrichment using turnip slices as the cellulose source could turn up with new type of cellulose-degrading bacteria.

In this project, I am using boiled turnip mash as a substrate to isolate cellulolytic bacteria. Congo Red stain is used to indicate cellulose hydrolysis, Cellulose Azure is used as substrate to do cellulose assay.

Materials and Methods

Enrichment, Culture conditions and media: Enrichment: The sources of bacteria were the gut of a terrestrial snail found in Dr. E. Leadbetter's backyard and Eel Pond sediment. Boiled and autoclaved turnip slices was used as carbon source.
Media: All media contained (g / l)

K_2HPO_4	1
NH_4Cl	1
$MgSO_4.7H_2O$	0.05
$CaCl_2.2H_2O$	0.02 add water to 1000 ml.

In enrichment broth, turnip slices were made by boiling 3 times and then autoclaved; in the isolation media, turnip paste were made by boiling turnip slices 3 ~ 5 times, throw the water each time, then use blend mash the turnip into very fine paste solution; Washed Agar were made by Add 25 g Agar to 5 l distilled water, stirred for 1 hour then replace water for one cycle, generally 5 ~ 6 cycles, original 25 g agar used for 1000 ml media. Cellobiose (2 mg / ml final concentration), cellulose (ball milled filter paper, 1 mg / ml), CMC (1 mg /ml) and xylan (1 mg / ml) agar plate were also made by adding these sugars to the base media given above. Cellobiose, glucose and CMC broth media were made by adding those sugar solution to the autoclaved base media before inoculation. 10% cellobiose and glucose stock solutions were filter sterilized

Culture Conditions: Aerobic isolates were cultured in 37°C incubator. Anaerobic isolate was culture in room temperature under $N_2 + CO_2$ in the anaerobic chamber.

Congo Red test: Follow protocol according to Teather, after appropriate growth period, flood the plate with the Congo Red Solution (1 mg / ml in water) for 15 minutes. For CMC, pour off Congo Red and flood plate with 1 M NaCl for 15 minutes. Visualized zones of hydrolysis can be stabilized for at least 2 weeks by flooding the agar with 1 M HCl, which will change the dye color to blue and inhibit further enzyme activity.

Oxidase test: Use spot test oxidase reagent form Difco lab. Pick up colonies from plate, put them on the filter paper, drop one or two drops of oxidase reagent to the colonies, check blue color for positive result after 5 minutes. Colorless is negative.

Electron microscopy: Cell were negatively stained for microscopy. Carbon coated grids were used to absorb cells from well prepared bacterial cell suspension for 2 minutes. Uranyl acetate solution (1% wt / vol, pH 4.5) was used. Negatively stained preparations was examined using a Zeiss 10CA transmission electron microscope.

In Situ 16s RNA Hybridization: Follow Dr. Sandra A. Nierzwicki - Bauer's Handout.

Cellulose Assay: Use cellulose Azure as a substrate. Suspend 100 mg of cellulose Azure in 10ml phosphate buffer with no cellulose. In microcentrfuge tube, put 1 ml cellulose Azure solution and 1 ml cell culture incubate for different period time at room temperature. Centrifuge for 3 ~ 4 minutes, measure supernatant absorption at 570 nm, DH_2O and cellulose were used as negative and positive controls.

Results and Discussions

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Enrichment and morphology Enrichments were streaked on the isolation plate put under aerobic and anaerobic conditions. there were on growth from sea sediment. From snail gut, three aerobic and one anaerobic strain were isolated

Colony and Morphology

isolates	colonies	morphology	congo red test	oxidase test	Gram stain	aerobic or anaerobic
IS1	large white, very convex	single, thin but long cells, terminal endospore former	+	-	+	anaerobic
IS2	white, flat, irregular edge, look like mycelial colonies	large rod form filaments, middle endospore former, gliding	+	+	- (?)	aerobic
IS3	tiny, white , convex	rod or cocci, nonmotile, single or pair, nonendospore former	-	-	-	aerobic
IS4	large.white glistening, regular edge	short rod, motile, single no endospore	-	-	-	aerobic

From the turnip plate, IS1 and IS2 gave clear zones around the colonies, so this two strains were chosen to do further studies.

Physiology Inoculate IS1 and IS2 on different plates which contain different carbon source

	CMC	cellobios	cellulose	xylan	unwashed agar	washed agar
IS1	++	++	+	+	+	-
IS2	++	+	-	+	+	-

++: grow very well
+: grow well
-: no growth

IS2 was taken from plate and inoculated to liquid broth medium under room temperature, and there were no growth. But if add 0.01% yeast extract, all of them grow rapidly, the negative control which has no carbon source also grow, but not as turbid as other tubes with carbon source in it. So IS2 may need some growth factor from yeast extract.

16s ribosome RNA hybridization and cellulase assay
12 different 16 rRNA probes were used to do the hybridization. For the anaerobic isolation IS1, all the probes are negative, even universal probe. the reason could be due to the old culture which contain all endospores instead of vegitative cells. It 's hard for the probes to

JH

get into the cell and gave the signals. For IS2, prime A which is universal probe, was the only positive result. maybe this organism is beyend the detect range of all probes. Besides, there are some immature aspests of 16s rRNA techenics
Only IS2 was performed of cellulase assay. the mechenism for this assay is cellulose azure which is cellulose derivtive that has azure binding to cellulose monomer, when cellulose azure is degradated by cellulase, the product show optical absorption at 570nm. This assay took very long time, the positive control turn pink after 4 days (86 hours). the assay was carried out in room temprature, the positive controwas made by adding 25 mg purifed cellulase to 1 ml phosphate buffer. Distilled water was used as negtive control, the sample is more like negtive control. overall, this assay was a very rough cellulase assay. And more this assay was designed for complete cellulase system. IS2 probably don't possose a complete cellulase system since it didn't grow on cellulase plate, but it can use cellobiose and CMC, so it may produce endoglucanases or β-glucosidases, not the complete system, those bactera, as suggested by Beguin, called "pseudocellulolytic" in contrast to few bacteria that synthesize the complete enzyme system that could result in extensive hydrolysis of the crystalline material found in nature, which is called "true cellulolytic". It is possible that IS1 could be "true cellulolytic" because it grow on cellulose plate. Further studies have to be done to define this bacterium.

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Reference

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Microbial Diversity Personal Project

Isolation and Characterization of Anaerobic Cellulolytic Fungi from Anaerobic Environment

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Introduction

Cellulose is the most abundantly produced biopolymer in terrestrial environment. Each year photosynthetic fixation of CO_2 yields more than 10^{11} tons of dry plant material worldwide and almost half of the material consists of cellulose. Another major source of cellulose is municipal wastes.

Most Cellulose is degraded aerobically, but 5 - 10% is degraded anaerobically. Thus, vast quantities of cellulose are degraded by cellulose- fermenting microorganisms in habitats anaerobic. In the past fifty years, aerobic cellulolytic bacteria have been intensively studied, including thermophilic and mesophilis fungi. By contrast, anaerobic fungi have been ignored for a long time and were first discovered in 1976. They were found in the rumen and hindgut of herbivores. They have a nice developmental cycle, that is observable in vitro and the possess cellulolytic and other hydrolytic capabilities.

Anaerobic fungi isolated from ruminants have been studied extensively (such as *Neocallimastix* spp.).In contrast to strains isolated from non-ruminants (such as *Piromyces* spp.). Anaerobic fungi isolated from ruminant and non ruminant herbivores have a high digestion capacity for cellulose (Teunissen et al.1991). Species isolated from ruminants have been shown to produce extracellular enzymes such as exoglucanase, endoglucanase, B-glucosidase and xylanase activities in vitro, when grown on cellulose. Production of the enzymes is often associated with the ability to produce an exoglucanase, which acts as a cellobiohydrolase. Fungi from the rumen such as Neocallimastix species have been shown to digest cellulose with rates varying between 0.04 and0.06 g/1 h^{-1} (Bauchop and mountfort 1981; lowe et al. 1987 b; Philllips and Gordon 1989). *Piromyces* species, isolated from non-ruminants, could digest cellulose at a rate comparable to *Neocallumastix* spp.

Although free-living anaerobic cellulolytic fungi similar to those found in the rumen (124) have not yet been found, Durrant et al reported isolating , using anaerobic culture conditions, two strains of morphologically andphysiologically distinct cellulose - fermenting fungi from soil. Both strains grow and utilize cellulose more rapidly when incubated under microaerophilic conditions, and one strain degrades cellulose most rapidly in well-aerated culture. Active cellulose and xylanase systems are produced by both strains, and enzymes are present in culture supernatant fluids. Clearly, fungi may play a significant role in the anaerobic degradation ofcellulose in soils and sediments, and further studies are needed to exploretheir potential contributions.

The goal of my project was to isolate anaerobic cellulolytic fungi from different anaerobic environments such as the termite gut and Cidar swamp sediments. I am going to look at

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cellulose activity, fermentation end product (possibly pathway!) as well as their interaction with methanogens by H_2 transfer!

Methods:

Chemicals and solid substrates. All chemicals were of Analar grade and unless stated otherwise were made up in distilled water. All vitamins and antibiotics were obtained from Sigma. Whatman no. 1 filter paper was used for cellulose substrate and was treated with phosphoric acid (Wood, 1971) and then pebble milled for 4 d at 4°C.

Preparation of media. Media were prepared, stored and inoculated using the aseptic, anaerobic techniques of Hungate (1950), Bryant (1972) and Miller & Wolin (1974). Except for the reducing agent solution (prepared and dispensed under oxygen-free N.), liquid media and solutions used to prepared media were dispensed under oxygen-free CO_2. Solid media were prepared by adding agar (final medium concentration, 18 g/1) to basal solution.

Medium A. Medium A. contained, per litre: basal solution A, 830ml; water (when insoluble substrates were used) or glucose solution (37.5g/1), 100 ml; Na_2CO_3 solution (80g /1), 50 ml; vitamin solution, 10ml; and reducing agent solution, 10 ml. Sometimes 60 ml of the water was replaced with 50 ml antibiotic solution and 10 ml lysozyme solution.

Base solution A contained: yeast extract, 2g; Trypticase peptone, 2g; clarified rumen fluid (Bryant & Robinson, 1961), 150ml; mineral salts solution [as described by Leedle & Hespell (1980) except that the nitrogen source was 0.54g NH_4Cl /1), 75 ml; K_2HPO_4 solution (6g/1; Leedle & Hespell, 1980), 75 ml; haemin solution, 10ml; fatty acid solution, 10ml; resazurin solution (1g/1), 1 ml. The pH of basal solution A was adjusted to 6.8 with 1 M-KOH and the volume was made up to 830 ml with water or a suspension of cellulose (final medium concentration 10 g/1 for the cellulose overlay plates. Haemin solution was prepared by dissolving 0.1 g haemin in 10 ml ethanol and adjusting the volume to 1 litre with 0.05 M-NaOH. The fatty acid solution was prepared by mixing 6.85 ml acetic acid, 3.0 ml propionic acid, 1.84 ml butyric acid, 0.55 ml 2-methylbutyric , 0.47 ml isobutyric acid, 0.55 ml valeric acid, and 0.55 ml isovaleric acid with 700 ml 0.2 M-NaOH. The pH of the fatty acid mixture was adjusted to 7.5 with 1 M-NaOH and its volume was adjusted to 1 litre with water.

Vitamin solution was prepared in 5mM-HEPES buffer and contained (g/1); 1,4-naphthoquinone, 0.25; calcium D-pantothenate, 0.2; nicotinamide, 0.2; riboflavin, 0.2; thiamin. HCl, 0.2; pyridoxine.HCl 0.2; biotin, 0.025; folic acid 0.025; cyanocobalamin, 0.025; and p-aminobenzoic acid, 0.025. The reducing agent solution contained 2.5 g Na_2S , $0.9H_2O$ and 2.5 g L-cysteine.HCl in 100 ml water. The antibiotic solution contained (g l-1): streptomycin sulphate, 2; penicillin G, 8; chloramphenicol, 6; oxytetracycline, 5; neomycin sulphate, 6. The lysozyme solution contained lysozyme (4 g /1) plus EDTA (disodium salt; 3 g /1).

Basal solution A, Na_2CO_3, glucose, water and the reducing agent solution were pre-reduced (boiled and gassed with oxygen-free CO_2).

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Sterilization and preparation of liquid and solid media: The Antibiotic, lysozyme, vitamin, Rumen fluid and 10% cellubiose solutions were sterilized by membrane filtration (0.22 μm pore diameter), All other solutions and Agar media were autoclaved at 121°C for 20 minutes. Liquid media were dispensed in 3.75 ml volumes in thick-walled glass tubes Cellulose medium was stirred when dispense in order to suspend cellulose fiber as even as possible. After autoclave and before inoculation following solutions were added to each tube separately!

	volume ml / tube
Rumen fluid	0.75
Antibio Sol.	0.25
lysozyme + EDTA Sol.	0.1
cellubiose	0.15

For the roll tube media, first melt the Agar media in boiling water, then put into 45°C water bath, every solution was added under N_2 flow using Hungate techenics

Samples and environment culture: Three different samples were taken from different source. Fresh termite gut . Volta flame Pond sedment ,Cow Rumen . First have very heavy inoculation from sample to cellobiose broth media, then inoculate 1 ml to each of tubes of cellulose and cellobiose agar and broth media. For the roll tubes, as soon as inoculation, put the tubes into ice, roll it horizontally until media solidified.

Enrichments of termite gut & sedinvent were incubated at 30°C incubator, cow fluid samples was incubated at 37°C. Roll tubes were incubated vertically and all broth tubes were incubated horizontally.

Result and Discussion:

After 5 days incubation, yeast cells (only guess, due to big, budding cells) were found in the cellubiose broth enrichment. Some filements endospore former were also found in the culture from microscope. There are lots of cells attached to the filements. Those cells could be methanogen, because bacterial antibiotics don't inhibit methanogen growth, and those methanogen could use H_2 released from fungi fermentation. This is also why we incubate the broth culture horizontally. In the sediment and cow fluid broth enrichment, there were no growth, no filement or even no cells found. All roll tubes had no growth or visible colonies. The possible reason for this phenomenon could be: Termite gut and sediment: 1) there are no anaerobic cellulolytic inhabited in termite gut, or even some, they are extremely hard to grow *in vitro*. 2) They don't like the media which provides an rumen-like environment. 3) Exposed to air! Cow Rumen fluid: The sample was taken 2 days before enrichment, and stored a long time in the fridge, this could be fatal to rumen fungi, because they really don't like low temperature. Generally, the rumen digesta were collected by suction into CO_2 filled tyermos flasks. Then enriched with milled barshey straw, the substrate could be another important reason. They probably prefer to use barhey straw other than pretty pure cellulose paper.

Morphologically, anaerobic fungi could be single cells. So the big budding cells could be another species of fungi. The possibility has to be further tested and those single cells cellulytic ability needs to be characteristicized!

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