Use of Canada Balsam as a Selective Agent and Possible Substrate in the Enrichment of Termite Hindgut Microbiota

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ABSTRACT

The termite gut microbiota naturally encounter high levels of resin compounds. I show that they are resistant to high levels of resin in culture, and that this resistance can be used as a selective technique for termite hindgut anaerobes. Suspended resin compounds are precipitated, solubilized, chemically degraded or metabolized in the course of culturing hindgut organisms. An attempt was made to determine whether metabolism was, in fact, occurring; results were inconclusive.

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INTRODUCTION

The intestinal tracts of most animals include oxygen-poor and anoxic environments suitable for the growth of anaerobes. Beyond this basic similarity, however, the gut environment varies: different animal species have distinctive diets which in turn provide a distinctive, species-specific gut environment.

This specificity has been used to enhance the success of culture techniques; for example, rumen fluid is used to culture the rumen microbiota of cattle (reference). For other animal gut cultures, this approach, while feasible, is less practical. In these cases, introducing compounds characteristic of the host diet in the culture medium may increase both specificity and yield of gut microbes. To do so, a compound should have the following characteristics: First, it should be something that the anoxic gut microbiota would actually encounter; that is, it should not be rapidly metabolized by the host early in the digestive process. Next, the compound should be characteristic of the standard diet, so that the microbiota are likely to be well-adapted to its presence. Ideally, active selection might be possible. In any diet, there are generally compounds present that have some level of anti-microbial activity; if these can be detected and employed, they might encourage growth of the "true," relatively resistant gut microbiota by concurrently inhibiting growth of sensitive adventitious organisms. Finally, added compounds might serve as a nutrient source for the gut microbiota. It should be noted that while this is the most obvious role for a medium-additive, it is not actually a necessary one; culture success depends on selectivity as much as upon adequate nutrition.

I have investigated the use of mixed resin compounds (Canada Balsam) in the culturing of obligate and facultative chemolithotrophs from the termite hind-gut. The termite diet is distinctive for the range of noxious resin compounds encountered; this, along with the exceptionally high concentration of cellulose, would serve to define the diet. Therefore, it is reasonable to expect that the gut microbiota survive in the presence of resin compounds. It is also possible that resin, which contains both volatile aromatics and larger, multicyclic organic compounds, serves as an auxiliary carbon source. A variety of acetogenic bacteria and a few methanogenic archaea are facultative chemolithotrophs, and are known to metabolize such simple compounds as sugars, alcohols, organic acids and amines (reviewed in Gottschalk, 1992, see also Schink 1994); among known termite-gut isolates, one, *Acetonema longum*, falls into this group.

Because resin derivatives have a wide range of uses not yet subsumed by petrochemical equivalents, metabolism of resins is potentially of wide interest. In art conservation, controlled removal of photochemically darkened resins and waxes might be useful; by contrast, conservation of old wooden structures requires that pine tars and pitch be preserved.

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METHODS

Enrichments

The hindgut of two termites (Woods Hole specimens, species undetermined) were dissected in a drop of AC21 reduced medium (Breznak reference). A methanogen-enriching primary culture was prepared using AC21 anoxic culture tubes (3 ml liquid medium under H_2/CO_2 gas phase at a pressure of approx. two atmospheres, incubated at 30°).

Upon evolution of methane, the culture was sub-cultured at high concentration (1:10 dilution) on AC21 bottle plates containing increasing concentrations of Canada balsam (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 % weight/volume Canada balsam). These plates were prepared by adding the appropriate volume of a 10% solution of Balsam in EtOH, then purging the EtOH (and balsam volatile compounds) by repeated gas exchange over the cooling agar medium, initially with moderate shaking to promote boil-off (at approx. 75-60°).

After colony formation, excess liquid medium from the base of the bottle plates (turbid run-off from plating) was used to inoculate further AC21 liquid cultures, in a limiting dilution series (10^{-2} to 10^{-10}). At most dilution levels, tubes were set up containing either .1% Canada balsam in EtOH, EtOH alone, or no additions. Finally, roll tubes were inoculated either by streaking or by addition of liquid culture from the (supposedly) limiting dilutions.

For bottle plates, CB was autoclaved with the medium either once or twice; for roll tubes, it was added prior to melting of the agar by boiling; and for liquid cultures, it was added without further heating. Heat-based hydrolysis of resin compounds could play a roll in rendering them metabolizable. In the future, all CB-containing media should be autoclaved after aliquotting and addition of CB.

Metabolic analysis

Evolution of methane was detected by gas chromatography (GC). Methanogens were also detected by characteristic A_{420} fluorescence. Potential metabolism of Canada balsam was quantified by extraction of both pellet and supernatant fractions (2.0 ml) with 1 or 2 ml methylene chloride (respectively). After vacuum evaporation of methylene chloride, constituents were re-suspended in the appropriate HPLC solvent phase and analyzed on a C18 column. As a variant on the ramped HPLC method of Kutney et al. (1988), two non-ramped HPLC runs were performed to elute different resin acid compounds. The two solvents used were 87% and 70% methanol containing .1% acetic acid as carrier, at a flow rate of 1.5 ml/min.

Hybridization

To predict possible drug resistance among the bacteria (a potentially useful aid to further directed purification of the enrichments), colony lifts were taken from one of the bottle plates and colonies were probed for Tet Q and Tet M resistance genes. Filter preparation was essentially as by standard methods (e.g. Sambrook et al.) with extended denaturation and (for one filter) autoclave lysis (1 min). Probe hybridization and detection were as described in the Dupont NEN Renaissance Kit (TM).

Gram Stain

Staining was done following the directions in the stain kit.

RESULTS

Canada Balsam is Tolerated

As hoped for, the termite gut contains microorganisms that are unusually resistant to high levels of Canada Balsam (CB). In fact, growth was observed on bottle plates up to a level of .3% CB. In comparison, when resin acids are used as a growth substrate or nutritional "challenge" for bacteria and fungi that metabolize them aerobically, the concentration used is in the range of .003-.004%. Because CB contains a range of acidic and non-acidic resins, it is unwise to assume that toxicity is equivalent. However, the resin acids are a considerable proportion of the ethanol-soluble fraction of CB (certainly more than one part in a hundred, and probably more than one part in ten). Anecdotally, it should be noted that observation of termite gut contents shows fluorescent globules that resemble the globules of CB seen under these culture conditions, further supporting the assertion that CB is a natural constituent of the termite hind-gut contents.

Canada Balsam Enhances Growth

The growth of methanogens and bacteria on the bottle plate with no CB was feeble. Eventually (> 10 days) a lawn of micro-colonies began to appear. By contrast, growth of individual colonies was vigorous on/in bottle plates containing .003 and .03% CB. It was more difficult to assess relative growth rates in liquid culture because CB forms a milky suspension in the liquid medium. CB also can interfere with microscopic analysis because the suspension includes many droplets that are in the size-range of bacteria. As mentioned above, these also fluoresce during A_{420} detection of methanogens. After several days (10+), growth was clearly no worse in CB tubes than in tubes without CB.

Canada Balsam is Selective

Upon observation of the (relatively) vigorous growth on/in CB-containing bottle plates, distinct colors and morphologies could be seen. When picked for microscopic analysis, the rare, larger, opaque cream-colored colonies were found to correspond to a methanogen (Fig. 1a). Additionally, the major methanogen seen (by A_{420} fluorescence) in the primary culture was enriched in the fluid "run-off" at the bottom of the .1% CB bottle plate, compared to the balsam-less plate (Fig. 1b). However, this selectivity has its limits, at least with respect to the methanogens. Methanogen-like colonies made up considerably less than 1% of the colonies on any of the plates. As expected from this number, methanogens were not brought into pure culture by a limiting dilution strategy (Fig. 1C, and dilutions beyond 10^{-8}--not shown).

Canada Balsam is Altered

Tubes inoculated with a dense culture of bacteria showed a clearing of the CB suspension within three days. Tubes inoculated with a more dilute culture "cleared" after longer incubation. Control tubes (no cells) never "cleared." At the same time, flocculent particles appear, and drop out of solution as a loose precipitate. One possibility is that the CB is somehow being metabolized (see discussion). To determine if metabolism was happening, HPLC analysis of the culture was carried out. Three vials from the dilution series were used. The 10^{-6} dilutions were chosen because growth had been vigorous but not instantaneous (assuring that effective selection could have taken place). These vials showed a range of cell types by microscopic examination (Fig. 1c) as did roll tube cultures derived from them (Fig. 1d).

Unfortunately, GC analysis was impeded by the finding that the gas phase over a supposedly pure CB and ethanol mixture was evolving a peak overlapping or corresponding to methane (Fig. 2a). The shift in the relative size of methane and other peaks, therefore, should be treated with caution. HPLC analysis was impeded by the too-small sample size. For both pellet and supernatant fractions, the CB-specific peaks were hard to detect relative to the baseline. One HPLC run was intended to show any drop in slower-eluting substrates; the other, to show any rise in the (generally) faster-eluting metabolites of these substrates. No reproducible change was seen for substrates (data not shown). The results for metabolites were only slightly more suggestive, though again signal-to-noise ratio was very low. However, there was a suggestion of a new or amplified peak (Fig. 2b). The composition of this peak remains unknown.

Gram Staining and Colony Hybridization can be Done

In the absence of positive controls, staining was inconclusive (Fig 1d). One set of spore-forming long rods may be positive, though the spores themselves are

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not. Colony hybridization was also negative. It was unclear whether this was due to the choice of probes, or to the fixation of adequate DNA on the filter. At a minimum, we now know that it is possible to lift colonies cleanly from a bottle plate. (It should be pointed out that this destroys the plate--at least, no re-growth was seen after 10 days additional incubation.)

DISCUSSION

Canada Balsam may turn out to be a useful selective agent. For pure culture of methanogens, it should probably be combined with a more standard antibiotic selection scheme (to enhance the proportion of archaea). A variety of spore formers seem to survive CB and ethanol selection. It is unlikely that they thrive both in the presence and absence of CB--if they were metabolizing efficiently, they presumably would not sporulate in large numbers. Because many of the (putative) spores were seen following a mixed culturing strategy (.1% CB plate run-off----> .1% CB liquid culture---->non-CB roll tube), it is hard to say where proliferation occurred and where sporulation occurred.

The whole question of Canada Balsam utilization is difficult to approach. For example, it would be unprecedented for methanogens to metabolize multi-carbon cyclic compounds. Yet, on CB plates, methanogens colonies grow reasonably well. In addition, the evolution of methane from the bottle plates is essentially undetectable, in spite of the presence of "methanogens." This could mean either that the colonies are not numerous enough to produce much methane, or that the methanogens have an alternate metabolism that they employ while growing on CB. Because all platings have been quite dense, it is (remotely) possible that diffusion from nearby colonies of bacteria could provide metabolically-limited methanogens with bacterially-produced CB metabolites. Due to the presence of a large volatile peak in the less-sparged ethanol-CB liquid cultures, evolution of methane from those cultures could not be meaningfully assessed.

HPLC analysis is both sensitive and flexible enough to approach questions of this nature; however, the run conditions must be optimized, and the size of the cultures used for analysis should be scaled up to at least 50 ml each to bring the signal associated with any one compound up above the general drift of the machinery and above the levels of generalized contaminants. Also, as a technical tip, it is important to flush the HPLC injector with the soluble phase at least 4 or 5 times between runs, as resin compounds stick avidly to a variety of surfaces (including glass).

Another interesting possibility is that resin compounds optimize some condition not directly related to metabolism. For example, they could improve adhesion t the agar surface, and also provide a non-aqueous substrate for growth in liquid culture. As a counter-argument, however, droplets of CB in liquid culture were

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not associated with attached microbial growth. Alternately, the effect on the microbiota and on the balsam could have been affected indirectly, perhaps by mutual contributions to a change in pH.

Finally, it is possible that CB supports microaerophilic growth, and that this growth helps to return the enrichments to truly anoxic conditions optimal for growth of methanogens and other obligate anaerobes--in other words, the effect is biologically mediated by transient growth of organisms that do not grow to (or remain at) high density, and would therefore not be observed at advanced time points.

REFERENCES

FIGURES

Figure 1
a) differential growth is already seen on the first set of bottle plates. methanogens are present primarily in the CB-containing plates (e.g. arrowhead, bottom left and bottom right), while filamentous chains are forming in the CB-less plate. This may correspond to the first step in spore formation seen in later cultures. Blue = fluorescence characteristic of methanogens; green = CB autofluorescence (as also seen in termite guts).

b) example of a methanogen colony "pick" from the .1% bottle plate--again, note blue fluorescence. Also note the internal structure (gas? inclusion?).

c) Serial dilutions aid selectivity of the enrichments. "-N" = power of the dilution; matched dilutions contain AC21 alone, AC21 + 1% EtOH, or AC21 + .1% CB and 1% EtOH.

d) Spore formers predominate following passage from CB to non-CB media; this also suggests possible selectivity in action, and growth followed by nutrient limitation.

Figure 2
a) In GC, methane is possibly evolved as a peak that is also present in CB directly; these are the main peaks present (not identified).

b) HPLC analysis shows a possible increase in an unidentified peak of the cells + CB + EtOH sample that is not present in cells alone or cells + EtOH. However, a similar peak is present when a dense culture grown in the absence of CB has CB added immediately prior to sample preparation. Therefore, if this is a degradation product, the degradation must happen quickly and (presumably) via an extracellular process.

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Bryant, M. and I. Robinson 1961. An Improved Non-Selective Culture Medium for Ruminal Bacteria and its use in Determining Diurnal Variation in Numbers of Bacteria in the Rumen. Journal of Dairy Science 44: 1446

Kutney, J. et al. 1988. Biotransformation of Dehydroabietic, Abietic and Isopimaric Acids by Mortierella isabellina Immobilized in Polyurethane Foam. Applied and Environmental Microbiology 54: 1015-1022

Mohn, W. 1995. Bacteria Obtained from a Sequencing Batch Reactor that are Capable of Growth on Dehydroabietic Acid. Applied and Environmental Microbiology 61: 2145-2150

All Other References are to course handouts and xeroxes, with general information taken from The Prokaryotes.

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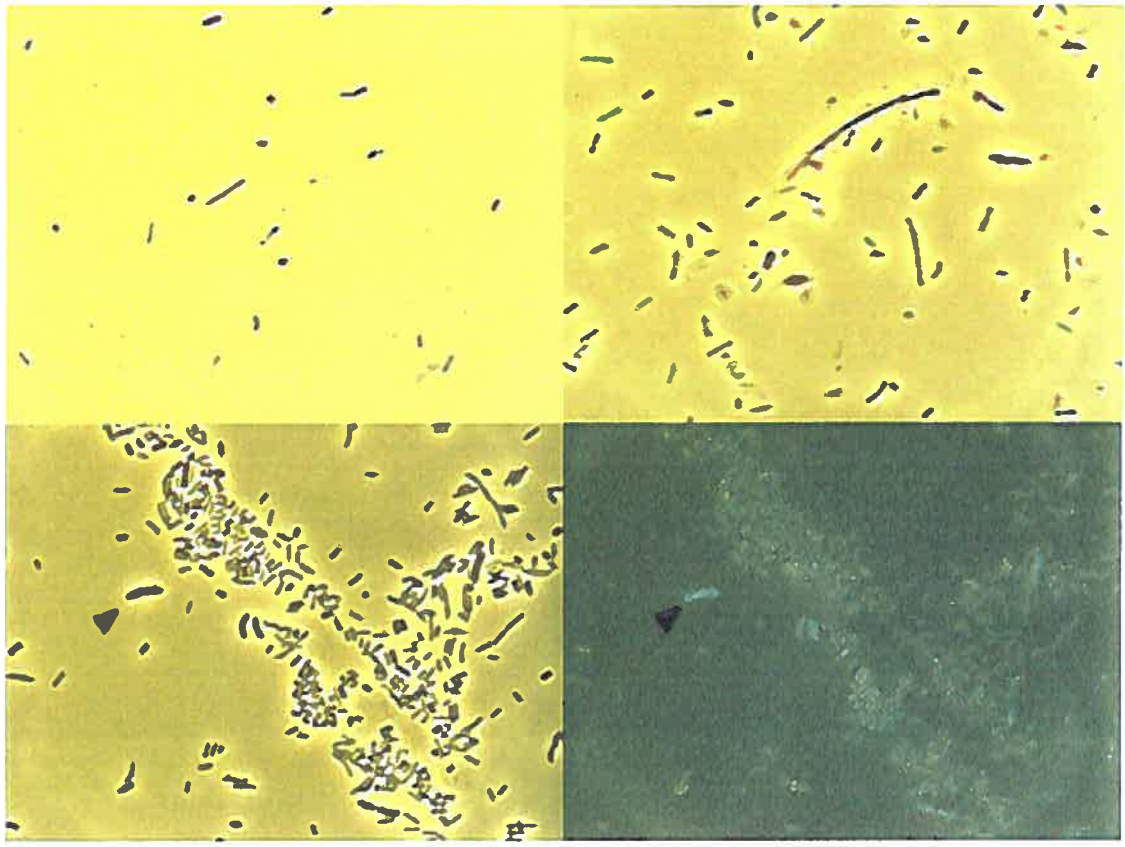
1a

bottle plate "Ø" no canada balsam

bottle plate .1 % canada balsam



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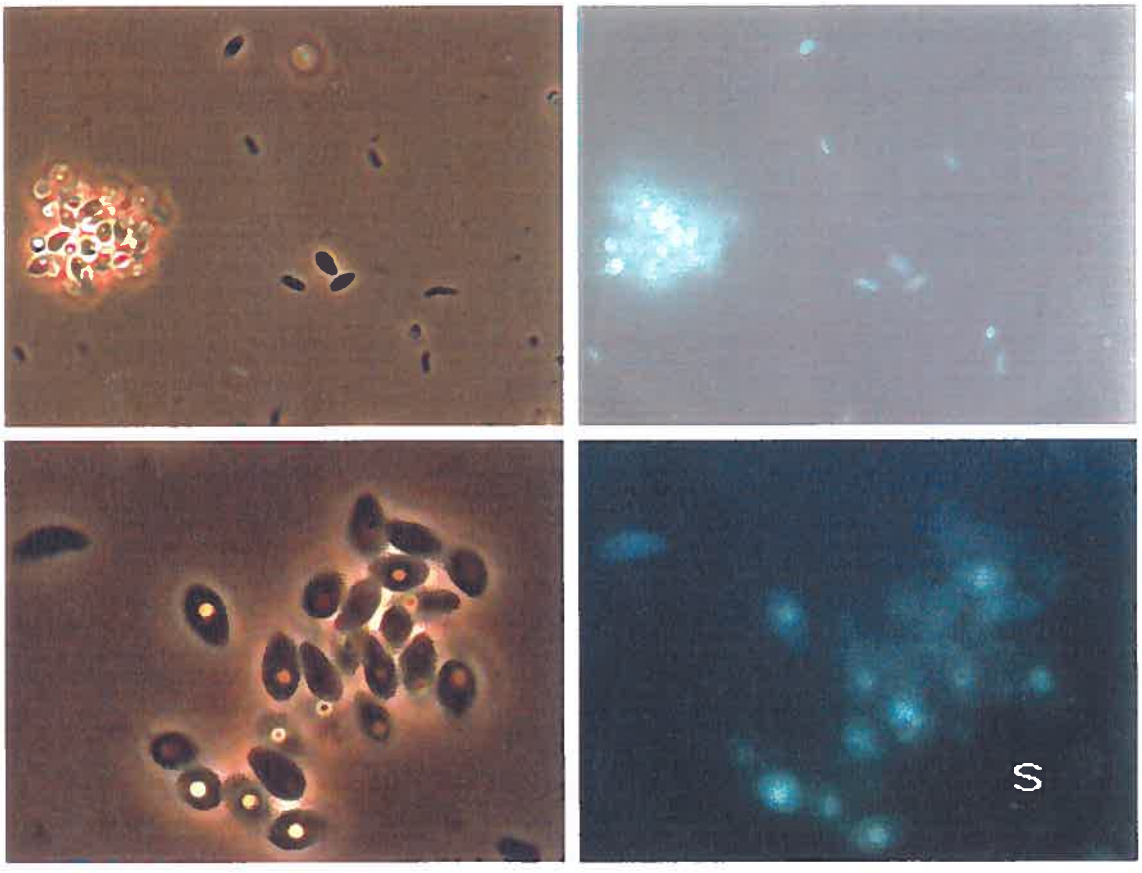

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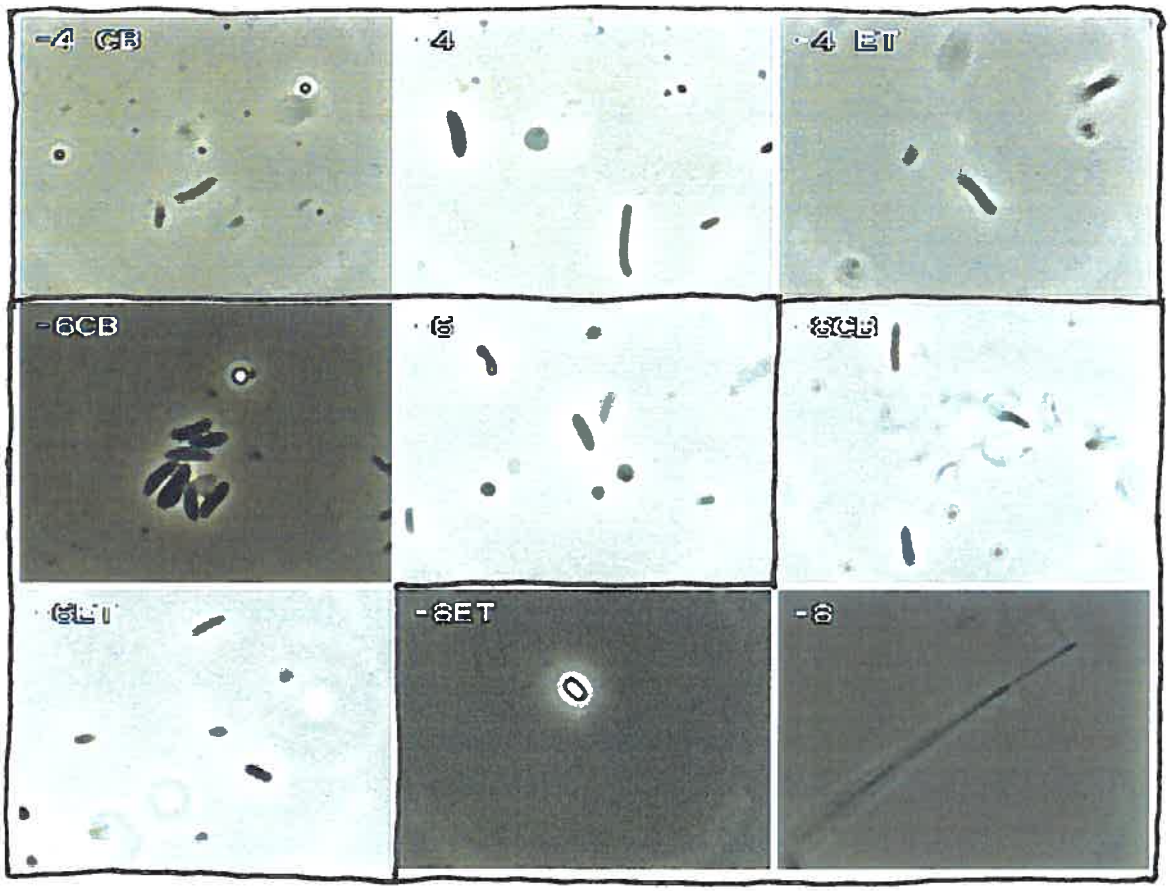
1c



1d

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liquid from non-CB roll tubes from CB liquid cultures gram stain

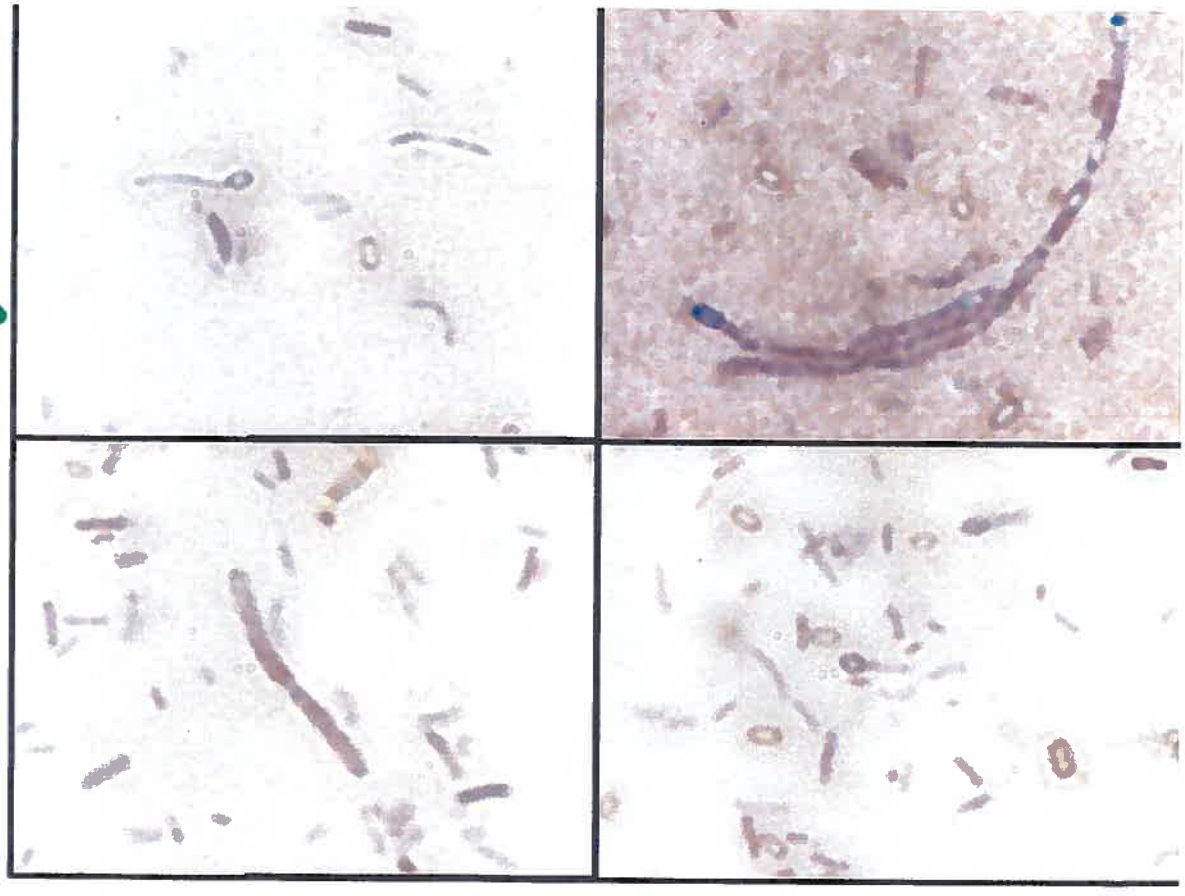

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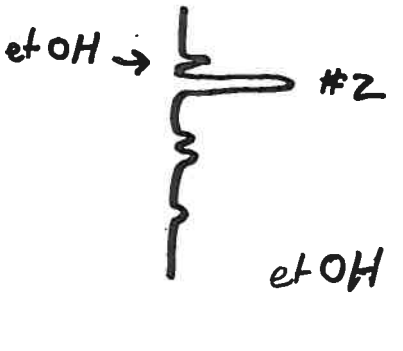
Figure 2

a)



± medium
± cells

.003 % CB ~1:1:1:1
.03 % CB ~3:4:2:2

b)