Degradation of lignin monomers and oligomers by a consortium of anaerobic bacteria

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Introduction

Lignocellulose constitutes more than 50 percent of the global carbon fixed by photosynthesis, but lignin is rarely preserved in marine and terrestrial sediments indicating extensive degradation under both aerobic and anaerobic conditions (Colberg, 1988). Lignin degradation has to be understood in terms of processes involved in the initial cleavage of ether-linked subunits and the subsequent degradation of substituted aromatic rings. Derivatives of syringic acid, vanillic acid, and cinnamic acid are the most important constituents of lignin (Colberg, 1988). Anaerobic cleavage of these aromatic monomers has been suggested to proceed in at least 3 different situations: (a) by bacterial sulfate reducers (Kaiser and Hanselmann, 1982); (b) in consortia with fermenting bacteria coupled to methanogenesis (Colberg, 1988); (c) by acetogenic bacteria (Daniel et al., 1988).

A general reaction scheme for the aromatic degradation has been outlined by Colberg (1988). Metabolism of monomers appears to take place by demethylation of methoxy substitutes and subsequent hydration of oxygen moieties to form hydroxy-substituted phenols (Fig. 1). Degradation then proceeds by fermentation of the benzene ring to 3 acetate molecules. In the presence of methanogenic bacteria, the final end products of this degradation sequence are CO_2 and CH_4. For bacterial sulfate reducers, metabolism of syringic acid is thought to proceed via demethylation of the two methoxy group to form gallic acid. Gallic acid is then further

decarboxylated to form pyrogallol (Hanselmann et al., 1995). In the presence of sulfate, the stoichiometry of these reactions may be summarized by the following reaction:

$$R\text{-}OCH_3 + 0.75SO_4^{2-} \rightarrow HCO_3^- + 0.25H^+ + 0.75HS^- + R\text{-}OH$$

where R indicates the substituted phenol ring. Pyrogallol is finally broken down to 3 molecules of acetate. The observed sequence of degradation products suggests a consortium of microorganisms that may sequentially degrade these aromatic compounds, whereby each degradation step is contingent upon the preceding breakdown reaction. It is uncertain whether the same organisms that are responsible for the initial demethylation of the substituted aromatic ring are also responsible for the production of acetate during the cleavage of the aromatic ring, or whether whether the same organisms is capable of metabolizing different aromatic acids. Furthermore, little is known about syntrophic relationships in the degradation of lignin.

While it is by now well established that many anaerobic bacteria are capable of degrading the aromatic monomers of lignin, there is remaining uncertainty about their ability to cleave the polymeric structure of lignin in order to release the aromatic monomers. Until recently, it was thought that white-rot fungi were responsible for the initial lignolytic step. However, a study by Colberg and Young (1985) showed that aromatic monomers could also be produced by methanogenic cultures, but it is not known whether sulfate-reducing bacteria or acetogens are also capable of cleaving the ether-linked oligophenols.

In order to establish whether complete degradation of aromatic monomers can be accomplished by a single group of organisms or whether complete degradation requires a consortium, incubations of bacterial sulfate reducers were prepared using inocubations that contain isolates of sulfate-reducing, methanogenic and acetogenic bacteria. Batch cultures were prepared to enrich for sulfate reducers, acetogens, and methanogens. Differences in the composition and concentration of the metabolic products were used to infer the ability of a specific microbial group to carry out specific degradation steps. Inferences about possible syntrophic relationships can be made by studying the metabolic products of isolates and comparing these with metabolic products obtained by groups of organisms.

Materials and Methods

1ml of an inoculum of EGSB granular sludge was used to enrich for sulfate reducers, acetogens and methanogens. Samples were incubated in an anaerobic medium with the following composition (g/l): NaCl (1.2), NH_4Cl (0.3), KCl (0.2), $MgCl_2.2H_2O$ (0.4), $CaCl_2.2H_2O$ (0.1), K_2HPO_4 (0.26g), $NaHCO_3$ (3.4g), rezarurin (0.5mg), $Na_2S{\cdot}9H_2O$ (0.2g), cysteine (0.2), $NiCl_2$ (0.27 μg), Na_2SO_4 (2.8), $FeSO_4$ (60 mg), vitamin solution (10ml), trace element solution (1ml). The salt solution was boiled and cooled down to room temperature under N_2. Aliquots were filled into serum vials, stoppered, crimped, and autoclaved. Bicarbonate, trace element solution, and vitamin solution were added after filtration through a 0.2μ filter. Next, the gas phase was changed to N_2/CO_2 (4:1) to eliminate oxygen from medium and headspace. Substrates (i.e., syringic acid, vanillic acid, and tannic acid) were added to a final concentration of 10mM. Introduction of substrate occasionally caused the introduction of oxygen to the vial. Excess oxygen was scrubbed using a 2.5% solution of Na_2S/cysteine. Enrichments for sulfate reducers were inoculated with 20mM Na_2SO_4 solution. Enrichments for acetogenic and methanogenic bacteria were incubated without Na_2SO_4. Acetogenic bacteria were enriched by suppressing methane formation from methanogens by the addition of 2-bromoethanesulfonic acid (BRES) to a final concentration of 5mM.

Samples were incubated at 65°C and checked for growth. After turbidity occurred, samples were transferred to a secondary enrichment. Growth occurred in the secondary enrichment of acetogens, methanogenic, and sulfate reducing enrichments. After growth occurred in the secondary enrichment after about 4 days, 1ml of the liquid enrichment was transferred into roll tubes containing the same concentration of substrate and Na_2SO_4 as the liquid enrichments. After the development of colonies, two colonies were picked from each roll tube and reinoculated in liquid enrichments with the same substrate concentrations.

The ability of this consortium of anaerobic microorganisms to metabolize aromatic acids of slightly different structure was tested by transfer of the syringic acid enrichment into an inoculum with vanillic acid as the substrate. Finally, to test the ability of these microorganisms

utilize oligomers of lignin, the cultures were incubated with tannic acid as the main carbon source and monitored for metabolic products.

Analytical methods

Syringic acid, vanillic acid, gallic acid, and pyrogallol, catechol, 3,5-dihydroxy-4-methoxybenzoic acid, protocatechuic acid were analyzed by HPLC on a Waters LC Module 1 equipped with a UV detector set at 254 nm. The flow rate was 0.2 ml/min, the column was a Waters Bondapak C18 column (length 3.9 x 150 mm, 125 Å). A mixture of methanol:H_2O: acetic acid in the ratio of 19:76:5 adjusted to a pH of 2.6 was used as eluent. Under these conditions it was not possible to separate gallic acid and pyrogallol so that quantification of these individual products was not possible. Elution times and concentrations were calibrated using standards of known concentrations. Degradation of vanillic acid resulted in an unidentified peak that was distinct from the other peaks. It is tentatively identified as catechol or protocatechuic acid. Similarly, an additional peak occurred in the degradation of syringic acid. This peak is distinct from pyrogallol and gallic acid. Based on literature it is possible that this peak represents 3,4-dihydroxy-5-methoxy-benzoic acid (Fig. 1). This compound has been recognized by various authors as an intermediate in the anaerobic degradation of syringic acid (Hanselmann et al., 1995; Colberg, 1988; Phelps and Young, 1996)

Actetate, proprionate, and butyrate were separated by HPLC using a Shodex Rspak column with a Rspak KC-G pre-column and were detected using a Shimadzu refractive index detector RID 6A. The eluent was a 0.2% solution of KH_2PO_4. Flow rate was 0.5ml/min. Elution times and concentrations were calibrated using standards of known concentrations.

Dissolved sulfate was quantified gravimetrically after acidification to pH=2 by precipitation with $BaCl_2$. Dissolved sulfide could not be quantified because the medium contained 1 mM sulfide and a 2.5 % mixture of Na_2S/cysteine was used to remove excess oxygen introduced during sampling of the enrichment.

Microscopy

Once turbidity was observed in the liquid enrichments, bacteria were inspected using wet mounts. Presence of methanogenic bacteria was tested by using the blue fluorescence of the F420 cofactor that is characteristic of methanogenic bacteria.

In-situ 16S rRNA hybridization of roll-tube enrichments

Isolated cultures in roll tube enrichments were hybridized using group-specific probes to establish their general phylogenetic position. 500 µl of the liquid enrichment was centrifuged at 13000 rpm for 5 minutes. The pellet was reconstituted in PBS and centrifuged twice. Reconstituted pellet diluted 1:50 with PBS. 15 µl of the mixture were applied to slides subbed in 0.1% gelatin and 0.01% $CrK(SO_4)_2 \cdot 12H_2O$ solution. Slides were dried at 37°C. Next, cultures were treated ethanol/formaldehyde (90/10 v/v) for 5 minutes at room temperature, and then washed twice iwth deionized water and dried at 37°C. After drying, hybridization mixes containing fluorescent group-specific 16S rRNA oligonucleotide probes characteristic for sulfate reducers, delta-bacteria, groups with low G+C content, and archaea were added to the slides. 40 µl of probe was applied to each spot on the slide. Samples were stored in the dark at 37°C overnight. The next morning, the slides were washed 3 times in 1 X SET for 20 minutes each, and dried afterwards at 37°C. Finally, mounting fluid was added to each spot on the slide and slides were covered.

PCR-amplification and sequencing of isolates from sulfate-reducer enrichment experiment

A more detailed identification of organisms present in the enrichments was attempted for a culture that was grown on syringic acid with Na_2SO_4 and isolated in a roll tube. However, PCR amplification failed - most likely because the cells did not lyse with the gene releaser used. Time constraints limited continuation of these experiments.

Results

Growth

Growth was observed in the secondary enrichments for sulfate reducers, methanogens and acetogens after 4-5 days. 1 ml of the liquid enrichment was transferred to a dilution series and diluted to a final concentration of 10^{-5} and 10^{-7}. 1ml of the diluted sample was inoculated in roll tubes at 65°C. After one week, small colonies appeared in the roll tubes of reducers enriched with Na_2SO_4/syringic acid, and with BRES/syringic acid. No colonies grew in enrichments for methanogens. 2 colonies each were picked from the roll tubes, and reinoculated in liquid enrichments. Isolated cultures grew within 5 days, and were inspected by bright light microscopy.

Phenotypic characterization

The enrichments contained small, non-motile rods, small filamentous bacteria, and spore-forming, rod-shaped bacteria. The secondary enrichments contained a mixture of these organisms indicating that the chosen media were not sufficiently selective for one group of organisms. Blue fluorescence characteristic of the enzyme F420 indicated the presence of methanogenic bacteria (Fig. 2). The same wet mounts also showed a number of bacteria that did not fluoresce suggesting sulfate reducers and/or acetogenic bacteria. Thus, the secondary enrichment containing syringic acid and Na_2SO_4 did not enrich exclusively for sulfate reducers which are thought to outcompete methanogens at high concentrations of dissolved sulfate. In contrast, roll tube enrichments contained only small, rod-shaped bacteria that did not fluoresce.

16S-rRNA in-situ hybridizations

Secondary enrichments on Na_2SO_4/syringic acid, Na_2SO_4/vanillic acid, and isolated cultures from roll tubes enriched with syringic acid/Na_2SO_4 were characterized using 16S rRNA in-situ hybridization (Nierzwicki-Bauer, lab protocol). A universal primer, and 3 group-specific primer labelling regions in 16s rRNA molecule each characteristic of archaea (methanogenic bacteria), sulfate-reducing bacteria, and bacteria with low G+C content (some acetogens). Unambiguous identification of sulfate-reducing bacteria was possible for the roll tube enrichment

containing syringic acid and Na_2SO_4 at the dilutions 10^{-5} and 10^{-7} (Fig. 3a). Similarly, hybridization was strong in the enrichment with vanillic acid/ Na_2SO_4 (Fig. 3b). Hybridization of ribosomal RNA with the other probes was much weaker, but nevertheless detectable. Cultures putatively identified as sulfate reducers isolated in roll tubes on Na_2SO_4/syringic acid also fluoresced with the low G+C probe. Desulfotomaculum has G+C contents between 20 % and 30% (Widdel and Hansen, 1992). It is therefore possible that the low G+C probe also hybridized to the 16s RNA of sulfate reducers. Similarly, a weak hybridization with the Archaea probe was observed in samples from the secondary enrichment with syringic acid and Na_2SO_4. In this case, however, it was also evident from fluorescence microscopy using the F420 fluorescence that the enrichments were not pure cultures and contained both methanogens and other bacterial groups. Roll tube picks from enrichments for acetogenic bacteria growing on syringic acid (dilution 10^{-5}) showed weak fluorescence with low G+C probes, Archaea probes, and probes for bacterial sulfate reducers. In order to better address the reliability of this technique, control samples and hybridization temperatures other than 37°C would be necessary. On the basis of these data it is not clear whether acetogenic bacteria are present in the enrichments. More unambiguous identification of acetogenic bacteria would have required PCR amplification and DNA sequencing using specific acetogenic primers.

HPLC-analysis

Table 1 summarizes the analytical results using HPLC, gas chromatography for methane analysis, qualitative analysis of free sulfide, and gravimetric analysis of dissolved sulfate. The dates listed above the columns indicate sampling dates. The initial substrate concentration was 10 mM for syringic/vanillic acid and 20 mM for Na_2SO_4 with the exception of M4 with vanillic acid where an error occurred during inoculation of the vanillic acid. The initial concentration of the substrate is not known for this sample. All enrichments on syringic acid and vanillic acid showed sequential decomposition of substrate. Syringic acid was first degraded to 3,4-dihydroxy-5 methoxy-benzoic acid, followed by degradation to gallic acid/pyrogallol. Vanillic acid was readily degraded to protocatechuic acid and further to catechol. Concentrations of residual sulfate can be used to determine whether breakdown of the aromatic ring has taken place.

Complete oxidation of syringic acid to CO_2 requires the transfer of 36 electrons, or an equivalent 4.5 moles of sulfate. For complete oxidation of syringic acid with an initial concentration of 10 mM, an equivalent of 45 mmoles of Na_2SO_4 is necessary. Complete degradation to 3,4-dihydroxy-5 methoxy benzoic acid requires an equivalent of 6.25 mmoles of sulfate. Continuing degradation to pyrogallol requires an additional 7.5 mmoles of sulfate. Thus, a total of 13.75 mmoles of sulfate is required to completely degrade all syringic acid to pyrogallol. Complete demethylation of vanillic acid to catechol requires an equivalent of 7.5 mmoles of sulfate. Degradation of the aromatic ring will have started when no intermediate degradation products are present, and sulfate concentrations are less than 6.25 mM and 12.5 mmoles in syringic acid and vanillic acid enrichments, respectively,. In the enrichments measured, these conditions are not met. Concentrations of dissolved sulfate are higher, and intermediate degradation products are still present. These observations do not preclude the potential breakdown of aromatic acids, but it is unclear whether sulfate-reducers, fermenters, or methanogens are responsible for this degradation step.

A surprising observation was the late detection of methane in inoculae from roll tube isolates that were thought to be sulfate reducers based on their fluorescence with 16S rRNA nucleotide probes. Methane was not detected when these enrichments first showed growth. One potential explanation is that methanogens grew slowly and were not detected when the samples were hybridized. On the other hand, a weak fluorescence was detected when an Archaea-specific probe was used to hybridize these samples. Individual colonies in the roll tubes were very small, and it is possible that several different colonies were picked when the roll tubes were.

Acetate was initially not detected in liquid enrichments from isolated sulfate reducers, which led to the hypothesis that production of acetate depended on a another group of organisms not extracted from the roll tubes. However, after another week modest amounts of acetate were detected in these liquid enrichments. By comparison, isolated cultures of putative acetogenic bacteria showed higher concentrations of acetate. Acetate was always detected in mixed cultures.

An additional experiment was started to test whether production of acetate would be stimulated if cultures of sulfate-reducing, methanogenic and acetogenic cultures were recombined in one liquid enrichment. After 3 days of inoculation, turbidity developed. After 5 days, the liquid was analyzed for residual aromatic acids. No residual compounds were detected,

but sulfate loss was relatively minor. Methane and acetate were detected in this enrichment, but not in concentrations that would account for the loss of substrate. Finally, an experiment was started to test whether isolates of sulfate-reducing bacteria would be able to grow on pyrogallol. After 7 days, no growth was observed, and no products had formed. Possibly, none of the isolated cultures were able to degrade the aromatic ring, or degradation proceeded at a slower rate than permitted by the length of the experiment.

Growth was observed in the inoculum of tannic acid after 2 weeks, but the eluting compounds were different from the intermediate products of monomeric aromatic acids and were not identified. Gallic acid/pyrogallol was detected at measurable concentrations, and sulfate loss was significant. However, determination of sulfate is affected by coprecipitation of tannic acid with $BaSO_4$ at the pH used.

Conclusions

This research project was initiated to address the following questions:

1. Are thermophilic anaerobic bacteria able to utilize substituted aromatic acids as substrate?
2. Can individual organisms carry out the degradation or is a consortium - possibly acting in syntrophy - required to degrade completely aromatic acids completely to CO_2 and CH_4?
3. Can sulfate reducers carry out the complete degradation of aromatic acids by producing biomass and HCO_3^-?
4. Do the organisms accept similar substrates, for example vanillic acid and syringic acid?
5. Are these organisms capable of utilizing larger-size oligomers of these aromatic acids, for example tannic acid?

The experiment showed that thermophilic bacteria enriched in this experiment grew rapidly on aromatic acids. Growth was usually observed within 4-5 days. An experiment with vanillic acid showed that all of the vanillic acid was converted within 10 days into demethylated phenols, acetate, and methane. It also obvious that the individual enrichments, while not completely exclusive, furthered the growth of either acetogenic bacteria, methanogenic bacteria,

or sulfate reducer, and thus affected the rates of decomposition. Enrichments for sulfate-reducing bacteria utilizing syringic acid produced methane and acetate in the later stages of growth indicating that the colonies picked from the roll tubes were not pure cultures. The sequence of demethylated intermediates compounds observed demonstrates that degradation of the aromatic monomers proceeds in the same manner as has been reported for enrichments from lake sediments (Hanselmann et al., 1995). Negative blank controls indicate that breakdown of these aromatic compounds is not catalyzed by the high temperature of these experiments. There is only circumstantial evidence for the breakdown of the aromatic ring. This evidence is based on the sum of the concentrations of the reactants and formed products which do not add up to the initial concentration of the substrate, but cannot be explained by loss of sulfate. No degradation of pyrogallol by isolates of sulfate reducers suggests that these organisms are not responsible for the aromatic ring cleavage. Breakdown of tannic acid by organisms from the same inoculum was observed over the course of this experiment. However, the products identified by HPLC were unlike the individual monomeric products formed by vanillic and syringic acid degradation, although pyrogallol was detected in the chromatographs. Based on the structure of tannic acid, it is unclear whether the aromatic monomers were utilized. It is also possible that the carbohydrate skeleton of tannic acid was utilized as substrate by the bacteria and individual monomers released during its breakdown. Determination of the individual degradation products of tannic acid could be accomplished by GC-MS analysis. Finally, to quantify the individual degradation steps by using an electron balance, better data for the quantification of residual sulfate are required. The present data are insufficient to assess the potential for syntrophic relationships between the individual organisms. However, removal of acetate formed by breakdown of the aromatic ring would strongly shift the equilibrium of this reaction towards the acetate side.

Table 1: Concentration of aromatic acids in liquid enrichments (@65°C): n.a. (not analyzed; n.d. (not detected)

	enrichment	7/18/96			7/27/96			7/10/96	7/26/96	7/28/96		7/28/96
syringic acid initial inoculum (10 mM)		**syringic a. mM**	**interm. mM**	**gallic a./ pyrogal. mM**	**syringic a. mM**	**interm. mM**	**gallic a./ pyrogal. mM**	**acetate mM**	**acetate mM**	**CH_4 ppm**	**H_2S**	**SO_4^{2-} mM**
S4 syringic acid	**secondary**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.3	0.6	56.5	+	12.5
	10^{-5}	5.2	0.5	n.d.	0.7	3.6	6.5	n.d.	n.d.	21	+	13.8
	10^{-7}	2.9	1.6	2	0.2	2.1	5.5	n.d	0.6	25	+	15
M4 syringic acid	**secondary**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.1	n.d.	98.6		n.a.
A4 syringic acid	**secondary**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.1	n.d.	56.7	+	n.a.
	10^{-5}	8.8	0.3	n.d.	n.a.	n.a.	n.a.	0.1	n.d.	102	+	n.a.
S4/S4/A4 syr. acid	**10^{-5};10^{-7};10^{-5}**				n.d.	n.d.	n.d.		0.2	60	+	18
pyrogallol initial inoculum (2.5 mM)												
S4 pyrogallol	**10^{-5} syr.**						2.5		n.d.	n.d.	-	n.a.
S4 pyrogallol	**10^{-7} syr.**						2.5		n.d.	n.d.	-	n.a.
vanillic acid initial inoculum (20 mM)		**vanillic acid**	**protocatechuic a. /catechol**		**vanillic acid**	**protocatechuic a./ catechol**						
S4 vanillic acid	**secondary**	7.0	3.7		n.d.	10.8		1.0	0.7	n.d.	+	(27.7)
M4 vanillic acid	**secondary**	(41.5)	2.7		17.8	1.9		0.4	n.d.	1	-	n.a.
A4 vanillic acid	**secondary**	10.2	2.4		n.d.	15.3		n.d.	n.d.	n.d	-	n.a.
tannic acid initial inoculum (10 mM)											-	
S4 tannic acid	**secondary**	**unidentified products**						n.a.	n.d.	n.a.	-	12.3
M4 tannic acid	**secondary**	"	"		"	"		n.a.	n.d.	n.a.	n.d.	n.a.
A4 tannic acid	**secondary**	"	"		"	"		n.a.	n.d.	n.a.	n.d.	n.a.

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Design of a continuous-flow chemostat to cultivate Beggiatoa spp. and Thiovulum

In order to cultivate and isolate sulfide-oxidizing bacteria *Beggiatoa spp.* and *Thiovulum* , an experiment was started to design a continuous flow apparatus. supporting growth under controlled conditions. The medium used was filtered deoxygenated seawater containing a 40mM solution of $NaHCO_3$ and 1mM solution $Na_2S{\cdot}9H_20$. 1ml of an 0.5M solution of rezarurin was added as oxygen indicator. The medium was kept O_2-free by continuously purging the solution with N_2. Flow was introduced from the bottom through a 6mm tube into a 25 cm long glass tube packed with 3 cm of glass beads to provide surface for growth. Medium was introduced into the glass tube by pressuring the medium reservoir with N_2 driving the medium through Teflon tubing into the inoculated glass tube. The flow rate was adjusted such that a pink coloration would appear halfway up the tube. Oxygen was introduced from the top through an open 6mm glass tube by pumping oxygen into the tube with a small aquarium pump. The outlet used was a 2mm wide tip of a syringe connected to Tygone tubing. The inoculum was added with a syringe from the top.

Further design of this setup was not continued because of large fluctuation in the N_2-flow. In general the N_2-flow was too high permit oxygenation of the medium in the glass tube. An improved design of the chemostat could be achieved either by passive diffusion of Na_2S solution across a dialysis membrane or finer tuning of the in- outflow to the glass tube.

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I would also like to acknowledge support from the Arthur Klofein Scholarship and Fellowship Fund and the William Morton Wheeler Family Founders' Scholarship. Without this financial support, participation in this course would not have been possible.

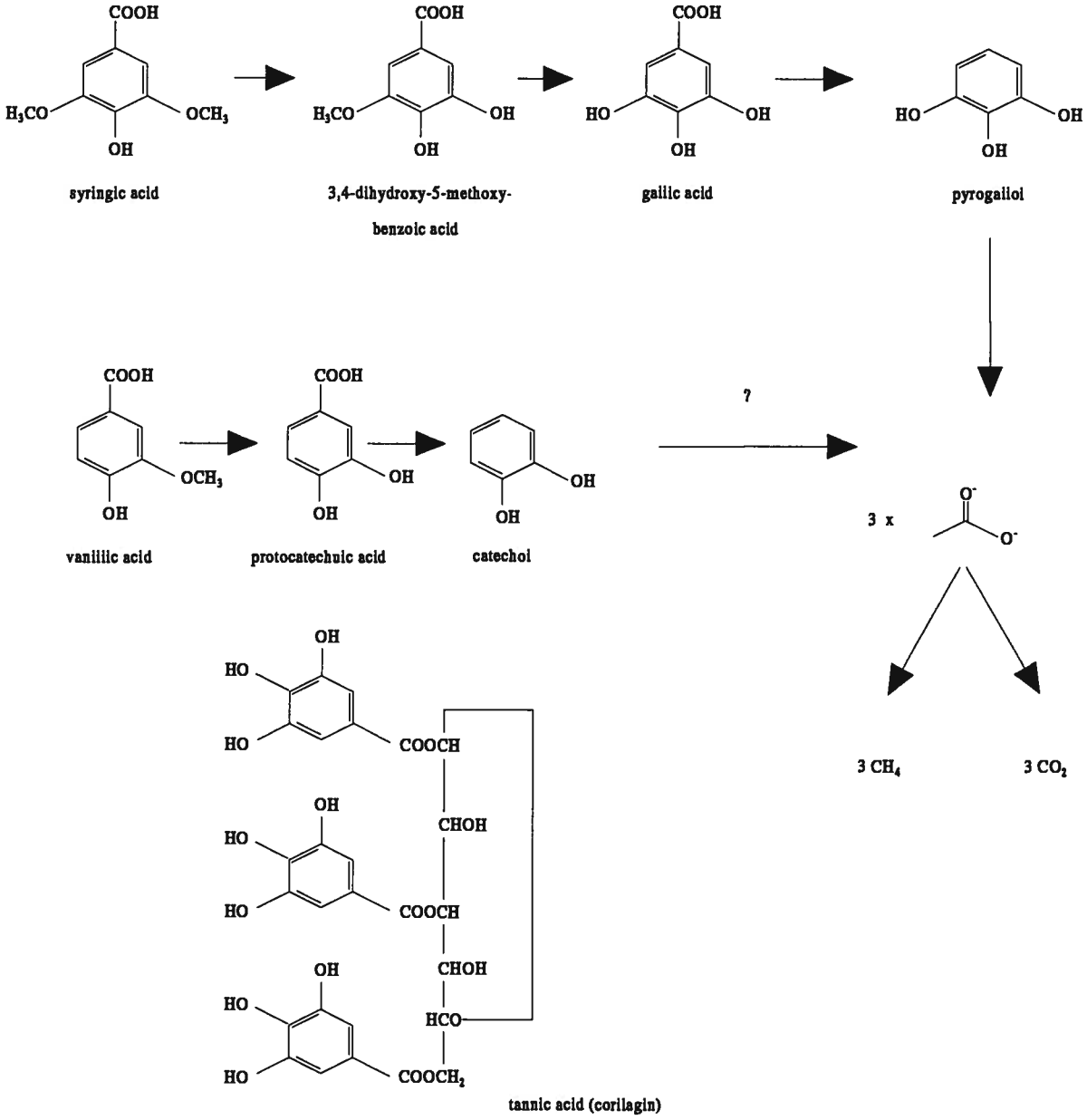
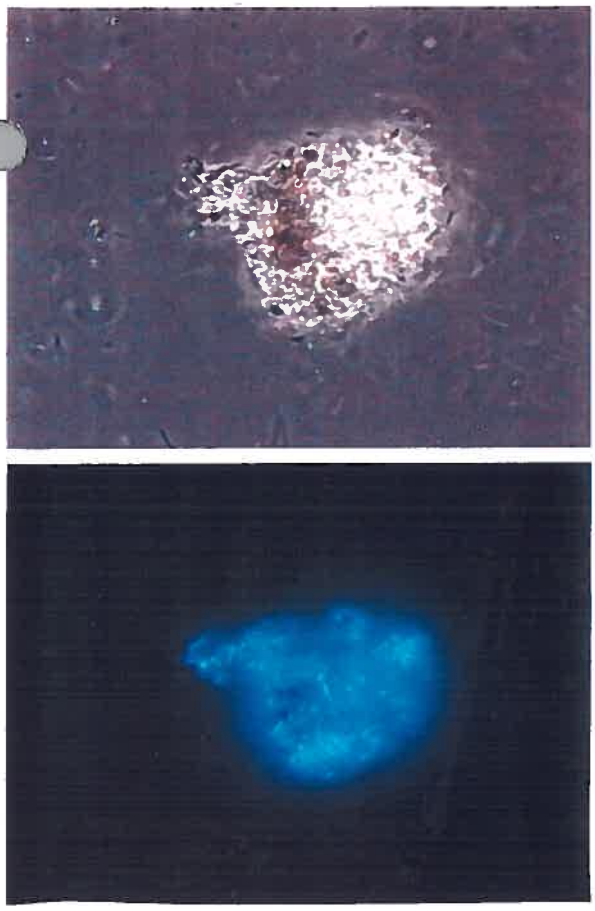
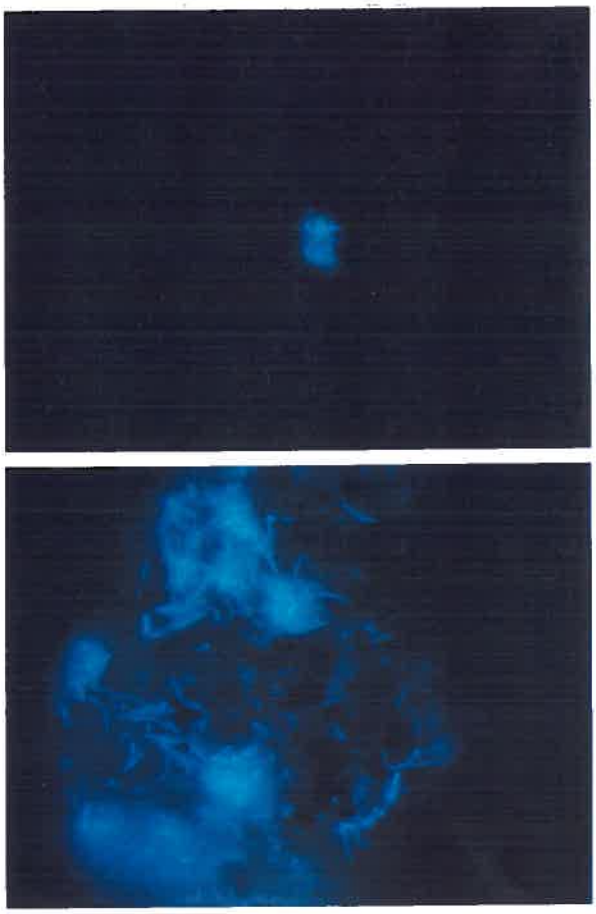

COOH
H3CO
OCH3
OH
syringic acid
3,4-dihydroxy-5-methoxy-
benzoic acid
HO
gallic acid
pyrogallol
vanillic acid
protocatechuic acid
catechol
?
3 x
O-
3 CH4
3 CO2
CHOH
HCO
COOCH2
tannic acid (corilagin)

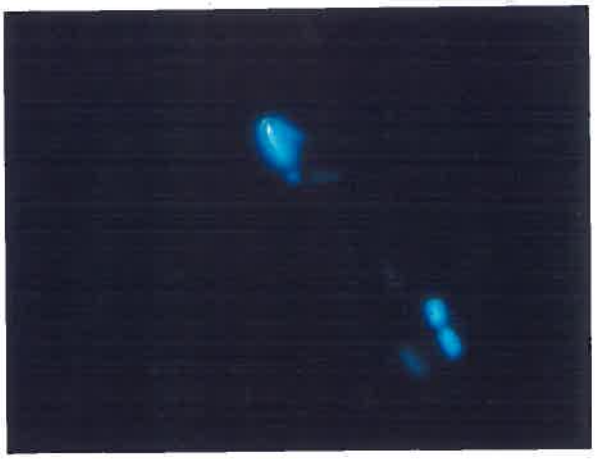
Fig. 1



syringic acid (sulfate reducer enrichment)

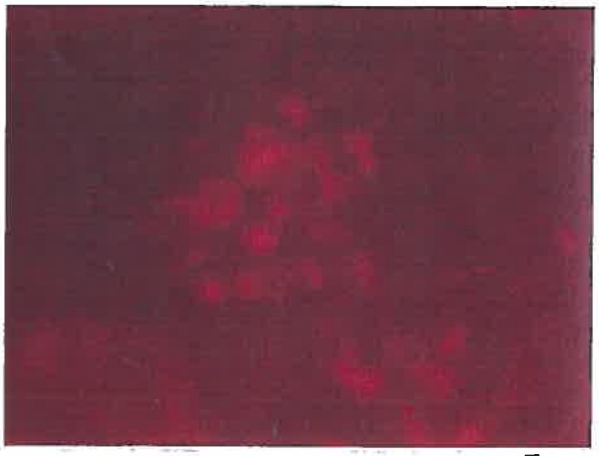


syringic acid (acetogenic bacteria enrichment)



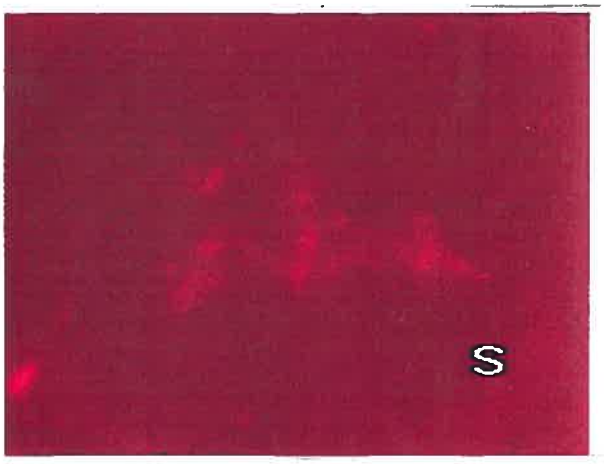
syringic acid (methanogenic bacteria enrichment)

Fig. 2



syringic acid; dilution 10^{-7}
SRB probe
sulfate reducer enrichment

vanillic acid; 2°- enrichment
SRB probe
sulfate reducer enrichment



syringic acid; dilution 10^{-5}
low G+C probe
acetogen enrichment

Figure 3