

Aromatic chemicals through anaerobic microbial conversion of lignin monomers

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Summary. Large efforts are directed towards production of ethanol from cellulosic biomass in order to reduce our dependence on petroleum based ethylene. No satisfactory process exists to date, however, which would make the aromatic molecules present in wood available to economic exploitation. A combination of physico-chemical pretreatment of lignocellulose and selective microbial conversion of the mixture of aromatic monomers into a few phenolic products is outlined. Anaerobic microbial communities are employed since they offer thermodynamic and physiological characteristics necessary for efficient conversion. Under anaerobic conditions most of the carbon and energy initially present in the substrate can be recovered as useful products; oxidative losses as CO₂ and H₂O are minimized. The 3,4-disubstituted aromatic lignin monomers are converted to catechol while 3,4,5-trisubstituted monomers are mineralized to CH₄ and CO₂. Further studies are directed towards an understanding of the physiological functions of the populations participating in the conversion process, the reason for catechol recalcitrance and the tolerance of the community towards phenolic endproducts.

The rate at which world oil reserves are presently being consumed is about 3.4% per year which means that consumption of proven reserves may be complete in 29 years³. This period of time could be extended by a few years if consumption would decrease further. But clearly within less than half a century we will have to find ways to change petroleum consuming civilizations to those that use alternative sources for chemicals and fuels. Although it might not be our first option, biomass will certainly be the only alternative in the long run⁴.

We consider in this presentation some microbiological aspects of producing chemicals from biomass, in particular the production of aromatic compounds from lignin by means of anaerobic microbial processes. Using microbial communities to produce valuable synthesis chemicals from substrate mixtures is to be encouraged. Fermentation processes in which aromatic products derived from lignocellulose would accumulate could become interesting economically for wood-rich countries. Furthermore, economically competitive production of lignochemicals could lessen our present dependence on petrochemicals.

Microbial metabolism of aromatics

To provide a useful source of aromatic synthesis chemicals, enzymatic or chemical processes that monomerize lignin must leave intact the aromatic nucleus and ensure microbial conversion of the resulting mixture of phenolics into a few separable compounds. In nature, degradation of lignin takes place through the sequential action of different microbial populations. This process and some of the organisms involved in the aerobic pathway have been widely studied⁵⁻⁹. Monomeric compounds found during these investigations in decaying wood and in culture media of fungi grown with isolated lignins include vanillic-, ferulic-, syringic-, p-hydroxybenzoic-, p-

hydroxycinnamic and 3-methoxy-4-hydroxyphenylpyruvic acids as well as some of their aldehydes. These products are converted to the corresponding diphenols by a sequence of 4 reactions⁶:

1. Elimination of methylgroups by O-demethylase or laccase forming formaldehyde and methanol respectively.
2. Oxidation of the C-3 side chain.
3. Oxidation of the alcohol- and aldehyd functions at C-1.
4. Hydroxylation of monophenols to diphenols.

Catechol, protocatechuic acid and gallic acid are the corresponding products for ring fission. Pathways for ring cleavage in the presence of oxygen are well established and their regulation has been studied in detail^{10,11}. Oxygenases play the major role in initial ring fission.

Based on the original report by Tarvin and Buswell (1934)¹² that certain aromatic compounds could be fermented to methane, pioneering work has been carried out on reductive ring fission under anaerobic conditions¹³⁻¹⁷. Anaerobic dissimilation of the aromatic ring is now known to be promoted in at least 4 different situations^{18,19}: through anaerobic photometabolism by certain Rhodospirillaceae; by *Pseudomonas* spp., a *Bacillus* sp. and *Moraxella* spp. and mixed microbial populations in presence of nitrate as electron acceptor; by microbial communities through fermentation to methane; and by certain sulfate reducing bacteria.

The ring is cleaved by reductive transformation of the aromatic nucleus into the corresponding cyclohexane followed by oxidative ring fission¹⁸. This mechanism is outlined by the postulated pathways for the degradation of syringic acid (fig. 1).

Interactions in microbial communities

Ferry and Wolfe²⁰ have made a microbiological ana-

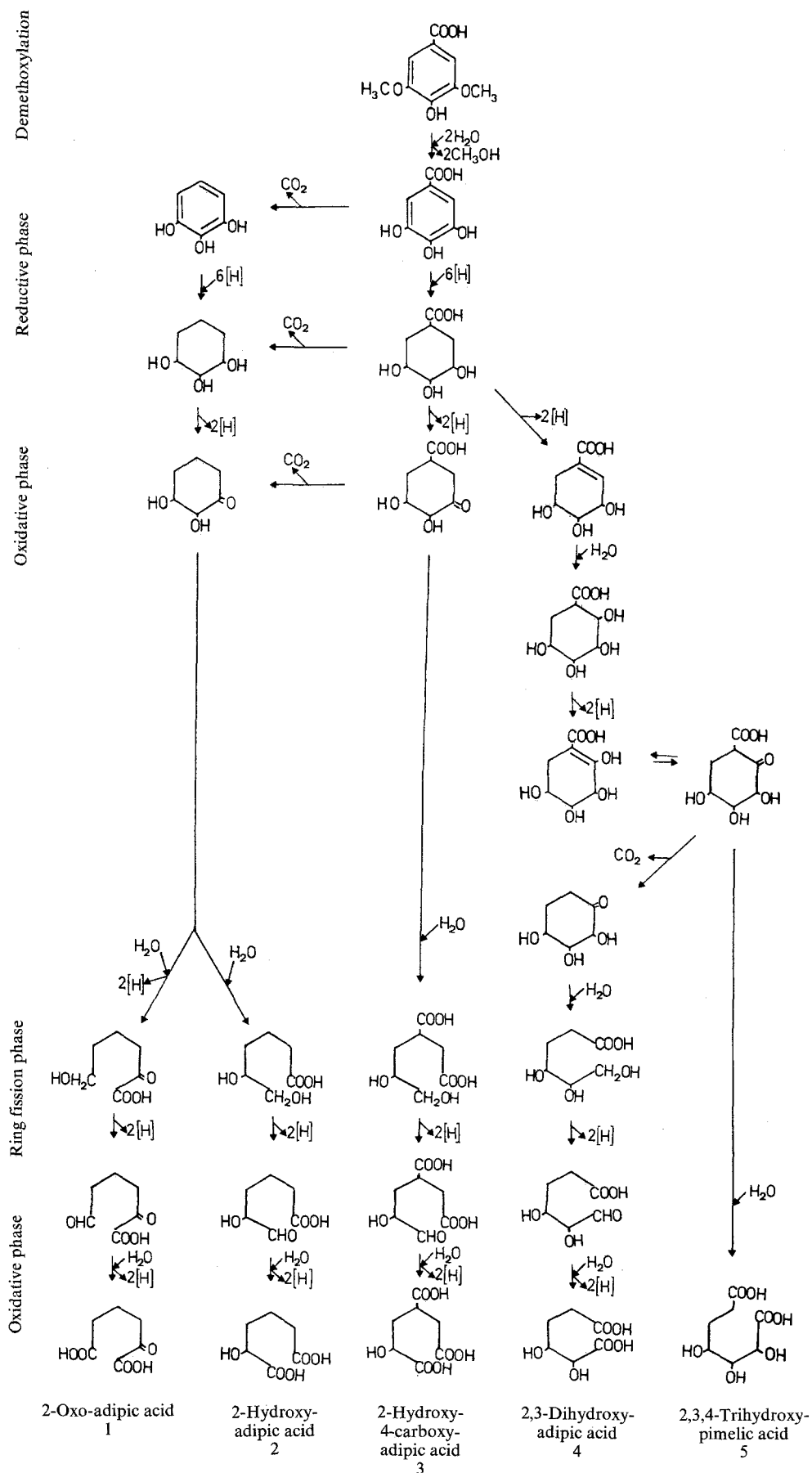
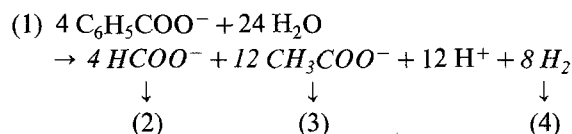
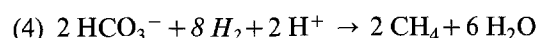
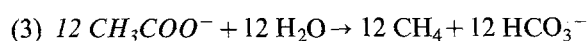
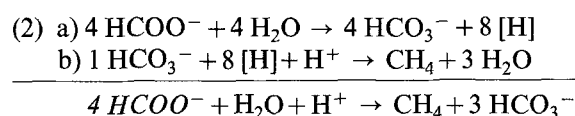


Figure 1. Hypothetical pathways for syringic acid degradation under anaerobic conditions.

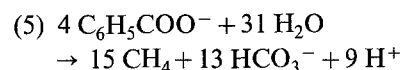
lysis of a benzoate degrading methanogenic consortium and postulated the following sequence of reactions:



Reaction (1) is carried out by a gram negative, facultatively anaerobic organism. It catalyzes the reaction only in the presence of symbiotic populations that continually remove the products (reactions 2-4) thereby maintaining favorable thermodynamic conditions for ring fission to proceed.



Reactions (2) and (4) are assigned to *Methanobacterium formicium* and *Methanospirillum hugatei* respectively which were isolated from the community. The acetate utilizing methanogenic organism could not be obtained in axenic culture. Complete mineralization of the aromatic ring (reaction 5) under anaerobic conditions, therefore, requires synthrophically interacting populations.



Healy and Young²¹⁻²³ have applied methanogenic communities to the degradation of lignin monomers which could be produced during alkaline heat treatment of wood. All of the 11 aromatic model compounds tested were degraded and methane-produced. Their communities also consisted of several different populations which were necessary to make the ring fission reaction exergonic. Ring fission could thus be prevented through regulation of certain populations of the community.

Conversion and mineralization of lignin derived aromatics

Enrichments from anaerobic freshwater lake sediments which we have obtained with syringic acid as the sole carbon- and energy source, mineralize syringic acid (fig. 2a) to CH_4 and CO_2 with temporary accumulation of acetate (fig. 3). The benzene ring is cleaved rapidly while the further catabolism of degradation intermediates is presumably limited by the size and activity of the appropriate companion populations. Vanillic acid and other 3,4-substituted aromatic

compounds are converted by our community to catechol (fig. 2b).

Syringic acid adapted cultures were fed with vanillic acid repeatedly and the products were allowed to accumulate. From figure 2b it may be seen that the absorption band at 251 nm, characteristic for vanillic acid, decreases while the new compound with absorption at 275 nm accumulates. Separation of acidified ether extracts with HPLC (fig. 4) shows that catechol

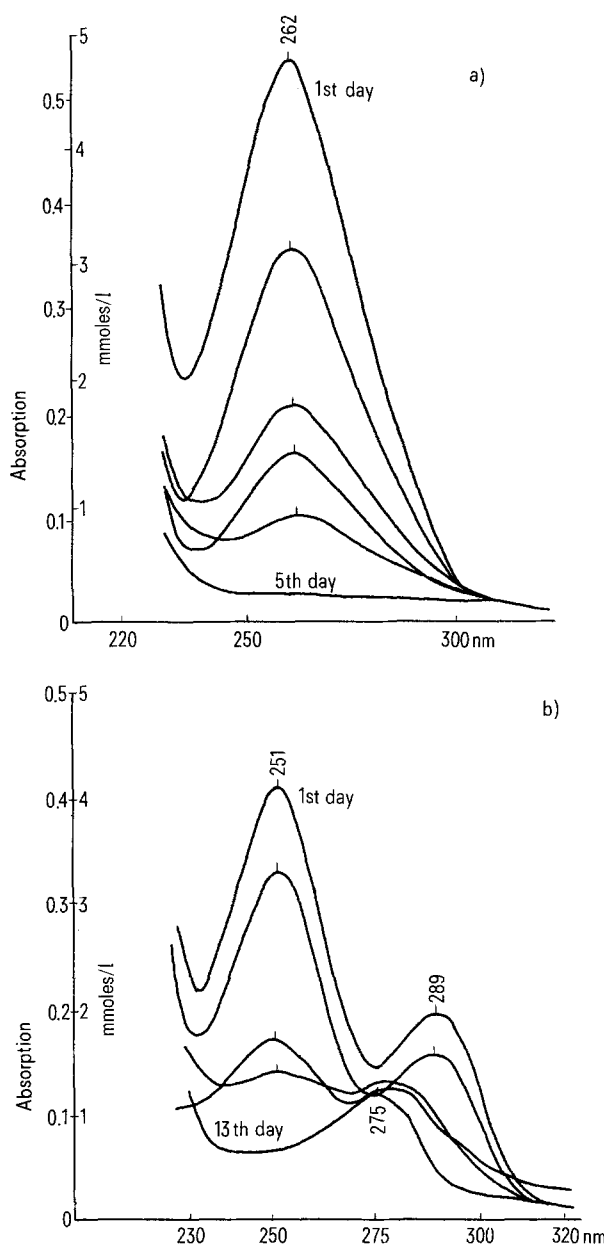


Figure 2. a Spectral changes during syringic acid mineralization. Syringic acid adapted cultures were fed 5 mmol/L syringic acid. Within 5 days all of the substrate is converted as indicated by the disappearance of the absorption band at 262 nm. Samples for absorbancy measurements were diluted 80-fold. b Spectral changes during vanillic acid conversion. Syringic acid adapted cultures were fed 5 mmol/L vanillic acid twice within 1 week. The substrate from the 1st feeding was degraded; the substrate from the 2nd one is converted into catechol (absorption band at 275 nm). Samples for absorbancy measurements were diluted 80-fold.

is formed from vanillic acid via protocatechuic acid as an intermediate. All of the syringic acid analogs with substituents in the 3,4 and 5 position of the ring were completely mineralized (fig. 5), while 3,4-substituted substrates tested were converted to catechol only (fig. 6). Ring fission of this intermediate did not take place with our communities under the strictly anaerobic conditions maintained. The refractory nature of catechol has been documented for aerobic⁹ and anaerobic conditions by other investigators^{14,24}. Healy and Young²¹ however, found complete mineralization. At present, we do not know why 3 consecutive substituents are necessary for mineralization to occur. The knowledge that microbial communities can metabolize one class of monomers while only converting another could become a useful tool in reducing the complex mixture of aromatic monomers obtained in lignin hydrolysis to a few recoverable products.

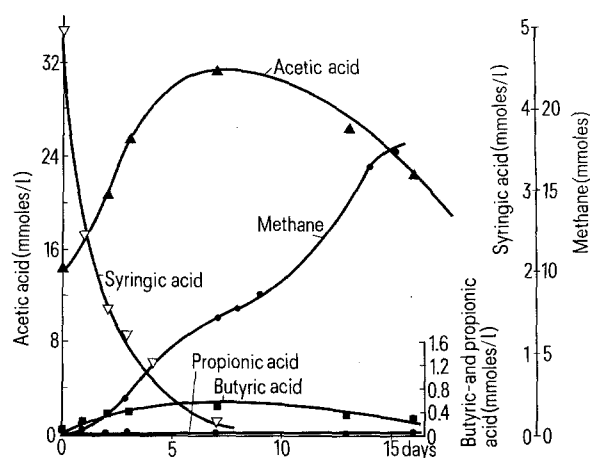


Figure 3. Intermediary products and methane formed by a syringic acid mineralizing culture. Syringic acid is converted within 1 week. Acetic acid accumulates and serves as a long lasting source for methane production.

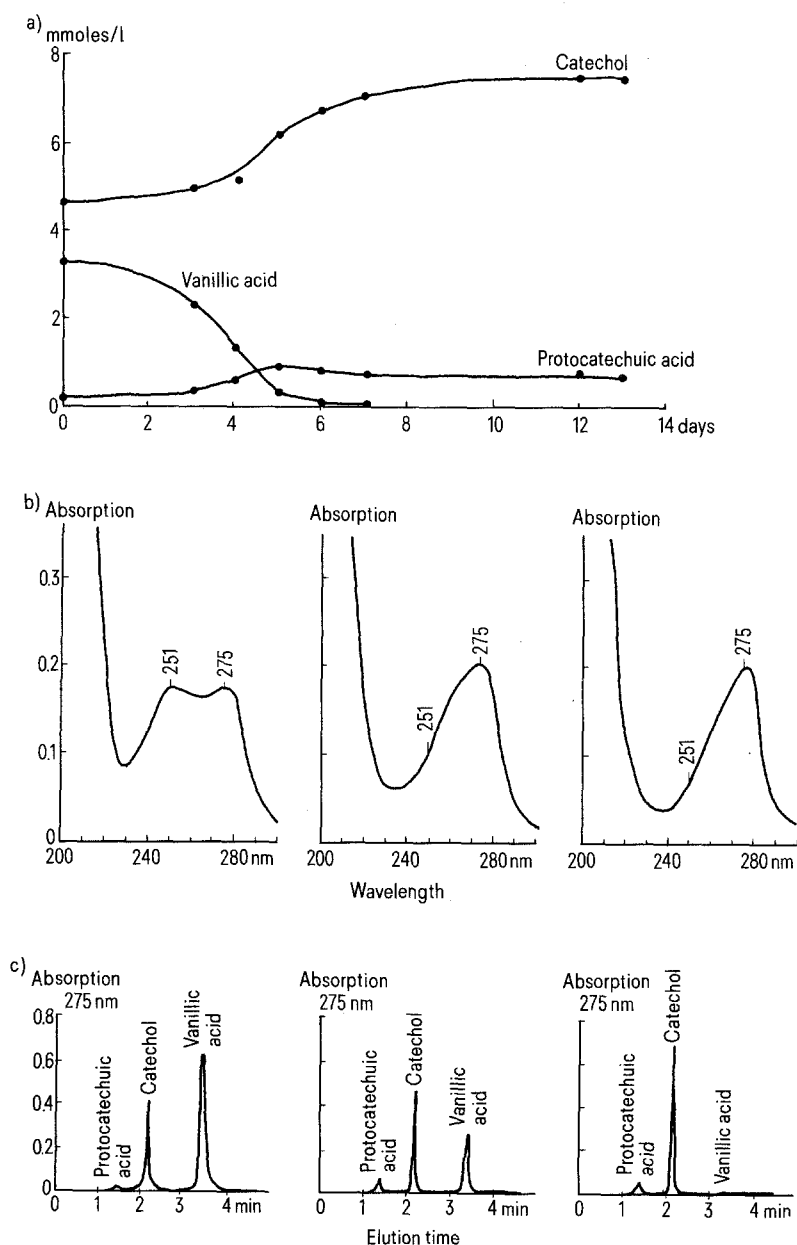


Figure 4. Time course (a), spectral changes (b) and product separation patterns (c) from a vanillic acid converting batch culture. Catechol was allowed to accumulate during previous conversions of vanillic acid. Protocatechuic acid appears as intermediate. Spectral changes (b) and separation patterns (c) are representative for days 1, 3 and 7.

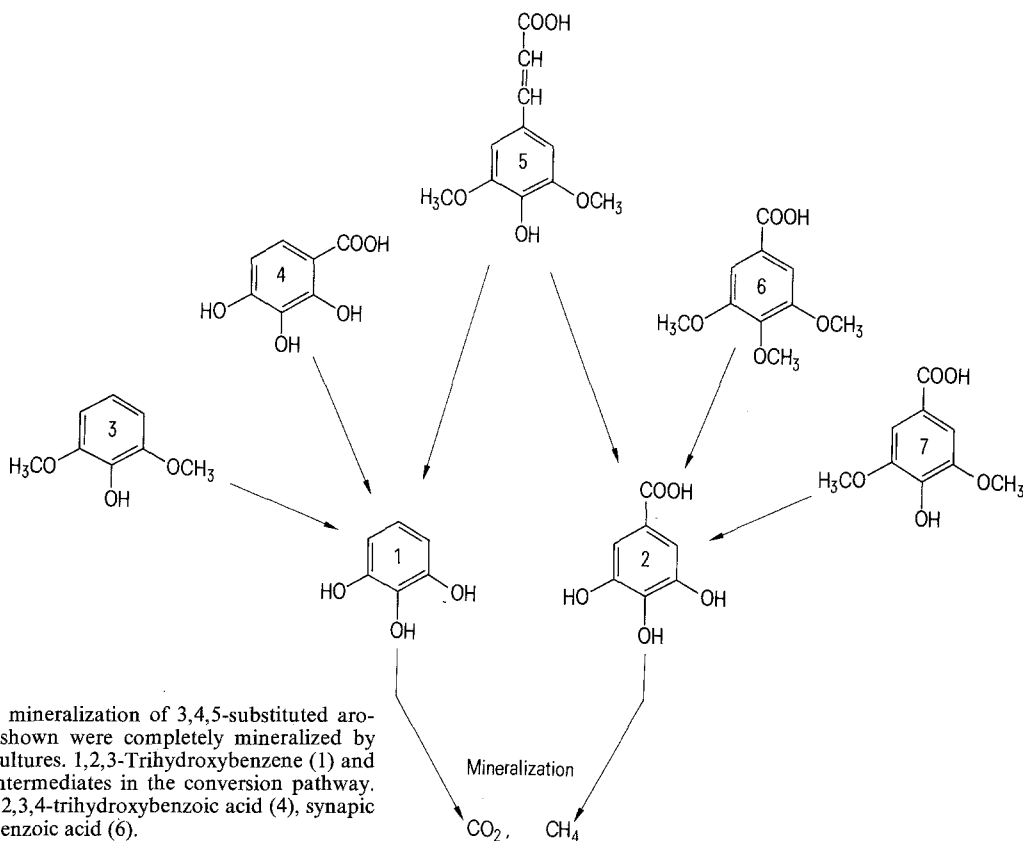


Figure 5. Conversion and mineralization of 3,4,5-substituted aromatics. The 7 substrates shown were completely mineralized by syringic acid (7) adapted cultures. 1,2,3-Trihydroxybenzene (1) and gallic acid (2) might be intermediates in the conversion pathway. 2,6-Dimethoxyphenol (3), 2,3,4-trihydroxybenzoic acid (4), synapic acid (5), 3,4,5-trimethoxybenzoic acid (6).

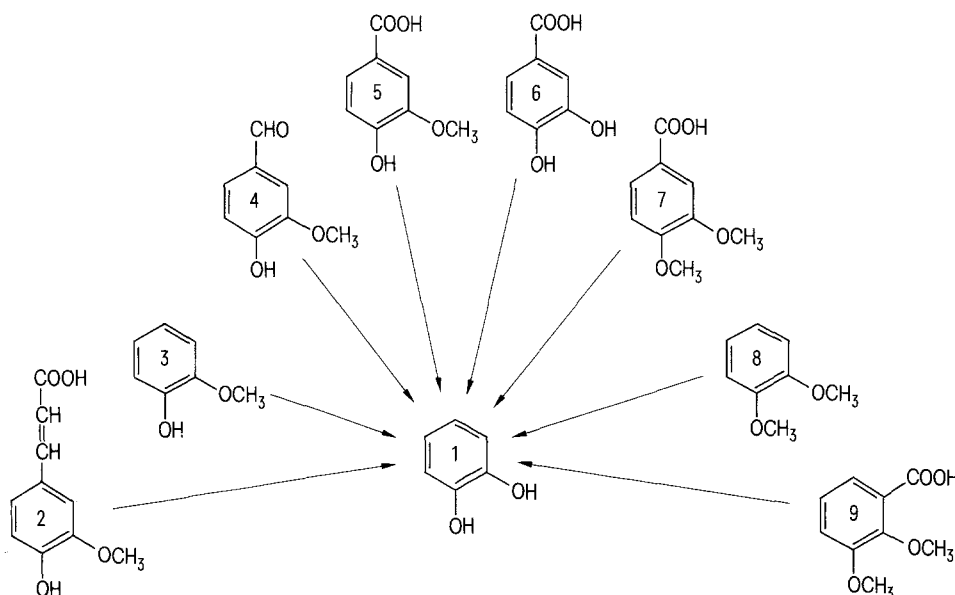


Figure 6. Conversion of 3,4-substituted aromatics into catechol (1) by syringic acid adapted cultures. No or only small amounts of methane were formed during metabolism of the 9 substrates listed. The aromatic ring was not cleaved. 4-Hydroxy-3-methoxycinnamic acid (2), 2-methoxyphenol (3), vanillin (4), vanillic acid (5), protocatechuic acid (6), veratric acid (7), 1,2-dimethoxybenzene (8), 2,3-dimethoxybenzoic acid (9).

Microbiological feasibility

Lignin fractions and phenolic compounds are used in herbicides, insecticides, fungicides, and as wood preservatives. Besides these man-made applications which are directed towards limiting the life span of certain organisms, many naturally occurring aromatic substances act similarly in natural ecosystems. Since their toxicity contributes to their recalcitrance, they tend to accumulate as humic- and fulvic acids in soil,

peat and sediments and, through the years, have been conserved under extreme environmental conditions as coal, oil and natural gas²⁵.

The continuous presence of these toxic substances, on the other hand, has led to selection of microorganisms capable of coping with them metabolically. We therefore find microbial communities today with abilities to convert and metabolize lignoaromatics anaerobically.

Three main characteristics distinguish naturally occurring degradative systems from those that are preferably employed industrially: substrates available in nature occur as complex mixtures; degradation is carried out by microbial communities; and degradation ultimately leads to mineralization in most cases. Industrially interesting processes require high substrate-to-product conversion from well-defined substrates by stable cultures, preferably consisting of one axenic population that can be dependably regulated. Degradation should stop at the level when a useful product is achieved that still contains most of the matter and energy present in the original substrate. Thus, there is still a large gap between the recognition of an ecologically functional pathway and its adaptation to an industrially interesting process.

Many organisms are able to oxidize completely organic polymers aerobically. No single organism has been found, however, that can achieve the same thing under anaerobic conditions. Only interacting microbial populations, each with limited metabolic abilities, can achieve together complete mineralization under anaerobic conditions. Symbiotic interactions are therefore responsible for the regulation and energetic efficiency of an overall process. They determine both, the kind and the amount of metabolic end products, and the conversion rates.

Although there are organisms with broad substrate utilization abilities, no single species exists in nature that can degrade all naturally occurring substances. Diversity in metabolic pathways reflects the diversity found in biomass composition. In an ecosystem, one expects a larger number of different populations as the substrate mixture increases in complexity. At the first levels of degradation, population diversity depends on the kind of intramolecular bonds that have to be hydrolyzed and the classes of monomers derived for fermentation. Glycosidic-, amin- and ester linkages are hydrolyzed easily while the different C-C bonds and the ether bonds in lignin require extremely oxidative conditions. Pretreatment is required, therefore, to make this biopolymer accessible to industrial fermentation.

Nature allows populations to become dominant for the conversion of a particular substrate and later to be replaced by other populations which attack other substrates. Thus, degradation of a complex substrate mixture is achieved through oscillating population activities in syntrophic associations. Microbial communities that show phases of adaptation and population fluctuations are more difficult to handle and seem less suited for industrial applications. We have tried to overcome some of the difficulties mentioned with our communities that degrade lignin monomers. A stable community, able to mineralize 3,4,5-trisubstituted aromatic monomers and to convert 3,4-disubstituted aromatics into catechol will continue to do so

in the presence of a mixture of syringic- and vanillic acid (fig. 7). Conversion to catechol seems to be stimulated by low levels of syringic acid. At higher concentrations vanillic acid conversion is inhibited until the concentration of syringic acid has dropped below a certain level. This experiment indicates that the great variety of monomeric substrates obtained through pretreatment of lignin could possibly be reduced to a few products through microbial fermentation. Certain classes of substrates would be converted into one common aromatic product which could be harvested more easily while certain companion substrates would be degraded further into products that would not interfere with the extraction process (e.g. acetic-, butyric acid) or which would escape as gases (CH_4 ; fig. 8). The principles of selective fermentation are presently employed, for example, in the recovery of pentoses from hardwood hydrolysates. The hexoses are fermented selectively to ethanol while xylose and arabinose remain unaltered and can be concentrated and recovered by crystallization.

Our cultures contain 4 to 5 different populations (fig. 9) which have been maintained through many transfers for almost 2 years as a balanced community. The interactions within this community stabilize the metabolic functions both thermodynamically and physiologically and maintain the environmental conditions for optimal fermentation. Although interactions lead to highly stable processes, failure of only one population disrupts the entire community. This fragility coupled with our limited knowledge about the behavior of mixed populations in general have prevented successful industrial applications (with the exception of the fermentation of certain foods). Research directed towards a better understanding of microbial communities could lead one day to economic exploitation of many more of the anaerobic processes which offer advantages over aerobic conversions and single population processes.

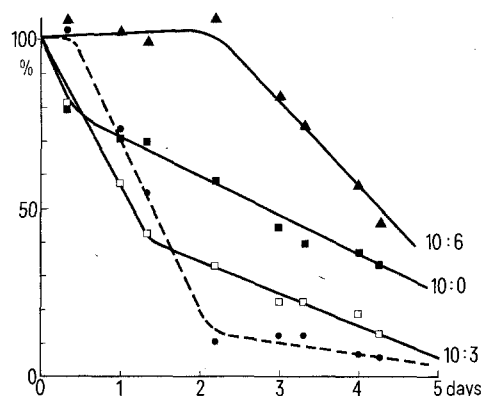


Figure 7. Microbial conversion of aromatics in substrate mixtures. Syringic acid adapted cultures were fed vanillic acid (10 mmoles/l) and 0 (■), 3 (□) or 6 (▲) mmoles/l syringic acid respectively. 1 culture was fed syringic acid only (5 mmoles/l, ●). Solid lines: disappearance of vanillic acid; dashed line: disappearance of syringic acid.

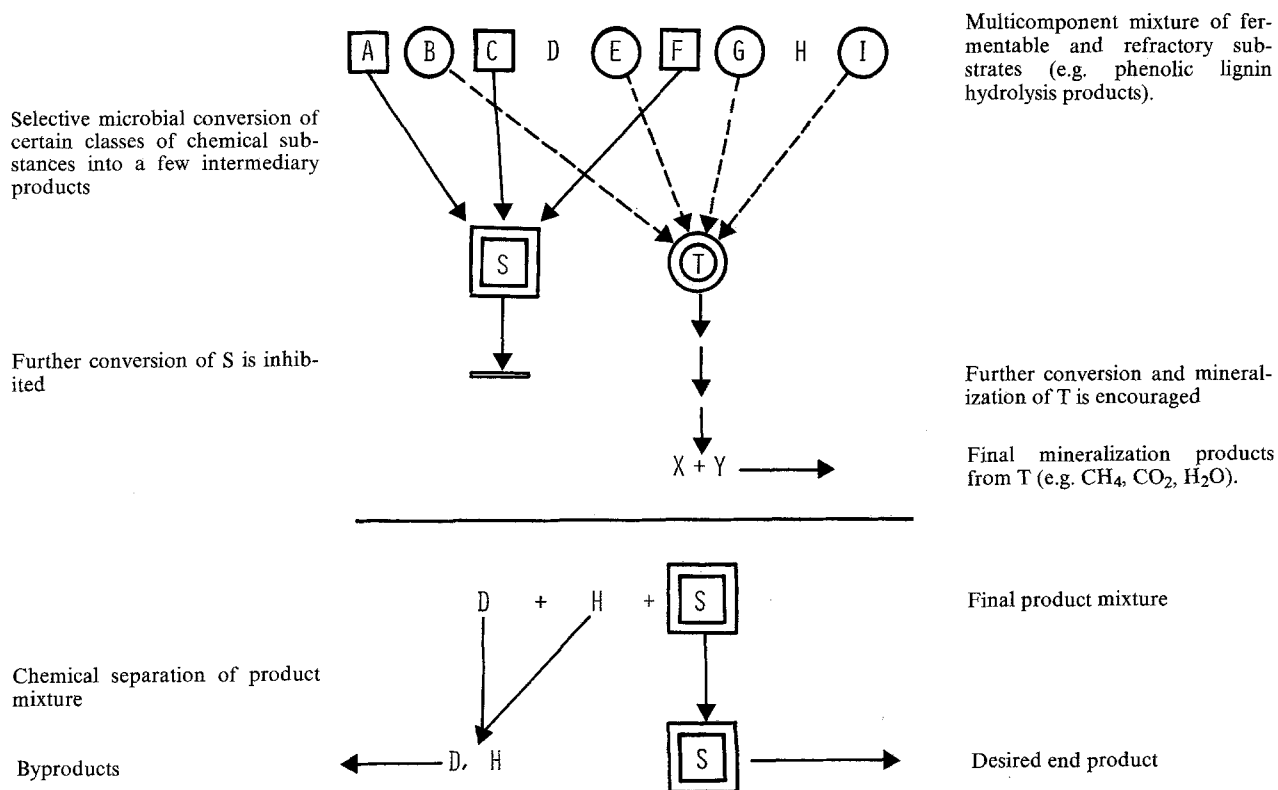


Figure 8. Major steps in selective fermentation with pure cultures or microbial communities.



Figure 9. Diversity of microbial populations in a community that degrades syringic acid fermentatively.

Many anaerobes produce large amounts of reduced end products. Product composition depends on the energy metabolism of the organisms involved and can be altered by adjusting growth conditions, by changing the environment, and by culturing together with other organisms. For example in the absence of an

inorganic electron acceptor (NO_3^- , SO_4^{2-} , CO_2), reducing equivalents are deposited in organic acceptors formed from the substrates offered. This leads to the production of reduced organic compounds which might be useful synthesis chemicals (table 1). Diversion of electrons to an acceptor other than the one that leads to the desired product results in decreased yields and product mixtures. The homolactic and the homoacetic fermentations where carbon and electrons

Table 1. Primary synthesis chemicals produced by anaerobic microbial fermentations

Chemical	Example of producing microorganisms
Ethanol	<i>Clostridium thermocellum</i> , yeasts
Butanol	<i>Clostridium acetobutylicum</i>
Isopropanol	<i>Clostridium butylicum</i>
Acetone	<i>Clostridium acetobutylicum</i>
Butanediol	<i>Enterobacter aerogenes</i>
Formic acid	<i>Enterobacter punctata</i>
Acetic acid	<i>Clostridium formicoaceticum</i>
Propionic acid	<i>Clostridium propionicum</i>
Butyric acid	<i>Clostridium butyricum</i>
Caproic acid	<i>Clostridium kluyveri</i>
Lactic acid	<i>Lactobacillus casei</i>
Succinic acid	<i>Ruminococcus flavofaciens</i>
Methane	<i>Methanobacterium thermoautotrophicum</i>
Hydrogen	<i>Rhodospirillum rubrum</i> , <i>Clostridium butyricum</i>
Carbon dioxide	<i>Clostridium butyricum</i> and many others

The microbially produced chemicals are present in fermentation broths. Their concentration depends on the substrate offered, the microbial species employed, the fermentation conditions and the tolerance of the population towards toxic effects of the accumulating endproducts.

are preserved in 2 moles of lactate (e.g. *Lactobacillus casei*) or 3 moles of acetate (e.g. *Clostridium thermoaceticum*) belong to the most efficient substrate-product conversion processes. Diversion of electrons to inorganic electron acceptors might be employed to regulate product formation or to prevent the buildup of undesired organic by-products. However, resulting reduced inorganic products (N_2 , HS^- , CH_4 , H_2O) might constitute an energy loss or inhibit certain populations. Thus, proper consideration should also be given to the composition of the biomass as a substrate and its possible influence on directing metabolic routes if minimal carbon- and energy losses and maximal product yields are expected. Wood pulping waste waters, for example, have been proposed as substrates for the production of chemicals^{26,27}. Feeding this resource (table 2) after chemical hydrolysis and adjustment of an appropriate C:N:P ratio to microbial cultures could create problems and unwanted side effects. The extremely high content of sulfur present in the sulfonate groups at the C-3 side chain would presumably be liberated during chain oxidation. In its oxidized state it could serve as an electron acceptor, thereby diverting energy into an inorganic, inhibitory and noxious end product and reducing the yield of the desired reduced organic products.

Further developments

Discussions have been limited to naturally occurring populations that can be selected for particular purposes from appropriate ecosystems. Mutation and selection of strains with improved conversion rates

and higher tolerance towards toxic products, cloning of hyperproductive, metabolically deregulated strains and the incorporation of plasmid associated metabolic functions into appropriate strains are possibilities that could widen applicability of anaerobic conversion processes²⁸. Also from studies about the ecology of the gut of termites and other wood eating insects we can learn more about microbial communities involved in anaerobic lignin degradation. One day it might become possible to synthesize microbial communities from defined strains and make industrial fermentation processes with mixed cultures more feasible.

Conclusions and outlook

Since the beginning of this century, microorganisms have been employed to produce chemicals, antibiotics, enzymes, vitamins and food and feed biomass. During times of inexpensive and seemingly unlimited supplies of fossil biomass, microbial processes have not remained competitive. Thus the study of many microbes with industrial potential, their physiology, the regulation of their metabolism, genetics and interactions with other organisms have been neglected. The encouraging success of genetic engineering in a time of concern about the dwindling resources of fossil biomass have resulted in a renewed interest in the biosynthetic and catabolic abilities of predominantly anaerobic organisms. Anaerobes are more difficult to handle since they must be maintained in an oxygen-free environment. They offer features, however, that make them attractive catalysts for efficient substrate-to-product conversions with high yields. Some of them have an energy metabolism which is most efficient in the presence of syntrophic companion organisms. Some have been found in extreme environments: in hot springs, at low pH and at high salt concentrations. These organisms might become particularly well suited for fermentations with high product accumulation, substrate compositions with low water activities or elevated process temperatures²⁹.

The decision whether to make a certain product through microbial fermentation, or through chemical synthesis, is primarily based on economic considerations. The price and the availability of raw materials, investments, energy costs, production rates and yields, recoverability of the products, their versatility and ecological impacts influence the decision. Two factors are worth emphasizing here. 1. The efficiency of anaerobic microbial processes depends largely on the energetic efficiency with which the organisms can dispose of reducing power. 2. Not all of the plant-derived raw materials are economically and politically suited as substrates. It would be unwise to base a large fermentation industry on a raw material which predominantly serves to feed mankind. Where the food- and the chemical industries compete for the same

Table 2. Composition of calciumlignosulfonates^a (as dry powder)^h

Dry substances (DS) ^b	92–96%
Lignin ^c	50–70% of DS
Other organic substances ^d	6–15% of DS
Inorganic salts ^e	8–14% of DS
Nitrogen as N ^f	0.80% of DS
Sulfur as S ^g	4–7% of DS
Methoxy groups (–OCH ₃)	6–10% of DS
C:N ^f	52.5
C:H	8.4
C:S ^g	6–10.5

^a Values calculated from analysis data supplied by Cellulose Attisholz AG., Switzerland;

^b dried at 103 °C;

^c polymers with mol. wts of 2000–30,000 daltons, corresponding to polymers with 11–170 phenolic monomers with an average mol. wt of 180 daltons;

^d reducing substances, hexoses, pentoses, small fibers, yeast residues;

^e after combustion at 800 °C; mostly calciumsulfate, calciumcarbonate and oxides of Ca, Mg, Na, K and Fe.

^f The high nitrogen content and the variability in the concentration of 'other organic substances' are due to addition of ammonia to the sulfite liquor from gymnosperms and its use for the production of ethanol and yeast biomass.

^g The high sulfur content stems from the acid used in the sulphite pulping process.

^h The powder is soluble in water. The pH of a 50% (w/v) aqueous solution varies between 3.5 and 5.

plant product the former must be given priority. A fermentation-based chemicals industry should therefore concentrate on raw materials that are less important in the human food chain. Even raw materials for which an established use already exists should not be diverted to the production of chemicals without due evaluation of the influence on other aspects of our living habits and on the economics of other industrial branches. Plant raw materials which have been underutilized in the past and those that can be grown for a particular new application are the most promising candidates to support a fermentation-based chemical industry. Lignocellulose from wood, the most abundant renewable biomass component produced by terrestrial ecosystems, has a good potential as raw material to support production of many chemicals³⁰. At first, research should be directed towards the production of lignochemicals with structures that cannot be obtained as petrochemicals. This minimizes economic competition for the same product while processing competence in handling lignin microbiologically can increase.

Today's efforts are directed towards efficient fermentation of the cellulose component of wood into chemical feedstocks³¹. Lignin is obtained as a by-product and supplies the energy needed to operate the pretreatment, the fermentation and the extraction processes^{32,33}. Energetically, this may lead to an eco-

nomic utilization of cellulose; it does not make use, however, of the synthetic work of plants which is stored in the chemically advanced structures of lignin monomers. Energy expenditure during resynthesis of the aromatic structures should also be considered before lignin is used as fuel only. Unfortunately, the microbial fermentation- and conversion processes for aromatic lignin monomers are not yet so highly developed that they already can be applied industrially.

A renewed effort is being made at present to search for new organisms and to study metabolic pathways and biochemical abilities of organisms and communities potentially useful for the production of chemicals. The field has been neglected during times of fossil biomass surplus and some time will be needed to reactivate it.

Note added in proof. After completion of this manuscript, Aftiring and Taylor³⁴ published evidence for anaerobic degradation of phthalate (1,2-benzene dicarboxylate) with nitrate as electron acceptor by a pure culture of a *Bacillus* sp., Bache and Pfennig³⁵ identified the organism responsible for anaerobic demethoxylation of lignin monomers as *Acetobacterium woodii*. In the presence of bicarbonate the methoxygroups are oxidized to acetate. Our data support these observations.

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Lignochemicals

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Introduction

Only 6-8% of the petroleum produced worldwide serves, at present, as raw material for the production of basic chemicals. But since petrochemicals supply 98% of the basic feedstocks used by the chemical industry, its dependence is obvious². Economical production of chemicals from natural oil and gas has been possible because of their relative purity and their continued availability at low cost. Highly developed technologies have evolved based on the well known and specific hydrocarbon composition of petroleum and natural gas. Changing from these sources to biomass, would require the development and adaptation of new process technologies. Wishart³ envisions 3 phases necessary to convert today's petroleum-based chemical industry to one based on biomass. During phase 1, chemicals will still be based on oil and gas resources, but they will be produced at a higher yield with improved processes and technologies. Phase 2 will be characterized by increased production of synthesis gas from heavy oil and coal to produce bulk chemical feedstocks such as ethylene and methanol. Production of chemicals from biomass will take place in phase 3 which is beginning to be developed now and which will be operative economically and with commercial significance later in the 21st century. A renewed interest in converting biomass components into organic chemical feedstocks is expressed by the increasing number of publications dealing with the subject⁴⁻¹². I would like to discuss here some aspects of physico-chemical pretreatment and microbial conversion for the production of aromatic synthesis chemicals from wood.

Development of microbial processes

The conversion of lignin monomers to catechol with microbial communities under anaerobic conditions is outlined in the preceding article (Kaiser and Hanselmann). According to Wishart's plan, the microbial conversion processes which are being studied and improved today will not have an immediate applicability. Therefore, the decision for, or against, their development should not turn solely on how economical the processes are at present. A proper evaluation should be based on a consideration of the changing raw material resources and the time required to develop industrially interesting microbial fermentation processes, as well as an appreciation of microbial abilities to convert biomass efficiently. Although the production of chemical feedstocks via microbial processes is at present not economically competitive with the production of petrochemicals, the development of these processes should no longer be postponed. We know little about many of the potentially interesting biochemical pathways in microbes and even less about their suitability to be employed in biotechnology. The spectacular hopes invested in genetic engineering can only become reality if we gain enough knowledge about the physiological basis of the metabolic sequences that look useful for biomass conversion.

Wood as a biomass resource

Wood represents a stored form of renewable solar energy and materials (fig. 1). It is the most abundant