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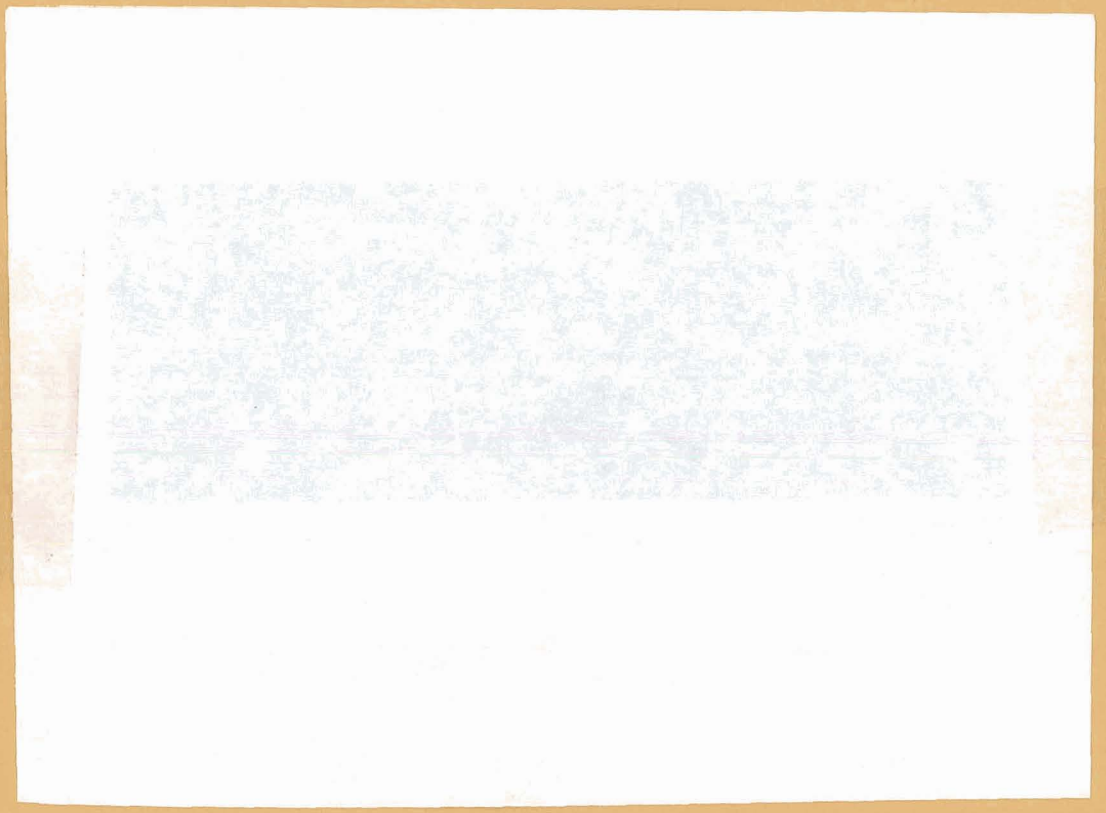

NOTICE

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FUSARIUM SPECIES: THEIR POTENTIAL FOR TRANSFORMING BIOMASS TO ETHANOL

by

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FUSARIUM SPECIES: THEIR POTENTIAL FOR TRANSFORMING BIOMASS TO ETHANOL

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ABSTRACT

The purpose of this study was to review existing literature and present some considerations pertaining to the use of Fusarium in degrading and fermenting certain biomass constituents to ethanol.

Energy stored in the carbon bonds of biomass can be extracted in a form usable as fuel by several fungal species. Members of the genus Fusarium (tuberculariaceous deuteromycetes) have demonstrated their ability to depolymerize macromolecular carbohydrates and lignin and to ferment monosaccharides to ethanol. Research has focused on decomposition of cellulose, hemicellulose, pectic substances and lignin by several formae speciales of Fusarium oxysporum, as well as on ethyl alcohol production by fermenting hexoses and pentoses. In this context, the exceptional capabilities of Fusarium species have been emphasized by students of fungal biochemistry. Unlike yeasts and other fungi, Fusaria can ferment both pentoses and hexoses (yeast can ferment only hexoses), and are able to saccharify the cell wall and middle lamella constituents and ferment the released sugar units.

Existing research data support well the idea of utilizing selected Fusarium strains to decompose and convert biomass to ethyl alcohol. Since ethanol blends and performs effectively with gasoline (as gasohol), its yield through the Fusarium fermentative action should be exploited. There are certain biological, technological, and economic limitations that constrain the application of biomass conversion to ethanol by Fusarium strains today on a large scale. These obstacles, however, could be overcome through additional research and development.

INTRODUCTION

It is well documented that Fusarium species are potential polysaccharide decomposers and simple sugar fermenters that ultimately produce ethanol. Since ethanol is the second major constituent of gasohol and by itself a powerful fuel, selected Fusarium members can be employed in alleviating the fuel shortage problem. It is therefore the purpose of this study to review existing research pertaining to plant biomass (phytomass) degradation and ethanol production through the saccharifying and fermenting action of Fusarium species.

The genus Fusarium includes individuals of imperfect (asexual) stages of certain ascomycetous (perfect - sexual) genera. Figure 1 depicts characteristic structures of Fusarium species. Fusarium members are organisms of a wide substrate range, cosmopolitan, mostly saprophytic, and, in some cases, dangerous plant pathogens. They are armed with a versatile series of enzymes that enable them to overcome adverse ecological conditions. These enzymes are mostly responsible for the Fusarium phytopathogenicity, competitive saprophytic ability and survivability. Fusarium species, aided by their powerful enzyme mechanism, utilize as their carbon source plant material decomposed to assimilative units. It has been demonstrated that Fusarium members can break down cellulose, hemicelluloses, pectins, and similar polysaccharides, as well as lignin and many more plant constituents. This investigation is confined to the decomposition of such plant compounds found in the cell walls and middle lamellae.

Extensive studies have revealed that the dry material of plant cell walls is made primarily of special polysaccharides and lignin. It has been calculated that the walls of cambial and soft tissue cells generally consist of 20-35% α-cellulose, 10-20% pectic substances, 35-50% hemicelluloses, 3-10% proteins, and 2-7% lipids (dry wall material) (29). On the other hand, estimates of the dry constituents of the woody cell walls in both hardwoods and softwoods have indicated an average of 43-45% cellulose content, 1-4% pectic substances; hemicelluloses averaged 40% in hardwoods and 30% in softwoods (29). Lignin, amounting to 30% in softwoods and 20% in hardwoods, is localized mostly (60-90%) in the middle lamella and primary wall (29). Cotton contains about 90% α-cellulose, 5% noncellulosic polysaccharides, and no lignin.

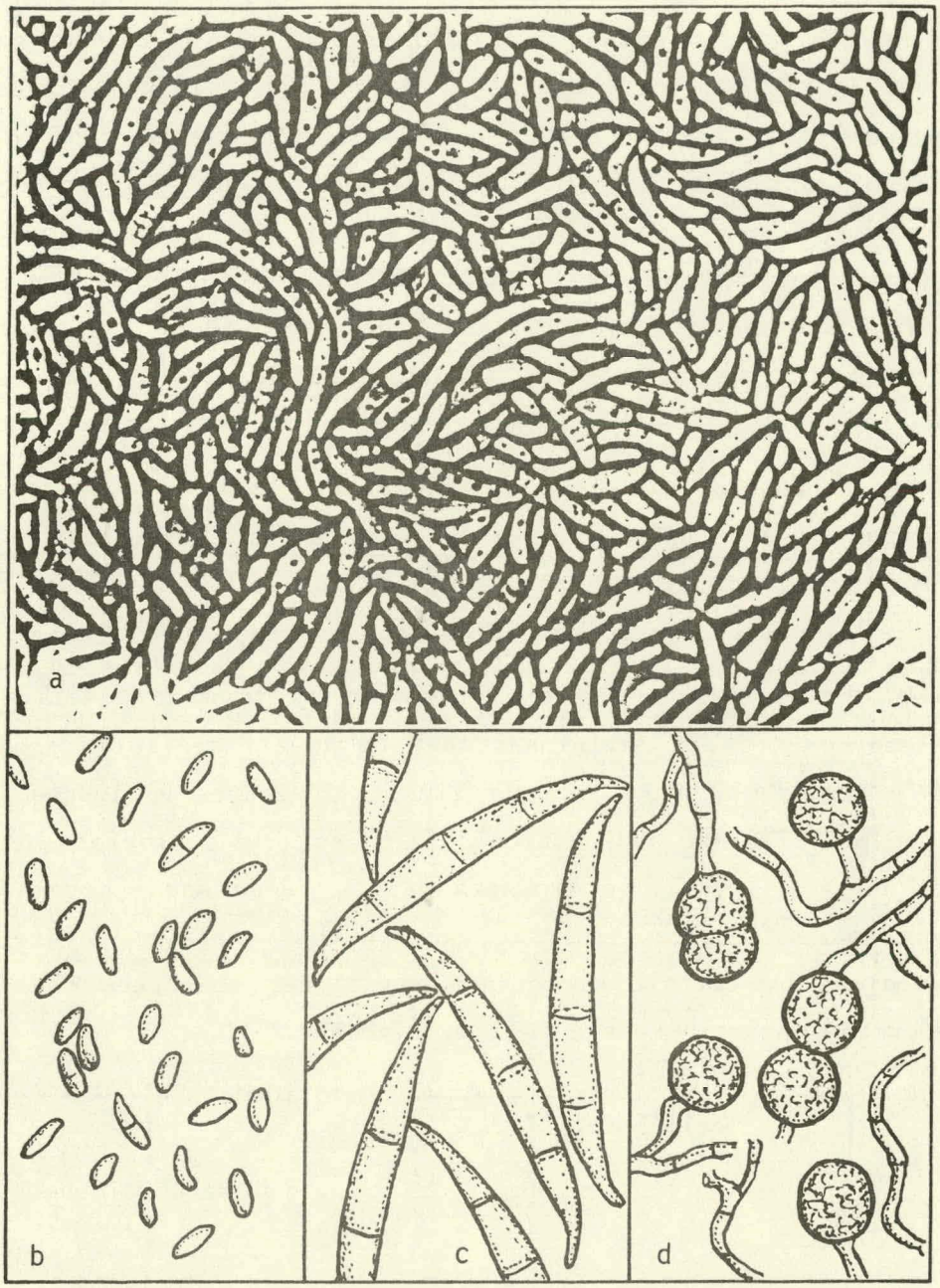


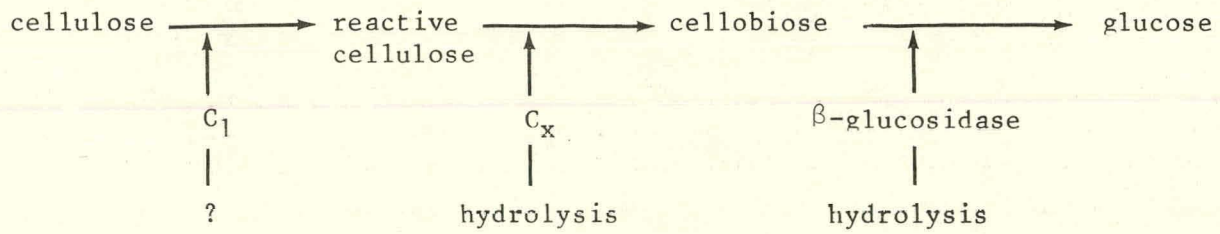
Fig. 1. Characteristic reproductive structures of Fusarium oxysporum: (a) a spore - macroconidial - mass as seen under the microscope (approx. X450); (b) microconidia, tiny uni- and bicellular spores (approx. X1,000); (c) macroconidia, the typical elongated, crescent-shaped, multicellular spores of the fungus (approx. X1,000); and (d) chlamydospores, the thick walled, spore-like vegetative structures that resist adverse conditions and aid the fungus to survive (approx. X1,000).

HYDROLYSIS OF CELLULOSE BY FUSARIUM SPECIES

Cellulose is a macromolecular polysaccharide made of β-1,4-linked D-glucose units (Fig. 2), with a degree of polymerization ranging generally between 8000 and 10,000 for wood cellulose and up to 15,000 for cellulose from unopened cotton balls (29). Native cellulose is made up of partly crystalline microfibrils which are aggregates of glucose anhydride chains arranged in a strictly (crystalline micelles) or less (paracrystalline and amorphous) parallel fashion (29). The cellulosic microfibrils are embedded in a noncellulosic polysaccharide matrix. Precise information is lacking on the existence of linkages between cellulose and lignin, or between hemicelluloses in the cell walls and the surrounding middle lamellae (23).

Fungi, because of their hyphal characteristic and continuous release of cellulases, are outstanding cellulolytic organisms. Many fungi representing imperfect stages of Ascomycetes as well as a plethora of Basidiomycetes are the main cellulose decomposers in natural environments.

Cellulose hydrolysis to its glucose units is made possible by the cellulase enzymes. These cellulases seem to be extracellular products of cellulolytic organisms, and are generally considered to be induced only in the presence of cellulose and particularly of soluble cellobiose or similar compounds having β-glycosidic linkages (25). Reese and others (33), and Mandels and Reese (25), in order to explain the attack and hydrolysis of native cellulose by certain microorganisms alone, suggested the C_1 - C_x cellulase system depicted in the following scheme:



The C_x-cellulase, a β-1,4-glucanase, can hydrolyze modified celluloses (soluble cellulosic derivatives, acid- or alkali-treated celluloses, ground celluloses, etc.), while C_1-cellulase initially attacks native crystalline cellulose by pushing apart the glucose chains and making the linkages accessible to the hydrolytic action of C_x-cellulase (29). The resulting cellobiose is hydrolyzed by β-glucosidase to glucose.

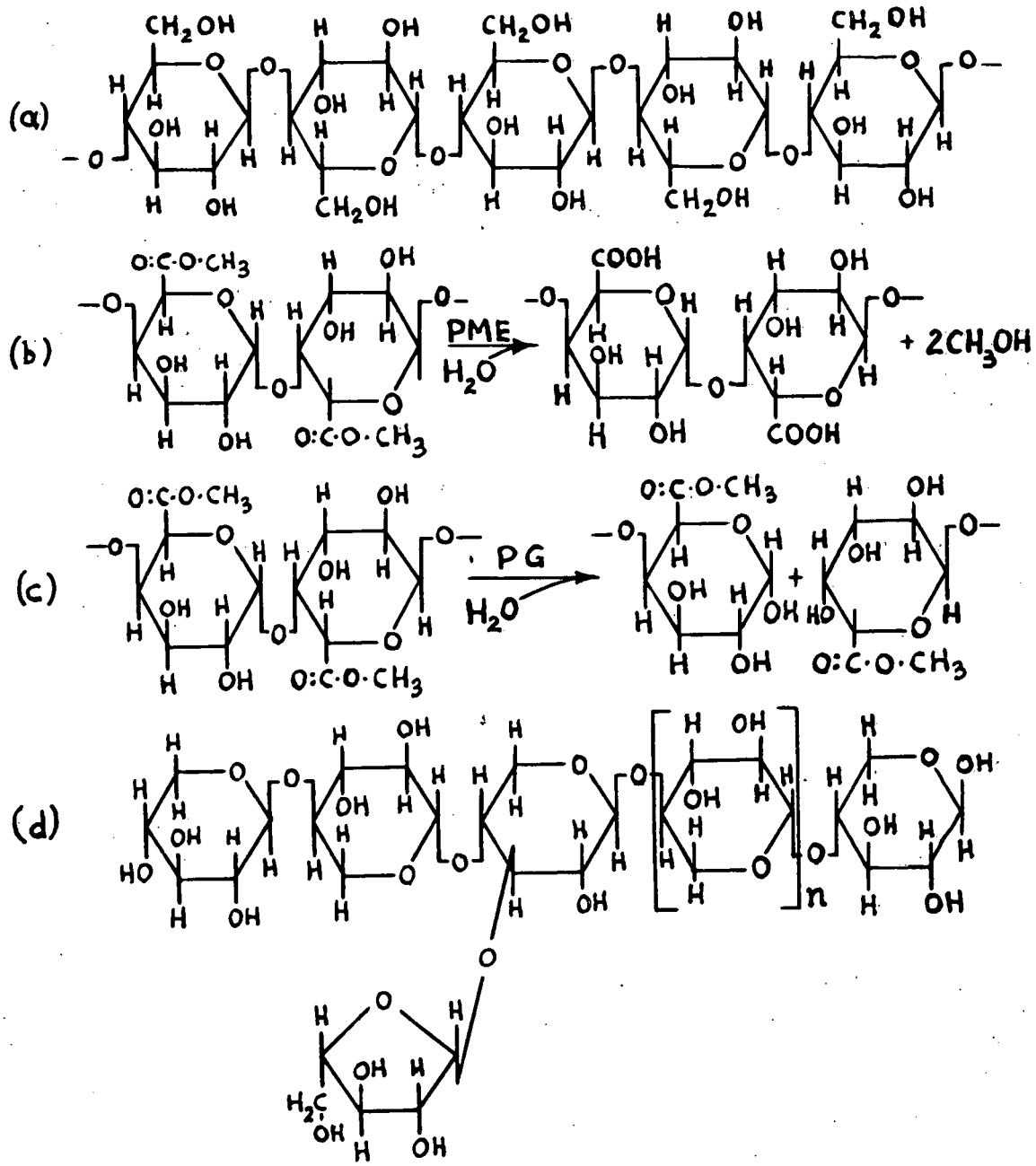


Fig. 2. Main constituents of plant cell walls and middle lamellae. (a) Cellulose (a linear polymer of β-1,4-linked D-glucose units); (b) hydrolysis of a pectic compound by pectin methylesterase (PME) to yield pectic acid and methanol; (c) hydrolysis of a pectic substance by polygalacturonase (PG); and (d) xylan (a hemicellulose made of a chain of xylose units bonded by β-1,4-linkages and of side-branched arabinoses).

Husain and Dimond (17, 18), in studying the phytopathogenic behavior of Fusarium oxysporum f. sp. lycopersici from tomato plants, found that the fungus showed cellulase activity on wood cellulose, paper, cotton linters, carboxymethyl cellulose, and on living tomato tissues. It was obvious that cellulase activity released nutrients for the F. o. f. sp. lycopersici. Davis and others (8) reported that F. o. f. sp. lycopersici yields detectable amounts of β-glucosidase within the infected plant tissue and is able to hydrolyze cellobiose.

White and others (38), assaying the cellulolytic potential of several fungi isolated from fabrics and related items exposed in the tropics, noted that Fusarium oxysporum Fla C-8 exhibited considerable ability to decompose cotton cellulose, although it was surpassed by Chaetomium spp., Myrothecium verrucaria, Trichoderma viride, and Thielavia sepedonium. Cellulase formation was detected by Deese and Stahmann (9), when Fusarium oxysporum f. cubense attacked stem tissues of both resistant and susceptible banana plants. The damping-off Fusarium moniliforme was found to secrete cellulase, and thus was able to disintegrate hardened and mature plant tissues (35).

The work of the aforementioned authors verifies the fact that Fusarium species utilize cellulose as a carbon source by means of cellulases released from the fungal cells. However, as Cochrane (7) emphasizes, two serious limitations characterize the reported results from cellulase studies: first, the native cellulose is to a certain extent resistant to microbial decomposition, and that is the reason most of the investigators have experimented with modified celluloses; and second, the studies have utilized crude and contaminated cellulase preparations.

In the case of the cellulolytic activities of Fusarium species, quantitative data are presently inadequate to support general theories pertaining to the potentiality of these fungi as cellulose decomposers and glucose producers. Furthermore, we do not have a clear picture of the C_1-C_x - cellulase system in the Fusarium members, and most importantly, we do not know of particular Fusarium strains that can release relatively high amounts of C_1-cellulase which in turn can easily attack native cellulose. In spite of this lack of information, there is reason to believe

that Fusarium strains able to readily attack native cellulose do exist, and new strains can be developed. Therefore, investigations should be initiated with the purpose of selecting and developing potential cellulolytic Fusarium strains to be employed for ethanol production.

PECTIC SUBSTANCES AND PECTOLYTIC ACTIVITIES OF FUSARIA

Pectic substances are the principal constituents of the middle lamellae that cement together plant cells. They are composed of D-galacturonic residues linked together through 1,4-α-glycosidic bonds, and have their carboxyl groups either esterified with methanol (pectic) or not esterified (pectic acids) (Fig. 2). Sugars such as arabinose, galactose, xylose, and rhamnose are variously bonded to pectin.

Fusarium species seem to be potential pectolytic microbes. It has been reported that Fusarium oxysporum f. sp. lycopersici (10, 26, 27, 32, 36), F. o. f. sp. cubense (9, 30), F. o. f. sp. pisi (31), F. solani f. sp. cucurbitae (14), F. s. f. sp. phaseoli (2, 31), and several other formae speciales constitutively or inductively form pectolytic enzymes such as protopectinase, pectin methylesterases, polygalacturonases, and polygalacturonate transeliminase that dissolve the middle lamellae and disintegrate the plant tissues. It is worth mentioning that pectin methylesterases hydrolyze pectin to pectic acid and methanol.

DECOMPOSITION OF HEMICELLULOSES BY FUSARIA

Hemicelluloses constitute the main component of the amorphous matrix in which the cellulosic microfibrils are embedded, the other components being pectic substances and lignin. The hemicelluloses form a heterogeneous group of polysaccharides that includes xylans, mannans, glucomannans, galactans, and arabans, as well as galacturonic acid, linked inside in a linear or branched fashion with some monomers or polymers of other sugars (Fig. 2).

Microbial hemicellulases can degrade hemicelluloses, e.g., xylanase hydrolyzes xylans and yields xylose. Simpson (34), in surveying the enzymatic action of 112 fungal isolates on a pentosan (of unspecified composition), found that filtrates from cultures of Fusarium strains were the most efficient pentosan hydrolyzers. Information pertaining to Fusarium spp. hemicellulolytic activities is insufficient, and additional research is needed.

DEGRADATION OF LIGNIN BY FUSARIUM SPECIES

Lignin is another important component of mature plant cells, particularly those of woody tissue. It is a complex three-dimensional polymer composed of derivatives of phenyl propane units which are methoxylated and form syringyl and guaiacyl units (Fig. 3). Lignin is generally deposited between and around the cellulose microfibrils, and heavily lignifies the middle lamella.

There is very little information on lignin degradation; most of what is known comes from studies on lignin decomposition by Basidiomycetes which cause wood decay. It is believed that the main effect of fungi upon lignin is the removal of methoxyl groups, thus modifying the lignin and exposing the adjacent cellulose microfibrils to cellulase action (19, 22).

There are a few reports concerning biodegradation of lignin by Fusarium species. Ledingham and Adams (20) investigated the decomposition of calcium lignosulfonate by 13 different species of Fusarium and found that these fungi were able to degrade up to 13% of the lignin contained in the medium. Fischer (11) reported that Fusarium lactis, F. nivale, and other unidentified Fusarium species were the most effective fungi (included in their investigation) in destroying phenol lignin (lignin extracted from wood with phenol).

In similar studies, Waksman and Hutchings (37) noted that soil-borne Fusarium species decreased the possibly recoverable lignin in the medium as much as 25%. As Kirk (19) mentions, Gulyäs (13) showed that pure cultures of soil-borne Fusarium spp. were able to decompose the lignin contained in wheat straw up to 20%. It appears from the aforementioned data that Fusarium species can degrade lignin and expose cellulose microfibrils to cellulase attack. These encouraging indications need further investigation, particularly from a fuel production point of view.

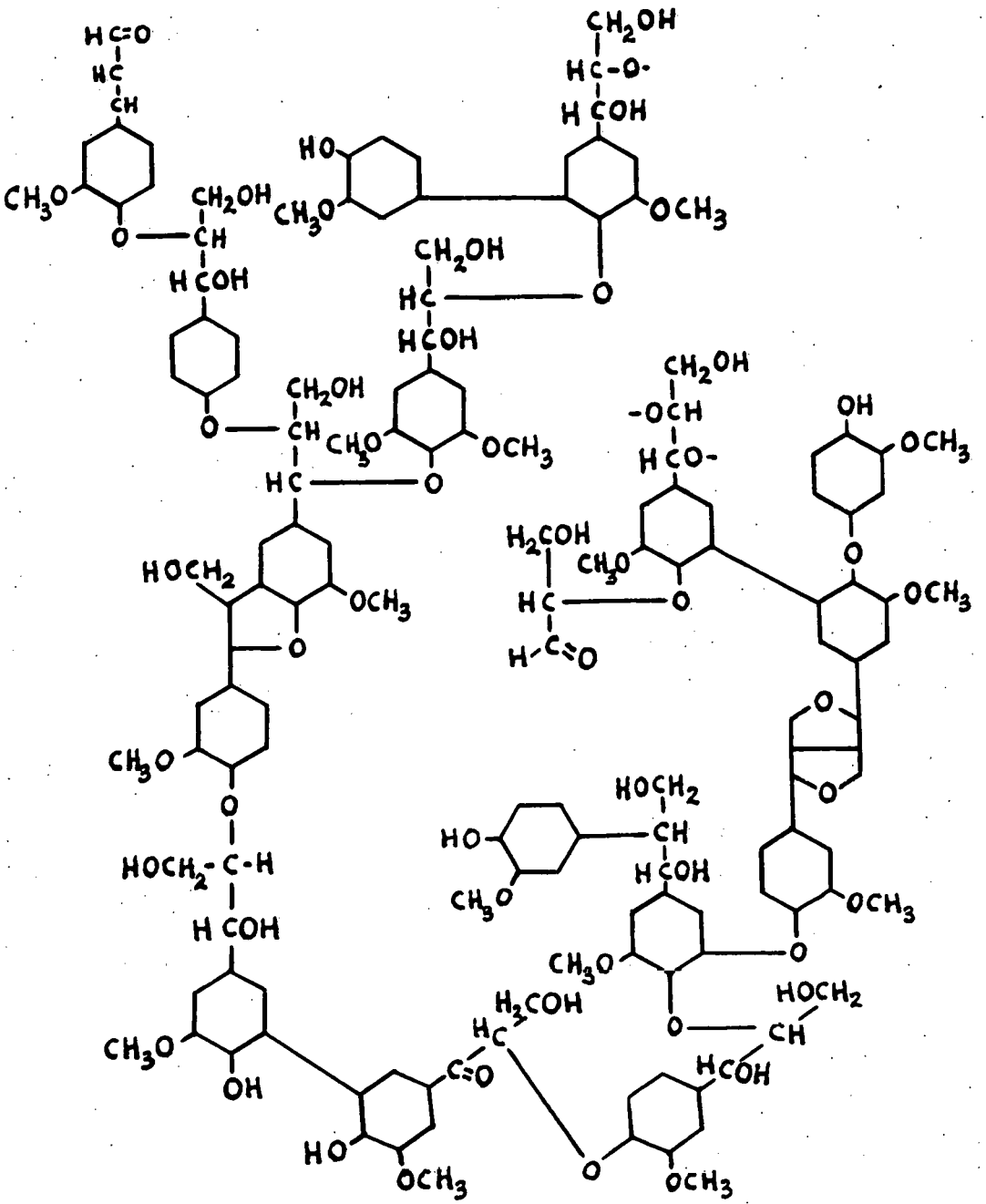


Fig. 3. Conifer lignin, a complex polymer of methoxylated phenylpropanoid units.

PRODUCTION OF ETHANOL BY FUSARIUM SPECIES

The ability of Fusarium species to ferment sugars to ethanol has been noted by many investigators ever since fermentation was put under scientific observation. The recent fuel shortage may be a sound reason for considering the ethanol-producing ability of Fusaria. It is imperative to mention here a summary of the biochemistry of fermentation by Fusaria.

Wilkinson and Rose (40) have defined fermentation as the anaerobic energy-yielding process in which organic substances act as both the electron donors and the electron acceptors, while anaerobic respiration is the process during which inorganic substances are the electron acceptors. (In aerobic respiration, oxygen acts as the final electron acceptor.) The main substrates of alcoholic fermentation are various hexoses and pentoses, but glucose fermentation is the most studied. Several physicochemical and biological methods are utilized in splitting polysaccharides to units available for fermentation.

Fusarium species, like many other fungi, possess the characteristic of releasing polysaccharide-splitting enzymes and have the ability of assimilating the derived monomers for their energy and carbon requirements. Furthermore, these fungi seem to be equipped with a highly efficient sugar-transport mechanism that enables them to glycolyze and readily ferment the simple sugars.

Various metabolic pathways of carbohydrates in microorganisms have been discovered, and pyruvic acid appears to be the key intermediate. Particularly in fungi, three glycolytic pathways are known: (a) the hexose diphosphate pathway, known as the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 4); (b) the hexose monophosphate (HMP) pathway; and (c) the Entner-Doudoroff (ED) pathway (5). All three pathways ultimately yield pyruvic acid which may serve as the terminal electron acceptor (fermentation), or may be oxidized via the citric acid cycle (aerobic). In the fungi, the EMP mechanism is the major glycolytic pathway and consequently the principal fermentative producer of ethanol, glycerol, lactic acid, etc.

Ethanol is formed by (a) the activation of pyruvic acid by the coenzyme thiamine diphosphate (TPP); (b) decarboxylation by the pyruvate

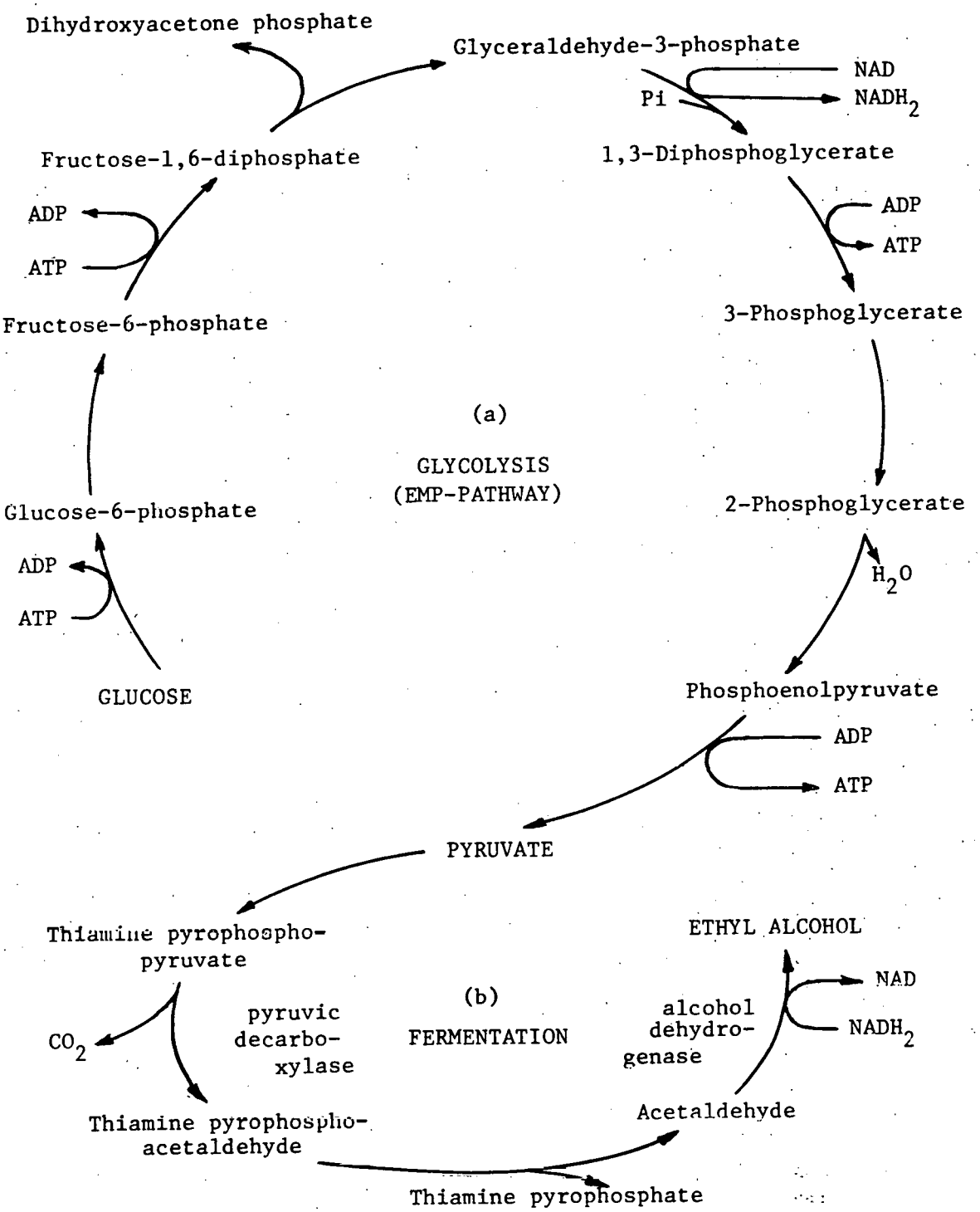


Fig. 4. From glucose to ethyl alcohol: (a) the Embden-Meyerhof-Parnas (EMP) pathway (glucose -- pyruvate); and (b) activation, decarboxylation and reduction of pyruvate to ethanol.

decarboxylase to form acetaldehyde; and (c) reduction of acetaldehyde by the alcohol dehydrogenase, finally to form ethyl alcohol (Fig. 4).

Strong evidence resulting from studies of carbon balance, enzymatic analyses, and isotope distribution indicates that the EMP mechanism functions anaerobically in the Fusarium spp. to produce ethanol (6, 15). Yeast and Fusaria have certain characteristics in common in regard to the way they ferment sugars to ethanol. It has been shown that these fungi produce alcohol and carbon dioxide from hexoses in the same ratio as that obtained from yeast (4, 28), although the carbohydrate metabolism to alcohol in Fusaria occurs somehow slower than that in yeast (28). Wirth and Nord (41) were able to isolate pyruvic acid (the precursor of ethanol via acetaldehyde) from four different Fusarium species metabolizing various hexoses and pentoses, and thus presented the only proof of Fusarium carbohydrate metabolism and ethanol production via the EMP pathway.

Birkinshaw and others (4) have found that all 23 species of Fusarium investigated give appreciable amounts of ethanol, and suggested that it is possible to separate a few species which have a rather unusual fermentative potential. The same authors recorded that with F. avenaceum, of 4.683 gm. of glucose carbon metabolized, 2.453 gm. appeared as "volatile neutral compounds," which means a yield of 52.4% ethanol; this is in contrast to the maximum yield of 60% ethanol obtained with species of Saccharomyces.

Hollis (16) reported that Fusarium oxysporum can survive under essentially anaerobic conditions up to 13 weeks, while F. eumartii declined after the third week.

Anderson and his coworkers (1), experimenting with Fusarium oxysporum metabolizing glucose, found that in 20 days the percentage of the original carbon in the form of ethanol was 49.21, which increased to 51.89% at the end of 46 days. From then on, it was noted that the amount of ethanol decreased to 47.88% at the end of the experiment, and the authors concluded that F. oxysporum utilizes ethyl alcohol only slightly.

Lockwood and others (24) examined 29 strains of Fusarium for metabolic products. In studying submerged cultures of Fusarium oxysporum in a 15% glucose nutrient solution, they obtained the following data at the end of the 14th day of experimentation (all values in grams):

Initial glucose	30.20
Final glucose	12.90
Alcohol (calculated as glucose = c. as g.)	4.70
Unidentified compounds (c. as g.)	1.23
Carbon dioxide (in NaOH)(c. as g.)	6.05
Volatile organic compounds (in H_2SO_4)(c. as g.)	2.23
Mycelium (c. as g.)	0.88
Total carbon accounted for (c. as g.)	27.99
Carbon not accounted for (c. as g.)	2.21

It is obvious from the above data that the ethanol yield was substantial.

Many investigators have studied the fermentative abilities of Fusarium oxysporum f. sp. lini (the cause of flax wilt). Anderson and others (1), in comparing the fermentative conversion of glucose to ethanol by F. oxysporum (in this case another forma specialis) and F. (oxysporum f. sp.) lini, found that, at the maximum point of ethanol production, F. lini converted 52.75% of the original carbon into alcohol. They also noted that at the end of the experiment the original carbon in the form of ethanol was only 29.51%, and concluded that F. lini utilizes ethyl alcohol as a carbon source. Ethanol oxidation in F. lini reveals the capacity of this fungus to oxidize primary and secondary alcohols (7).

Cochrane (6), using resting cells of F. lini metabolizing glucose, was able to confirm earlier claims that glucose is converted, as in yeast, to two moles of ethyl alcohol and two of carbon dioxide. He found aldolase (one of the enzymes which is characteristic of the EMP pathway), triose phosphate dehydrogenase, and ethanol dehydrogenase in extracts of Fusarium lini. Cochrane concluded that glucose fermentation by F. lini follows the EMP pathway.

Pentose fermentation by Fusarium species has been considered as the model mechanism possibly occurring in several other species of fungi. Resting cells of F. lini have been noted to ferment xylose in this sequence:

xylose → triose phosphate + acetyl phosphate; triose phosphate → ethanol + carbon dioxide; acetyl phosphate → acetate (6, 12). White and Willaman (39) and Letcher and Willaman (21) have observed that while fermentation of hexoses with F. lini yields equal amounts of carbon in ethanol and carbon dioxide as do the yeasts, with pentoses (which yeasts cannot ferment) the ratio is nearly 1:1. Like yeast, F. lini can directly and indirectly ferment the disaccharides maltose and galactose (28).

Through carbon balance methodology, White and Willaman (39) have tabulated their results from the fermentation of arabinose by F. lini as a percentage carbon distribution that follows:

Age of culture (days)	Mycelium	Carbon Dioxide	Alcohol	Lead precipitate	Sugar	Total Carbon
5	0.8	0.6	7.6	---	90.6	99.6
10	3.4	4.4	7.6	0.6	85.2	101.2
15	4.6	6.1	6.6	1.0	80.4	98.7
25	4.0	9.4	3.3	1.5	81.2	99.4
40	10.4	20.8	9.9	1.7	55.2	98.3

Letcher and Willaman (21) have reported that not all isolates of a species are equally efficient in yielding ethanol. Eight isolates of F. lini produced varying quantities of ethanol on the same medium. They also found traces of acetaldehyde in their culture media, indicating that acetaldehyde possibly originated from the alcoholic fermentation.

CONCLUSIONS

This review of literature reveals the ability of Fusarium species to both degrade phytomass and yield ethanol via fermentation. It is worth emphasizing that many authors have pointed out both the quantitative and qualitative similarities in fermentative activities of Fusaria and yeasts. They have also noted the superiority of Fusaria over yeasts in degrading cellulose, lignin, and other plant constituents, as well as in fermenting pentoses. Furthermore, Fusaria can compete successfully with most of the microorganisms in degrading and fermenting phytomass simultaneously. Members of the genus Fusarium are associated with vigorous ethanol formation even under aerobic conditions and that is why Birkinshaw (3) called them "the alcohol formers par excellence."

It is obvious that further research is needed to elucidate certain biochemical, genetic, and ecological matters concerning Fusarium strains before considering them for lignocellulose decomposition and ethanol production. Efforts should be concentrated on the isolation, selection, and development of Fusarium strains having the ability to economically degrade and ferment phytomass to ethanol. Furthermore, consideration should also be given to the economic, technological, environmental, and sociopolitical issues relevant to this technology.

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