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Ethanol Production Via Fungal Decomposition and Fermentation of Biomass

Phase II (FY 1981)
Annual Progress Report

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ARGONNE NATIONAL LABORATORY
Energy and Environmental Systems Division

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ANL/EES-TM-162

ARGONNE NATIONAL LABORATORY
9700 South Cass Avenue
Argonne, Illinois 60439

ETHANOL PRODUCTION
VIA FUNGAL DECOMPOSITION AND
FERMENTATION OF BIOMASS

Phase II (FY 1981)
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ANL/EES-TM--162

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by

Antonios A. Antonopoulos and Edward G. Wene
Energy and Environmental Systems Division

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PREFACE

This annual progress report focuses on research efforts and results during the second phase (FY 1981) of the Ethanol Production via Fungal Decomposition and Fermentation of Biomass program at Argonne National Laboratory. This work is sponsored and funded by the U.S. Department of Energy through the Solar Energy Research Institute (SERI).

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ETHANOL PRODUCTION
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ABSTRACT

This program has as its main goal the isolation and development of Fusarium strains that can efficiently and economically decompose plant polysaccharides to pentoses and hexoses and ferment them to ethanol for fuel purposes. During Phase II (FY 1981) of this program, more than 800 new Fusarium isolates were isolated and screened. All showed cellulolytic activity. The Fusarium mutant ANL 3-72181 (derived after UV exposure of ANL 22 isolate) produced 2.45 IU cellulase after 14 days. This cellulase activity was achieved in the presence of 0.7 mg/mL extracellular protein. In separate tests, the use of both proteose peptone and yeast extract with 1% cellulose increased the production of extracellular protein three times over that on cellulose alone. Initial fermentation by Fusarium strains on 1% glucose produced up to 4.2 mg/mL ethanol in 48 hours. All Fusarium isolates and mutants found during this period were screened for xylose fermentation. Ethanol production during early experimentation required from 120 to 144 hours to yield 4.0 to 4.5 mg/mL ethanol from 1% xylose solutions. Through continuous selection of isolates, this time was reduced to 66 hours. By recycling Fusarium cell mass, fermentations of 1% xylose yielded 4.0 to 4.3 mg/mL ethanol in 48 hours. Consecutive fermentations of 2% xylose produced an average of 8.1 mg/mL ethanol in 48 hours. Fermentation of a 4.5% xylose + 2% glucose solution produced 21 mg/mL ethanol and 0.8 mg/mL acetic acid, while fermentation of a 7% xylose + 2% glucose solution yielded 25.5 mg/mL ethanol and 0.85 mg/mL acetic acid; these fermentations were aerated at a rate of 0.03 v/v-min.

1 EXECUTIVE SUMMARY

It has been demonstrated that several Fusarium strains possess the necessary enzymatic mechanisms to degrade plant macromolecules (celluloses, hemicelluloses, pectic substances, and lignins) to their simplest units, and to ferment both five- and six-carbon sugars to ethanol. Since ethanol blends

well with gasoline to form gasohol, the main goal of this program is to isolate and develop Fusarium strains that can decompose plant polysaccharides to pentoses and hexoses and then ferment these monosaccharides to ethanol efficiently and economically. This summary presents the research efforts and results of Phase II of the program (which coincides with FY 1981).

Research efforts during Phase II were a continuation of those made in Phase I (FY 1980), focusing mainly on the isolation, selection, and development of Fusarium strains capable of degrading and fermenting plant constituents to ethyl alcohol. Efforts also continued on the characterization of enzymes involved and on the optimization of parameters that affect hydrolysis and fermentation.

Both selective and nonselective media were employed to identify more than 800 Fusarium isolates, most of which belonged to the oxysporum type. It was noted that the most effective isolating medium was dextrose-peptone-rose bengal-streptomycin-PUNB.

All Fusarium isolates were initially screened for cellulolytic activity by employing media in which cellulose was incorporated and by using various specific cellulase assays, as previously reported (Antonopoulos and Wene, 1980). The presence of pentoses in liquid media was found to be inhibitory to the production of cellulase by the isolates tested. All the Fusarium isolates screened have shown at least some cellulolytic activity. About 60 of the isolates appear to have higher cellulase activity and these are being examined further.

During evaluation of the Fusarium cellulase complex by filter paper assay (FPA), a temperature of 50°C and a pH of 5.3 were determined to be the optimal parameters for this method, and therefore all FPAs were conducted under these conditions. A 1% Solka Floc SW-40 concentration was found to be optimal for cellulase production. An end-product inhibition of cellulase production was noted, and restricted aeration in the fermentor was found to increase Fusarium cell mass and filter paper activity.

The IU CMCase/IU FPase ratio was seen to remain nearly constant (around 4:1 during four to eight days of hydrolysis) for the Fusarium isolates tested.

A Fusarium mutant (Strain #ANL 3-72181) derived from a culture (ANL 22) exposed to UV irradiation was observed to produce 2.45 IU cellulase after 14 days. Both inoculum and nutritional parameters are under optimization in order to accelerate cellulase production by this mutant.

The highest cellulase activity (2.45 IU/mL) achieved by tested Fusaria occurred at a level of 0.7 mg/mL of extracellular protein. Since extracellular protein appears to be related to cellulase production, experiments were conducted to stimulate the production of extracellular protein by Fusarium strains during cellulose hydrolysis. In separate tests, the use of both proteose peptone (PP) and yeast extract (YE) with 1% cellulose increased the production of extracellular protein three times over that achieved on cellulose alone. Filter paper activity in these tests was increased five times with PP and YE. When either PP or YE was used in combination with cellobiose, xylan, or carboxymethyl cellulose, a further increase in production of

extracellular protein and filter paper activity was seen. Tests are currently under way to determine the nature of the stimulatory effect of the above substances, to optimize the parameters of extracellular protein production by *Fusaria*, and to search for *Fusarium* isolates or mutants that have a higher ratio of cellulase released per unit of extracellular protein.

Screening was continued to identify lignolytic and hemicellulolytic strains. Several *Fusarium* isolates grown on xylan media formed large numbers of fusoid-to-spheroid cells and exhibited restricted mycelial growth. Since these characteristics may increase overall cellular surface and enhance fermentation potential, several of these isolates were grown on different types of media. Isolates in liquid culture containing cellulose retained the fusoid-to-spheroid morphology, and cell mass increased.

Much effort was put into screening the available *Fusarium* isolates for fermenting effectiveness. Several hundred isolates were tested, and all were able to ferment glucose to ethanol to some degree. Of the isolates tested, several yielded 4.3 mg/mL of ethanol within 48 hours in 1% glucose solutions, a yield representing a conversion efficiency that is approximately 90% of that achieved in yeast fermentations. Ongoing experimentation is being conducted in an attempt to improve this efficiency.

Emphasis was placed on identification of *Fusarium* isolates that could effectively and efficiently ferment five-carbon sugars (particularly xylose) to ethanol. All *Fusarium* isolates and mutants found during this phase were screened for xylose fermentation. It was noted that the isolates that show the highest production levels of cellulase are also among the best xylose fermenters. Initially, ethanol production was relatively slow, requiring from 120 to 144 hours to yield 4.0 to 4.5 mg/mL ethanol from 1% xylose solutions. Through continuous selection of isolates, this time was reduced to 66 hours.

Because mycelial growth of *Fusarium* will not likely be able to compete with yeasts and bacteria in reproduction rate, a technique was developed and utilized to enhance xylose fermentation and increase ethanol yields. *Fusarium* inoculum made mostly of spores, germinated spores, and fusoid-to-spheroid cells (no mycelium), and centrifuged and pelleted after each consecutive fermentation, was reused to ferment xylose. By recycling *Fusarium* cell mass in this manner, fermentations of 1% xylose yielded 4.0 to 4.3 mg/mL ethanol in 48 hours.

Consecutive fermentations of 2% xylose by several *Fusarium* isolates produced an average of 8.1 mg/mL ethanol in 48 hours. Studies on 3% xylose showed that the fermentation rate was slow. When glucose was added along with higher xylose concentrations, the rate improved significantly. Fermentation of a 4.5% xylose plus 2% glucose solution produced 21 mg/mL ethanol in 72 hours. When 7% xylose plus 2% glucose solution was fermented, the overall productivity reached 25.5 mg/mL in 116 hours. These fermentations were aerated at the rate of 0.03 v/v-min. With the higher concentrations of xylose, aeration was found to increase the rate of fermentation. Acetic acid levels in these fermentations at the end of the runs were 0.8 (for the 4.5% xylose + 2% glucose aerated fermentation) and 0.85 (for the 7% xylose + 2% glucose aerated fermentation) mg/mL.

Many UV-induced Fusarium mutants have been obtained and tested for cellulolytic and fermenting ability. One new isolated Fusarium mutant produced less-than-detectable levels of acetic acid during aerated fermentations.

Several Fusarium isolates were tested for their ethanol tolerability. All were able to grow and reproduce in the presence of up to 55 mg/mL ethanol. Screening is continuing in order to evaluate higher ethanol tolerance by Fusarium isolates under fermentation conditions.

It is anticipated that, during Phase III (FY 1982) of this program, research will focus primarily on the following areas: (1) isolation of Fusarium strains that are capable of degrading plant macromolecules; (2) selection of Fusarium strains that show superior lignocellulolytic ability; (3) genetic manipulation (including mutagenesis) of Fusaria to develop superior strains; (4) determination of optimal parameters for better biomass saccharification and fermentation; (5) identification of concentrations of end product(s) tolerable by Fusarium strains; (6) identification and quantification of enzymes involved and stimulation to increase and accelerate enzyme yield by Fusaria; and (7) identification of synergistic and/or complementary actions of selected Fusarium strains and other microorganisms in saccharifying and fermenting biomass.

2 INTRODUCTION

It has been recorded that Fusarium isolates can effectively: (1) degrade several plant (biomass) constituents (cellulose, hemicelluloses, pectic substances, and lignin); (2) stimulate production of ethylene which appears to augment polysaccharide hydrolysis; and (3) ferment simple sugars (both pentoses and hexoses) to ethanol.

The main purpose of this program is to produce ethanol via fungal decomposition and fermentation of biomass. Specific objectives are to (1) isolate and develop Fusarium strains by exploring their parasexuality and mutagenic reactions that effectively delignify, saccharify, and ferment biomass to ethanol; (2) optimize the parameters that dictate maximum ethanol production via decomposition and fermentation of biomass by the selected Fusarium strains; (3) identify the type and quantity of enzymes released by the strains, and explore the use of stimulants that could increase and accelerate the enzyme yield; (4) investigate the synergistic action of Fusarium strains with other fungal and bacterial isolates that are known to degrade and ferment biomass; and (5) make comparative economic studies of this process and other known processes for obtaining ethanol. Phase I (FY 1980) covered parts of the first two objectives, and research findings have been reported (Antonopoulos and Wene, 1980). FY 1981 included Phase II of the program, and this report presents research conducted and results obtained during this phase.

The genus Fusarium includes individuals of imperfect (asexual) stages of certain ascomycetous (sexual) genera. Members of this genus are organisms with a wide substrate range. They produce a series of enzymes that enables them to break down most of the plant-tissue constituents for use as a carbon source.

Delignification and saccharification of natural cellulose are the main prerequisites in obtaining energy stored in plant tissues. Fungi, because of their hyphal (mycelial) nature and continuous release of cellulases, are outstanding cellulose hydrolyzers. Many fungi, representing imperfect stages of Ascomycetes and many Basidiomycetes, are the main cellulolyzers in natural environments. Research has shown that such cellulolyzers can be found among Fusarium strains. Husain and Dimond (1958, 1960) found that Fusarium oxysporum f. sp. lycopersici showed cellulase activity on wood cellulose, paper, cotton linters, carboxymethyl cellulose, and living tomato tissue. Davis et al. (1953) reported that the same fungus yields detectable amounts of β -glucosidase within the infected plant tissue and is able to hydrolyze cellobiose.

White et al. (1948), 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. Fusarium moniliforme was found to secrete cellulase, and thus was able to disintegrate hardened and mature plant tissues (Venkata Ram, 1956). Fusarium solani was also reported to be a cellulase producer (Wood and McRae, 1978). Geethadevi et al. (1978), as well as Sedha et al. (1978), in screening fungal isolates for cellulase production, identified several strains of Fusarium that released C_1 - and C_x -cellulases. Svabova et al.

(1980), in exploring the enzyme apparatus of the genus Fusarium, noted that all 35 strains belonging to sixteen different Fusarium species produced C_x-cellulase, C₁-cellulase, β -D-glucosidase, xylanase, and β -D-xylosidase.

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, quantitative data are presently inadequate to support general theories pertaining to the potential of these fungi as cellulolyzers. Despite this lack of information, there is reason to believe that Fusarium strains that readily attack native cellulose do exist, and that new strains can be developed.

During Phase I of this program, more than 500 Fusarium strains had been isolated; most of these were of the oxysporum type (Antonopoulos and Wené, 1980). Isolated Fusaria were tested for cellulase activity by several methods on various types of cellulose substrates. All of the isolated Fusarium strains showed cellulolytic activity. Forty-five of those tested showed increased activity.

In addition to cellulose, the hemicelluloses are also important biomass constituents. They are of interest as sources of fuel, since they can be decomposed to simple sugars that can be further fermented to ethanol, etc. Information pertaining to hemicellulolytic activities of Fusarium is insufficient, and additional research is needed. Simpson (1950), 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. Gascoigne and Gascoigne (1960), in studying the xylanase activities of 13 fungal species, found that Fusarium roseum possesses an effective extracellular enzyme system made of xylanases and trans-pentosylases that catalyzes the hydrolysis of esparto xylan to xylose and other compounds.

Lignin is another important component of mature plant cells, particularly those of woody tissue. It is generally deposited between and around the cellulose microfibrils and heavily lignifies the middle lamella. There are few reports concerning biodegradation of lignin by Fusarium species. Norris (1980) noted that an isolate (AF-W1) of Fusarium solani significantly degraded lignin in sound wood. Ohta et al. (1979) reported that F. solani M-13 was the most vigorous microbial degrader of synthetic lignin (a dehydrogenation polymer of coniferyl alcohol) among organisms that they isolated from soils. Ledingham and Adams (1942) investigated the decomposition of calcium lignosulfonate by 13 species of Fusarium and found that these fungi were able to degrade up to 13% of the lignin contained in the medium. Fischer (1953) reported that Fusarium lactis, F. nivale, and other, unidentified, Fusarium species were the most effective fungi (included in their investigation) in destroying phenol lignin, i.e., lignin extracted from wood with phenol. In similar studies, Waksman and Hutchings (1936) noted that soil-borne Fusarium species decreased the potentially recoverable lignin in the medium by as much as 25%. As Kirk (1971) mentions, Gulyas (1967) showed that pure cultures of soil-borne Fusarium spp. were able to decompose up to 20% of the lignin contained in wheat straw.

During Phase I of the present study, experimentation with sodium-lignosulfonate substrates inoculated with several Fusarium strains yielded encouraging results, but further research on lignolysis by Fusarium strains was needed (Antonopoulos and Wene, 1980). Additional experiments demonstrated that many Fusarium strains grown on xylan show a characteristic morphological and growth change, i.e., the mycelial type disappears and the cells change to fusoid and spheroid forms and increase in number (Antonopoulos and Wene, 1980).

The ability of Fusarium species to ferment sugars to ethanol has been noted by many investigators. These fungi seem to be equipped with a highly efficient sugar-transport mechanism that enables them to glycolyze and readily ferment the simple sugars. Strong evidence resulting from studies of carbon balance, enzymatic analyses, and isotope distribution indicates that the Embden-Meyerhof-Parnas (EMP) mechanism functions anaerobically in Fusarium spp. to produce ethanol (Cochrane, 1956; Heath et al., 1956). Yeasts and Fusaria have certain characteristics in common in the way they ferment sugars to ethanol (Birkinshaw et al., 1931; Nord and Weiss, 1951).

Birkinshaw and others (1931) found that all 23 species of Fusarium investigated yielded appreciable amounts of ethanol and suggested the possibility of separating a few species that have a rather unusual fermentative potential. The same authors recorded that with F. avenaceum, of 4.683 g of glucose carbon metabolized, 2.453 g of carbon appeared as "volatile neutral compounds," which means a yield of 52.4% (carbon percentage) ethanol; this is in comparison to the maximum yield of 60% (carbon percentage) ethanol obtained with species of Saccharomyces.

Anderson and coworkers (1933), experimenting with Fusarium oxysporum in metabolizing glucose, found that in 20 days the percentage of the original carbon in the form of ethanol was 49.21 and increased to 51.89 at the end of 46 days.

Many investigators have studied the fermentative abilities of Fusarium oxysporum f. sp. lini (the cause of flax wilt). Anderson and his co-workers (1933), 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. Cochrane (1956), using resting cells of F. lini to metabolize 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.

During Phase I of this program, several strains of Fusarium, selected on the basis of their cellulolytic potential, produced up to 4.2 mg/mL of ethanol in 48 hours in 1% glucose solutions (Antonopoulos and Wene, 1980).

Pentose fermentation by Fusarium species has been considered as the model of the mechanism that possibly occurs in several other species of fungi. Resting cells of F. lini were reported to ferment xylose in this sequence: xylose → triose phosphate + acetyl phosphate; triose phosphate → ethanol + carbon dioxide; acetyl phosphate → acetate (Cochrane, 1956; Gibbs et al., 1954). Therefore, one mole of fermented xylose produces one mole of

ethanol + one mole of carbon dioxide + one mole of acetic acid. Letcher and Willaman (1926) and White and Willaman (1928) observed that, while fermentation of hexoses with F. lini yields the same ratio of carbon in ethanol and carbon dioxide as do the yeasts, with pentoses (which yeasts cannot ferment [Barnett, 1976]) the ratio is nearly 1:1.

Batter and Wilke (1977), in a study of the fermentation of xylose to ethanol by Fusarium oxysporum, estimated that the ethanol yield factor was 0.41 g/g xylose fermented. Wilke (1978) and his coworkers further studied the xylose fermentation by F. oxysporum in connection with their investigation into the bioconversion of cellulose to ethanol. Rosenberg et al. (1980) investigated the utilization of a Fusarium oxysporum f.sp. lini strain for xylose fermentation to ethanol; they also determined the growth rate, ethanol tolerability, and other characteristics of this strain.

During Phase I of the present study, experiments with Fusarium strains in fermenting xylose showed that, of 150 strains tested, seven isolates yielded appreciable amounts of ethanol (Antonopoulos and Wene, 1980). As an example, Fusarium strain #ANL-10-680 produced 4.0 mg/mL of ethanol after 66 hours of 1% xylose fermentation.

The literature review revealed the ability of Fusarium species to degrade phytomass and yield ethanol via fermentation. 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. Members of the genus Fusarium are associated with vigorous ethanol formation, even under aerobic conditions (Birkinshaw, 1937). Results from research conducted during Phase I of this program were more than encouraging (Antonopoulos and Wene, 1980). It became obvious during Phase I that further research was needed to elucidate certain matters concerning Fusarium strains before considering them for lignocellulose decomposition and ethanol production. Therefore, future efforts will be concentrated on the isolation, selection, and development of Fusarium strains having the ability to economically degrade and ferment phytomass to ethanol.

3 RESEARCH EFFORTS AND RESULTS

During Phase II (FY 81) of this program, research efforts focused on the isolation, development, and screening of Fusarium strains that had a high ability to decompose plant polysaccharides in order to liberate simple sugars (such as glucose and xylose) and ferment them to ethanol. Efforts also continued on the characterization of enzymes involved and on the optimization of parameters that affect hydrolyses and fermentations by Fusaria. The following sections cover the research conducted and results obtained during this period.

3.1 ISOLATION AND DEVELOPMENT OF FUSARIA ABLE TO DECOMPOSE AND FERMENT BIOMASS

Efforts were concentrated on the isolation, selection, culturing, screening, and maintenance of Fusarium isolates that were capable of decomposing and fermenting biomass constituents. The isolates were obtained from various sources, and the media and techniques used for isolation and handling were similar to those reported in the previous phase (Antonopoulos and Wene, 1980). Conventional nonselective media, including potato-dextrose-agar (PDA), water agar (WA), and soil-extract agar, were used for isolation. In addition, selective media such as dextrose-peptone-PCNB-streptomycin-rose bengal, oxgall-rose bengal, and oxgall-rose bengal-streptomycin (Antonopoulos and Wene, 1980) were employed to isolate Fusarium strains. Several other media containing selective microbial-growth retardants were also tested. The purpose in using different media types was to exploit the selective advantages of the media for isolating strains of Fusarium species that exhibit a varied preference for nutrients and other physicochemical factors.

More than 800 new Fusarium isolates were identified, screened, and further investigated during FY 1981. The most effective isolating medium was found to be dextrose-peptone-rose bengal-streptomycin-PCNB-agar, while the Phosphon (tributyl [2,4-dichlorobenzyl] phosphonium chloride) media completely inhibited the growth of the tested Fusaria. Most of the new Fusarium isolates belonged to the oxysporum type. Cultures of Fusarium isolates and mutants were maintained on PDA media.

Ultraviolet irradiation was used to induce mutation in cultures of Fusarium in an attempt to develop mutants superior to the parent isolates in degrading polysaccharides and fermenting sugars to ethanol. Samples of three-to-five-day-old liquid cultures of Fusaria grown in xylose or cellulose substrates were mildly centrifuged, causing most of the spores to be retained in the top of the centrifuge tube. This spore suspension was then separated and centrifuged for 15 min at 3000 G. The spores were then washed and suspended in a 0.1-M magnesium sulfate solution, and the spore count was adjusted to 4×10^6 per mL with a hemacytometer.

Five mL of the same spore suspension was placed in a glass petri dish and exposed to an ultraviolet lamp (254 nm). Irradiation time was varied in order to achieve a survival rate (spore germinability) near 0.1%. Irradiated spore suspensions of Fusaria were diluted and spread on cellulose agar plates

to obtain a maximum density of 30 colonies per plate. Plates were incubated for three to five days at 30°C, then placed at 50°C overnight, and examined the next day for clearing zones around developing colonies. Colonies that showed large zones of clearing in the cellulose medium were then subjected to further screening for both cellulolytic and fermenting ability.

3.2 SEARCHING FOR CELLULOLYTIC FUSARIA

All Fusarium isolates were initially screened for cellulolytic activity, employing agar media in which cellulose was incorporated. Various specific cellulase assays were used, as previously reported (Antonopoulos and Wene, 1980). In particular, the cellulose test-tube clearing technique was utilized for rapid evaluation of the Fusaria's cellulolytic capability. The presence of pentoses in liquid media was found to inhibit the production of cellulase by the isolates tested. Several Fusarium isolates performed well in dye-release cellulase assay procedures; however, this performance did not continue in liquid cultures of cellulose substrates. All tested Fusarium isolates have shown at least some cellulolytic activity. About 60 of the isolates appear to have higher cellulase activity and these are being examined further.

During this phase, research focused on the evaluation of the Fusarium cellulase complex. Inoculum for cellulase production was grown in 250-mL flasks in a reciprocating water bath at 30°C. Flasks were inoculated by surface-scraping petri-dish cultures and growing them for three to five days in a basic mineral medium with 1 g/L of ammonium nitrate, yeast extract, and proteose peptone. This culture was used as inoculum for studies in a 500-mL fermentor. The basic medium for the fermentor consisted of either ammonium nitrate, ammonium sulfate, or urea, proteose peptone, yeast extract, Tween 80, (the concentrations of the preceding chemicals were varied), and basic minerals (consisting of 1 g KH_2PO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.0 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ per liter). Cellulose (Solka Flock SW-40 [Brown Co., Berlin, N.Y.] or BW 200) was tried as a carbon source. The cellulase activities were expressed in international units (IU/mL). Filter paper activity was based on the procedure of Mandels et al. (1976) and measured the release of reducing sugar produced in one hour by 1 mL of enzyme from 50 mg of Whatman No. 1 filter paper treated with 1 mL of a pH 5.3 acetate buffer. Incubation temperature was 50°C. Reducing sugars were determined by the dinitrosalicylic acid (DNS) procedure of Miller (1959), using glucose as a standard.

To evaluate the Fusarium cellulase complex, it was necessary to establish the optimum assay conditions. Therefore, the filter paper assay was conducted at temperatures of 30°, 40°, 50°, and 60°C to determine the optimum temperature for Fusarium cellulase production. The results of this experimentation indicated that 50°C was optimum for filter paper cellulase activity for the strains tested, as Figure 1 shows for strain #ANL 22-760. A pH range of 3.8 to 5.8 was also examined in order to determine optimum pH at 50°C. As Figure 2 indicates, for strain #ANL 22-760 and other strains tested, a pH of 5.3 was optimal for the expression of filter-paper cellulase activity. Based on these findings, all subsequent filter paper assays were conducted at 50°C with the assay buffer at pH 5.3.

Fig. 1 *Fusarium* (Strain #ANL 22-760) cellulase yield affected by various temperatures during filter paper assay. Optimum temperature was shown to be 50°C.

Fig. 2 *Fusarium* (Strain #ANL 22-760) cellulase yield affected by pH during filter paper assay. Optimum pH was shown to be 5.3.

The first *Fusaria* tested for cellulolytic activity were those isolates that showed the greatest depth of clearing in the test-tube-clearing assay, and those that had the highest rates of ethanol fermentation. Initial enzyme production in shake flasks ranged from 0.2 to 0.8 IU/mL after 14 days. Cellulose (Solka Floc SW-40) concentrations of 1% were optimum for cellulase production. At these levels of cellulase yield, the influence of yeast extract, proteose peptone, and Tween 80 was not apparent.

End-product inhibition was also tested by growing a single isolate (ANL 22-760) with 1% Solka Floc (SW-40), 1% Solka Floc (SW-40) plus 1% xylose, and 1% xylose. After 20 days the yield in the 1% Solka Floc flasks approached 1 IU/mL cellulase, while the other two flasks produced less than 0.4 IU/mL cellulase (Figure 3). This same type of experiment was repeated with 1% SW-40, 1% SW-40 and 0.25% xylose, 1% SW-40 and 0.25% xylan, and 1% SW-40 and 0.25% cellobiose (Figure 4). At these lower levels, there was no inhibition by any of the end products. At the 24th day, the filter-paper-cellulase (FP'ase) units obtained were 1.5/mL of 1% SW-40 + 0.25% xylan medium, while those for the other combinations were approximately 1.1 IU/mL. Although end-product inhibition was suspected in this system (by suppression of cellulase production), the tested *Fusaria* seemed able to metabolize these end products, and the inhibition was precluded.

Results of the end-product-inhibition experiments indicated the existence of a substantial time lag between the inoculation of the culture and the release of enzyme (cellulases) into solution (Figure 3). In order to decrease this time lag, cultures were grown in a 500-mL fermentor with aeration. Aeration increased the cell mass and, subsequently, filter-paper enzyme activity. Figure 5 shows cellulase production on 1% Solka Floc (SW-40) with an aeration rate of 0.08 v/v-min, by three different *Fusarium* mutant strains (ANL 8-72181, ANL 7-72181, and ANL 3-72181).

Fusarium mutant strain #ANL 3-72181, derived from a culture of ANL-22 exposed to ultraviolet irradiation, created larger clearing zones on cellulose agar plates than did the parent isolate. When grown in a medium with basic minerals and 1 g/L of ammonium nitrate, a two-week-old culture produced only 0.3 IU/mL cellulase. Figure 6 depicts the results when this isolate was grown with 1 g/L ammonium nitrate, 1 g/L yeast extract, and 1 g/L proteose peptone. Production was 2.45 IU/mL cellulase after 14 days and 2.65 IU/mL after 16 days. It is apparent that there is still a long time lag during which small amounts of extracellular cellulase are produced. Work is now in progress on optimizing inoculum and nutritional requirements in order to reduce the lag time for cellulase production.

Carboxymethyl cellulase was also measured by the release of reducing sugar (during 30- and 60-min periods from 0.5 mL of enzyme mixed with 1 mL of 2% carboxymethyl cellulose (Sigma Co., St. Louis, Mo.) in pH-5.3 acetate buffer, at an incubation temperature of 50°C. The ratio between carboxymethyl cellulase production and filter paper activity (in CMCCase/FP'ase IU) was found to be nearly constant. As Figure 6 shows, in the early stages (four to eight days), the ratio was approximately 4:1; at 14 days it was near 7:1. This ratio remained constant over many fermentor runs.

Fig. 3 Cellulase Production (in IU/mL) by Fusarium Strain #ANL 22-760 Grown for 20 Days on Various Media during End-Product Inhibition Tests

Fig. 4 Cellulase Production (in IU/mL) by *Fusarium* Strain #ANL 22-760 Grown for 24 Days on Various Media during End-Product Inhibition Tests

Fig. 5 Cellulase Production by Fusarium Strains #ANL 3-72181, ANL 7-72181, and ANL 8-72181 Grown on 1% Solka Floc with an Aeration Rate of 25 mL/min

Fig. 6 Fluctuation of the Ratio between Carboxymethyl Cellulase Production and Filter Paper Activity (IU CMCase/ IU FP'ase) of Fusarium Strain #ANL 3-72181 vs. Time

The highest cellulase activity (2.45 IU/mL in 14 days, and 2.65 IU/mL in 16 days, as Figure 6 shows) achieved by a UV-mutant strain of Fusarium was produced in the presence of 0.7 mg/mL of extracellular protein. Extracellular protein was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as the standard. Samples were centrifuged for 15 min at 10,000 rpm, and protein was not precipitated. We have not yet compared the extracellular protein production of the UV mutant to the parent strain to determine whether the difference in activity is due to more activity per unit of protein or to an increased rate of protein production. Efforts are under way to optimize fermentor conditions in order to increase the rate of extracellular protein production and to search for isolates or mutant strains of Fusarium that have a higher ratio of cellulase per unit of extracellular protein.

The isolates with the highest levels of cellulase production were also among the best xylose fermenters. This may reflect the production of extracellular protein, i.e., those isolates that produce the most extracellular protein produce more enzymes that, in turn, degrade more cellulose and ferment the by-products to ethanol.

3.3 INVESTIGATION OF LIGNOLYTIC AND HEMICELLULOLYTIC STRAINS

During Phase II, screening was continued for lignolytic and hemicellulolytic strains. Several Fusarium isolates grown on solid xylan-agar media formed large numbers of fusoid-to-spheroid cells and exhibited restricted mycelial growth. Since these morphological characteristics may increase overall cellular surface and enhance fermentation potential, several isolates that exhibited this growth pattern were cultured on different types of liquid media.

Isolates grown in liquid culture containing cellulose retained the fusoid-to-spheroid spore morphology; in addition, hyphal growth was restricted and cell mass was increased. The lack of mycelial formation is important if it can be maintained during fermentation.

Xylanase activity of Fusarium isolates tested was measured by the release of reducing sugar in 15 min from 0.5 mL of enzyme and 5 mL of 0.5% xylose in pH-5.3 acetate buffer and incubated at a temperature of 50°C.

3.4 SCREENING FOR EFFECTIVE SUGAR FERMENTERS

During Phase II, much effort was put into screening the available Fusarium isolates for fermenting effectiveness in producing ethanol from glucose and xylose. Cultures of Fusaria were maintained on PDA in petri dishes. Inoculum for 500-mL fermentations was grown for three days in 250-mL flasks, then 10 to 150 mL was centrifuged and the resulting pellet used for inoculum. The fermentor medium was adjusted to pH 5.0-5.2 with hydrochloric acid after sterilization and left uncontrolled for the duration of the fermentation. Fermentations were conducted without controlled aeration, unless otherwise indicated. Ethanol and acetic acid determinations were made on a Varian 3700

gas chromatograph with a 6-ft-long, 0.25-in.-O.D. glass column packed with Porapak Q. Injection temperature was 210°C, column temperature was 163°C, and the temperature of the flame ionization detector was 220°C.

3.4.1 Glucose Fermentation

Of the isolates tested, several yielded 4.3 mg/mL of ethanol within 48 hr in 1% glucose solutions. This yield represents a conversion efficiency that is approximately 90% of that achieved in yeast fermentations. Figure 7 shows representative results obtained with Fusarium strain #ANL 99A-780 (three repetitions with different subcultures) that fermented 1% glucose solution. Similar glucose fermentations with new isolates and mutants are under observation.

3.4.2 Xylose Fermentation

One of the main aspects of Phase II was the conversion of pentose sugars to ethanol. Emphasis was centered on identification of Fusarium isolates and mutants that could effectively and efficiently ferment five-carbon sugars (particularly xylose) to ethanol. Optimization of fermentation parameters was also stressed.

More than 1000 Fusarium isolates and mutants have been screened for pentose (xylose) fermentation. These screenings were conducted in 50-mL flasks with intermittent agitation and no pH control. Further tests were conducted in 500-mL flasks at 30°C with continuous agitation. The most promising isolates were then tested in a 500-mL Bioflo fermentor.

Original batch fermentation experiments utilizing the 500-mL Bioflo fermentor produced varying results when testing separate isolates. Ethanol production was relatively slow, requiring from 120 to 144 hr to produce 4.0 to 4.5 mg/mL ethanol from a 1% xylose solution. By screening and selecting the best fermenters, this time was reduced to 66 hr (still relatively slow).

Recognizing that mycelial growth of Fusaria will not likely be able to compete with yeasts and bacteria in reproduction rate, we investigated different inoculation techniques. By plating 0.5- to 1.0-mL portions of actively growing fermentation media onto agar substrates containing 1% xylose, mycelial growth was restricted and large numbers of microconidia were produced. At the same time, Fusarium isolates were grown in a liquid medium containing cellulose (Solka Floc-SW-40). Inoculum produced in this medium had restricted mycelium, and most of the cell mass consisted of spores (both macroconidia and microconidia), germinated spores with short germ tubes, and fusoid-to-spheroid cells.

Employing a Fusarium inoculum that consists mainly of spores and fusoid-to-spheroid cells, rather than mycelium, has several advantages. This inoculum is easily handled, causes fewer problems with clogging tubes, and does not restrict agitation by collecting on and clinging to fermentor baffles and other internal projections.

Fig. 7 Ethanol Yield (mg/mL) vs. Time (hr) during Fermentation by Three Subcultures (I, II, and III) of Fusarium Strain #ANL 99A-780 in 1% Glucose

Therefore, it was decided to utilize the following inoculation procedure to produce Fusarium inoculum to ferment xylose. Four plates of 1% xylose agar were inoculated with 0.5 mL of a Fusarium isolate liquid culture and grown for two to three days at 30°C. The four plates were then surface-scraped into 250 mL of a 1% cellulose (Solka Flow-SW-40) suspension. This suspension was maintained at 30°C with agitation from a Teflon stirring bar (without agitation, extensive mycelium developed). After two to three days, approximately 150 mL of this culture was mildly centrifuged and the pellet (40 to 50 mL volume) was used as inoculum for a 500-mL fermentation in a Bioflo fermentor.

Applying the above inoculation procedure, 500-mL volumes of 1% xylose produced up to 4.2 mg/mL of ethanol within 48 hr.

After 48 hours of fermentation, agitation in the fermentor vessel was stopped and the medium was allowed to settle for 30 min. The lower 175 to 200 mL was removed and centrifuged to provide inoculum (final volume 40 to 50 mL) for the next fermentor run. By recycling cells in this manner, seven consecutive 500-mL fermentations of 1% xylose yielded 4.0 to 4.3 mg/mL ethanol in 48 hr.

The same procedure was used to ferment 1% xylose solutions. Five consecutive fermentations by the same Fusarium isolate (ANL 22-760) produced an average of 8.0 mg/mL ethanol in 48 hours. Figure 8 illustrates a single fermentor run that yielded 8.2 mg/mL ethanol from 2% xylose within 48 hours. Also shown is the performance of the same Fusarium isolate when a 3% xylose solution was fermented.

Initial studies on 3% xylose fermentation by *Fusaria* showed that the rate of the process was slower than that of the 2% fermentations. When glucose was added along with higher xylose concentrations, the rate improved. Figure 9 presents the results of a 4.5% xylose plus 2% glucose (and, for comparison, of a 2% xylose) fermentation by strain #ANL 22-760. This fermentation produced 21 mg/mL of ethanol in 72 hours, plus 0.8 mg/mL acetic acid (while the 2% xylose fermentation produced 8.1 mg/mL of ethanol in the same time).

When 7% xylose and 2% glucose solutions were fermented by the same strain, the overall productivity reached 25.5 mg/mL in 116 hours (14.5 mg/mL in 72 hours), plus 0.85 mg/mL of acetic acid, as Figure 10 indicates. Comparing the results obtained from the fermentations of 4.5% xylose + 2% glucose and 7% xylose + 2% glucose, it appears that the fermentation rate was slower with the additional 2.5% xylose.

The last two fermentations were aerated at the rate of 0.03 v/v-min. It was found that, with the higher concentrations of xylose, aeration increased the fermentation rate. Although ethanol fermentation is considered to be an anaerobic process, there is still some requirement for oxygen. One new isolated Fusarium mutant produced less-than-detectable levels of acetic acid during aerated fermentation.

Fig. 8 Ethanol Yield vs. Time during Fermentation of 2% and 3% Xylose by Recycled Cell Mass of Fusarium Strain #ANL-22-760

Fig. 9 Ethanol Yield (mg/mL) vs. Time (hr) during Fermentation of 4.5% Xylose plus 2% Glucose, and 2% Xylose, by Fusarium Strain #ANL 22-760

Fig. 10 Ethanol Yield (mg/mL) vs. Time (hr) during Fermentation of 2% Glucose plus 7% Xylose by Fusarium Strain #ANL 22-760

3.5 OTHER RESEARCH EFFORTS

Another experiment was designed to test the ethanol tolerance of Fusarium isolates that have shown substantial xylose fermenting ability. The tested isolates were able to grow and reproduce in the presence of up to 55 mg/mL ethanol. This level indicated that xylose solutions of up to 8% might be fermented efficiently by these isolates.

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4 RESEARCH PERSPECTIVES

It is anticipated that, during Phase III (FY 1982) of this program, research will focus primarily on the following areas: (1) isolation of Fusarium strains that are capable of degrading plant macromolecules; (2) selection of Fusarium strains that show superior lignocellulolytic ability; (3) genetic manipulation (including mutagenesis) of Fusaria to develop superior strains; (4) determination of optimal parameters for better biomass saccharification and fermentation; (5) identification of concentrations of end product(s) tolerable by Fusarium strains; (6) identification and quantification of enzymes involved and stimulation to increase and accelerate enzyme yield by Fusaria; and (7) identification of synergistic and/or complementary actions of selected Fusarium strains and other microorganisms in saccharifying and fermenting biomass.

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REFERENCES

- Antonopoulos, A. A., and E. G. Wene, Ethanol Production via Fungal Decomposition and Fermentation of Biomass, Phase I, Progress Report, Argonne National Laboratory Report ANL/EES-TM-147 (1980).
- Anderson, A. K., E. L. Everitt, and P. D. Adams, The Carbon Metabolism of *Fusarium oxysporum* on Glucose, J. Agric. Research 46:473-482 (1933).
- Barnett, J. A., The Utilization of Sugars by Yeasts, in Advances in Carbohydrate Chemistry and Biochemistry, R. S. Tipson and D. Morton (eds.), 32:125-234, Academic Press, New York (1976).
- Batter, T. R., and C. R. Wilke, Study of the Fermentation of Xylose to Ethanol by *Fusarium oxysporum*, Lawrence Berkeley Laboratory-University of California at Berkeley Report (1977).
- Birkinshaw, J. H., Biochemistry of the Lower Fungi, Biol. Revs. Cambridge Phil. Soc. 12:357-392 (1937).
- Birkinshaw, J. H., et al., Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products Formed from Glucose by Species of *Fusarium*, in Studies in the Biochemistry of Microorganisms, H. Raistrick et al., Philosophical Transactions (London) 222(-467B):93-98 (1931).
- Cochrane, V. W., The Anaerobic Dissimilation of Glucose by *Fusarium lini*, Mycologia 48:1-12 (1956).
- Davis, D., P. E. Waggoner, and A. E. Dimond, Conjugated Phenols in *Fusarium* Wilt Syndrome, Nature 172:959-961 (1953).
- Fischer, G., Untersuchungen uber den biologischen Abbau des Lignins durch Mikroorganismen, Arch. Mikrobiol. 18:397-424 (1953).
- Gascoigne, I. A., and M. M. Gascoigne, The Xylanases of *Fusarium roseum*, J. Gen. Microbiol. 21:242-248 (1960).
- Geethadevi, B. R., et al., Screening of Fungi for Single Cell Protein and Cellulose Production, Indian J. Microbiol. 18:85-89 (1978).
- Gibbs, M., et al., Fermentation of D-Xylose-1-C¹⁴ by *Fusarium lini* Bolley, Arch. Biochem. and Biophys. 50:237-242 (1954).
- Gulyas, F., On the Role Played by Several Soil Fungi in the Microbiological Decomposition of Lignin, Agrochem. Taloytan 16:137-150 (Recorded by T. K. Kirk, q.v.) (1967).
- Heath, E. C., D. Nasser, and H. Koffler, Biochemistry of Filamentous Fungi, III, Alternative Routes for the Breakdown of Glucose by *Fusarium lini*, Arch. Biochem. and Biophysics 64:80-87 (1956).
- Husain, A., and A. E. Dimond, Production of Cellulase by *Fusarium oxysporum* f. *lycopersici*, Phytopathology 48:263 (1958).

Husain A., and A. E. Dimond, Role of Cellylolytic Enzymes in Pathogenesis by Fusarium oxysporum f. lycopersici, Phytopathology 50:329-331 (1960).

Kirk, T. K., Effects of Microorganisms on Lignin, Ann. Rev. of Phytopathology 9:185-210 (1971).

Ledingham, G. A., and G. A. Adams, Biological Decomposition of Chemical Lignin, II. Studies on the Decomposition of Calcium Lignosulfonate by Wood Destroying and Soil Fungi, Can. J. Research 20(C):13-27 (1942).

Letcher, H., and J. J. Willaman, Biochemistry of Plant Diseases, VIII, Alcoholic Fermentation of Fusarium lini, Phytopathology 16:941-949 (1926).

Lowry, O. H., et al., Protein Measurement with the Folin Phenol Reagent, J. Biol. Chemistry 193:265-275 (1951).

Mandels, M., K. Andreotti, and C. Roche, Measurement of Saccharifying Cellulase, Biotech. and Biophysics Symposium 6:21-33 (1976).

Miller, G. L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chemistry 31:426-428 (1959).

Nord, F. F., and S. Weiss, Yeast and Mold Fermentations, in The Enzymes, J.B. Summer and K. Myrback (eds.), Vol. 2, Part 1, pp. 684-790, Academic Press (1951).

Norris, D. M., Degradation of ¹⁴C-Labeled Lignins and ¹⁴C-Labeled Aromatic Acids by Fusarium solani, Applied and Environ. Microbiol. 40:376-380 (1980).

Ohta, M., T. Higuchi, and S. Iwahara, Microbial Degradation of Dehydroconiferyl Alcohol, a Lignin Substrate Model, Arch. Microbiol. 121:23-28 (1979).

Rosenberg, S. L., et al., Hemicellulose Utilization for Ethanol Production, Lawrence Berkeley Laboratory paper (1980).

Sedha, R. K., H. K. Tewari, and D. S. Chalal, Synthesis of Cellulases in Aspergillus terreus and Fusarium sp., Indian J. Microbiol. 18:93-96 (1978).

Simpson, F. J., Microbial Pentosanases, I. A Survey of Microorganisms for the Production of Enzymes that Attack the Pentosans of Wheat Flour, Can. J. Microbiol. 1:131-139 (1950).

Svabova, M., et al., Enzyme Apparatus of the Genus Fusarium, Folia Microbiol. 25:233-237 (1980).

Venkata Ram, C. S., Studies on Cellulolytic Activity of Fusaria with Reference to Bacterial and Other Cellulose Substrates, Proc. Nat. Inst. Sci. India 22:204-211 (1956).

Waksman, S. A., and I. J. Hutchings, Decomposition of Lignin by Microorganisms, Soil Sci. 42:19-30 (1936).

White, M. G., and J. J. Willaman, Biochemistry of Plant Diseases, X. Fermentation of Pentoses by Fusarium lini, Biochem. J. 22:583-591 (1928).

White, W. L., et al., Assay of Cellulolytic Activity of Molds Isolated from Fabrics and Related Items Exposed in the Tropics, Mycologia 40:34-84 (1948).

Wilke, C. R., Process Development Studies on the Bioconversion of Cellulose and Production of Ethanol, Lawrence Berkeley Laboratory and Univ. of California (1978).

Wood, T. M., and S. I. McCrae, The Cellulase of Trichoderma koningii, Purification and Properties of Some Endoglucanase Components with Special Reference to Their Action on Cellulose when Acting Alone and in Synergism with Cellobiohydrolase, Biochem. J. 171:61-72 (1978).