Appendix I-D

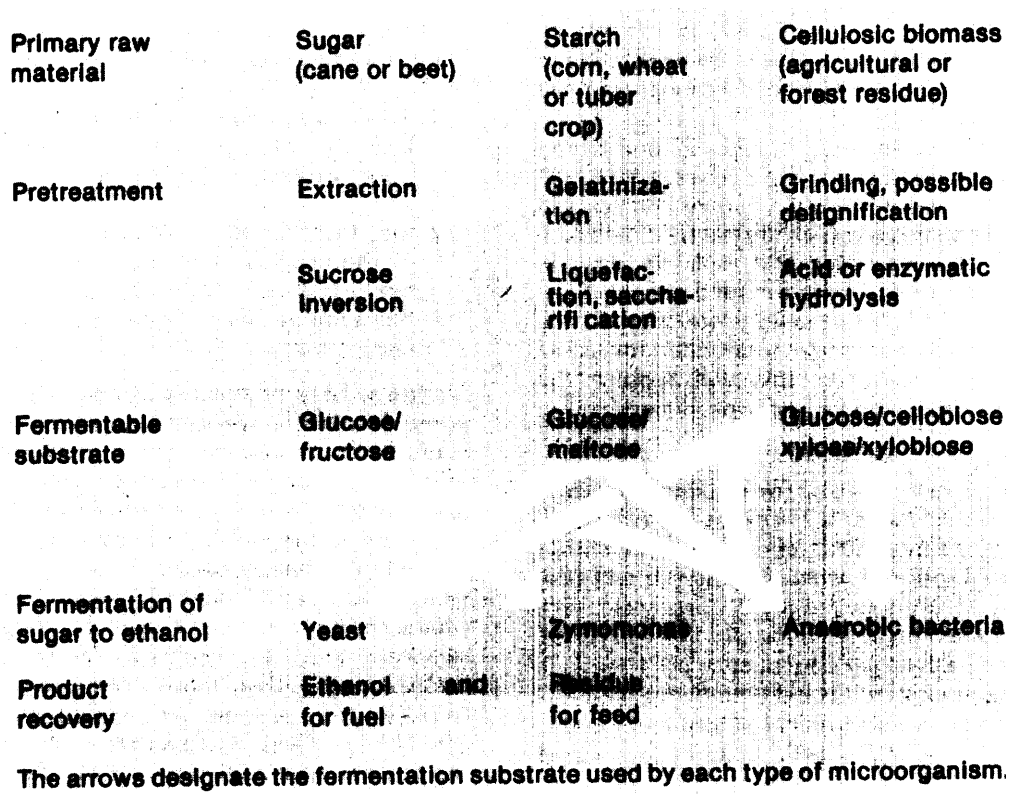
The Impact of Genetics on Ethanol—A Case Study

Objective

This study examines how genetics can and will affect the utilization of biomass for liquid fuels production. There are two major areas where genetics are applicable. One is in plant breeding to improve availability (both quantity and quality) of biomass resources (with existing and previously unused land); the second is in the application of both classical mutation and selection procedures and the new genetic engineering techniques to develop more efficient microbial strains for biomass conversion. Examples of goals in a plant breeding program would include improvements in photosynthetic efficiencies, increased carbohydrate content, decreased or modified lignin content, adaptation of high productivity plants to poor quality land, improved disease resistance, and so forth. However, the focus here is entirely on the second area, the use of genetics to improve microbial-based conversion to produce ethanol.

In order to assess the type and extent of improvements in micro-organisms that might benefit ethanol production, its process technology and economics must first be examined. An overview of the biomass conversion technology is presented in figure I-D-1; processes are defined mainly on the basis of the primary raw material and the type of pretreatment required to produce mono- or disaccharides prior to fermentation. In addition, there are several alternative fermentation routes to produce ethanol; these are characterized by the type of micro-organisms and will be examined with the in-

Figure I-D-l .—An Overview of Alternative Routes for Conversion of Biomass to Ethanol



SOURCE: Massachusetts Institute of Technology.

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tent of quantifying the potential impact of genetic improvement on each one. It is interesting to note that each type of organism has its substrate restrictions, **and only the anaerobic bacteria such as *Clostridium thermosaccharolyticum* and C. *thermohydrosulfuricum* can utilize all of the available substrate.**

Substrate pretreatment

Pretreatment refers to the processing that is required to convert a raw material such as sugarcane, starch, or cellulosic biomass to a product that is fermentable to ethanol. In most cases, the pretreatment is either extraction of a sugar or hydrolysis of a polysaccharide to yield a mono-or disaccharide.

EXTRACTION OF SUGAR

Sugar crops **such as sugarcane, sugar beets, or sweet sorghum are highly desirable raw materials for producing ethanol. These crops contain high amounts of sugars as sucrose. In addition, the yield of fermentable material per acre is high; sugarcane and sugar beets yield 7.5 and 4.1 dry tons of biomass per acre, respectively. '**

Sugar is extracted from cane or beets with hot water and then recrystallized. The resulting sugars are utilized directly by organisms having invertase activity (to split sucrose to glucose plus fructose). Molasses, a sugary byproduct of the crystallization of sucrose, may also contain sucrose although in most cases it is inverted with acid.

The primary use for sugar crops is food sugar. Sugar sells for over 20 cents/lb. Molasses, which currently sells for about $100/ton (about 10 cents/lb sugar) is used extensively as an animal feed. Substantial amounts of both sugar and molasses are imported into the United States for food uses and are therefore unavailable for ethanol production, There are proposals to increase sugar production for use as an energy crop; however, this will require the development of new land for sugar production.

STARCH

The primary raw material for ethanol fermentation in the United States is cornstarch. Corn processed by wet milling, yields about 36 lb of starch from each 56 lb bu; this amount of starch will produce 2.5 gal of absolute ethanol. Corn yields are typically 80 to 120 bu/acre so that 200 to 300 gal of ethanol can be derived per acre of corn per year.

Pretreatment of starch is initiated by a gelatinization step whereby a starch slurry is heated for 5 min at 105° C. After cooling to 98° C, œ-amylase is added to break down the starch to about 15DE (dextrose equivalents). This process of liquefaction reduces the viscosity such that the solution can be easily mixed. After further cooling to 30° C, glucoamylase is added along with a starting culture of yeast so that saccharification and fermentation proceed simultaneously. The resulting fermentation, to produce typically 8 to 10 percent ethanol (volume per volume), requires 42 to 48 hr for completion. This compares with a 16- to 20-hr fermentation if sugar as molasses or cane juice is used as the substrate. Thus, the use of starch requires the addition of enzymes prior to and during fermentation, as well as large fermenter capacity as a consequence of the slower fermentation time compared with sugar substrates.

Improvement in the economy of ethanol fermentation based on starch is possible by developing a micro-organism that can produce ɤ-amylase and glucoamylase and thus eliminate the need to add these enzymes. Since the rate of fermentation depends on the rate of starch hydrolysis, increased levels of glucoamylase may enhance the rate of starch hydrolysis and thus increase the rate of ethanol production, This would lower the capital requirements as well as the cost of enzyme addition.

CELLULOSIC BIOMASS

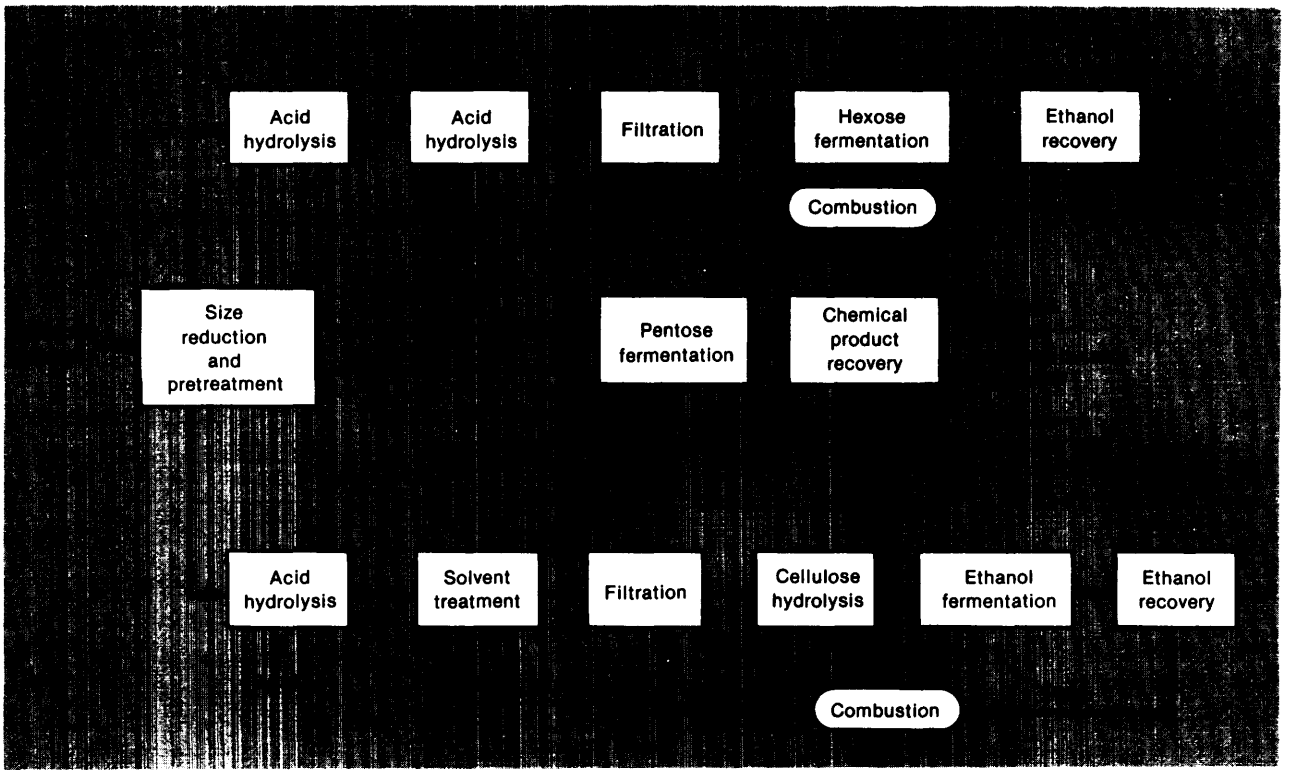
Processes for the utilization of cellulosic biomass to produce liquid fuels all have three features in common:

1. **They employ some means of pretreatment to at least effect some initial size reduction and, more often, cause a disassociation of lignin and cellulose;**
2. **they involve either acid or enzymatic hydrolysis of the cellulose and hemicellulose to produce mono- and disaccharides; and**
3. **they employ fermentation to produce ethanol or some other chemical.**

A wide variety of process schemes have been proposed for the conversion of cellulosic biomass to liquid fuels; a summary of the major steps in two acid hydrolysis and three enzymatic hydrolysis schemes in shown in figures I-D-2 and I-D-3. The initial size reduction is required to increase the amount of biomass surface area that can be contacted with acid, solvent, steam, enzymes, or chemicals that might be used to disassociate the cellulose and hemicellulose from the lignin. Pretreatments that have been investigated to facilitate the process are summarized in table I-D-1. The problems with pretreatment are that they require energy, equipment, and often chemicals; they result in an irretrievable loss of sugar, and in undesirable side-reactions and byproduct forma-

Paul B. Weisz and John F. Marshall, Science 06:24, 1979.

Figure I-D-2.– Alternative Schemes for Acid Hydrolysis of Cellulosic Biomass for Ethanol Production



SOURCE: Massachusetts Institute of Technology.

tion. Furthermore, if acids, alkali, or organic chemicals are used, they must be recycled to minimize cost or disposed of in order to prevent pollution.

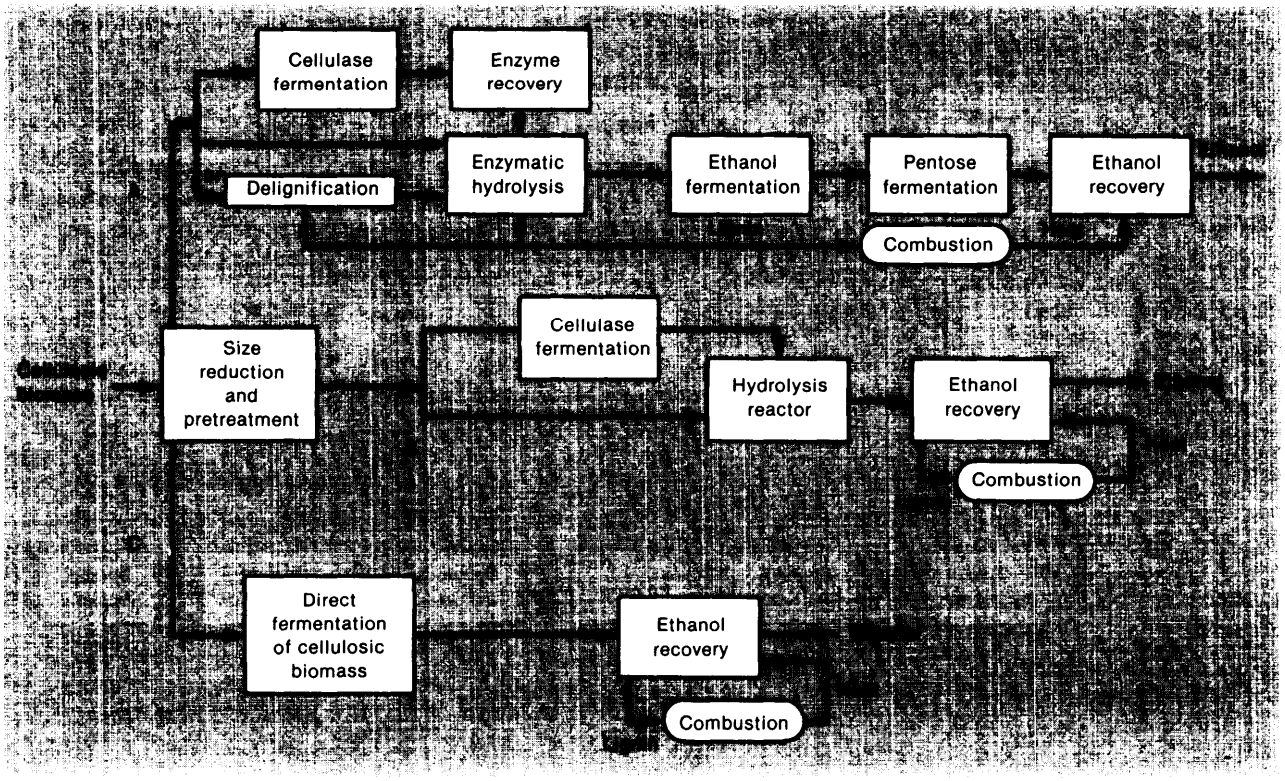
In starch processing, prior to ethanol fermentation, mechanical grinding, steam, and enzymes are employed. The energy requirements are small and contribute relatively little to the final ethanol cost. The objective in the development of cellulose-based processes should be to minimize both energy and chemical requirements. The development and scale-up of effective pretreatment technology are under active investigation and require continued financial support to better develop several alternative routes. The most promising routes are: steam treatment, solvent delignification, dilute acid, cellulose dissolution, and direct fermentation.

Several different acid hydrolysis schemes have been proposed. However, most appear as in flow scheme A or B in figure I-D-2. Dilute acid is used to hydrolyze the hemicellulose to pentose sugars primarily and then stronger acid at higher temperatures is used to cause cellulose hydrolysis (scheme A). A major problem with this approach is the irreversible loss of sugars to undesirable side-product formation. After separation of residual solids (mostly lignin), which can be burned to provide energy for distillation, the sugar solution is fermented by yeast to ethanol. The pentose sugars also can be fermented, but by organisms other than the ethanol producing yeast, to other chemicals, some of which could be used as fuels (e.g., ethanol, acetic acid, acetone, butanol, 2,3-butanediol, etc.).

An alternative (scheme B, figure I-D-2) to the above is to use a solvent, after pentose sugar removal, to dissolve the cellulose, allowing its separation from lignin. This cellulose solution is easily and efficiently hydrolyzed to sugars. The advantage of this approach over the direct acid hydrolysis is that the yield of sugar is much higher. In the harsh acid hydrolysis, considerable sugar is destroyed. However, the major disadvantage of both these schemes is that they require recycling or disposal of acids and solvents. A second problem is that almost nothing is known about how to scale-up some of the newly de-

'*Proceedings of 3rd Annual Biomass Energy System Conference*, National Technical Information Set-vice, SERI/TP-33-285,1979.

Figure I-D-3.—Alternative Schemes for Enzymatic Hydrolysis of Cellulosic Biomass for Ethanol Production



SOURCE: Massachusetts Institute of Technology.

Table I- D-1 .-Alternative Pretreatment Methods for Lignocellulose Materials

Chemical methods	Physical methods
Sodium hydroxide (alkali)	Steam
Ammonia	Grinding and milling
Chemical pulping	Irradiation
Ammonium bisulfite	Freezing
Sulfite	
Sodium chlorite	
Organic solvents	
Acids	

SOURCE: Office of Technology Assessment.

veloped technology, such as that developed by groups working at Purdue University, New York University, and Dartmouth College. There are several engineering problems involving both heat and mass transfer and acid/solvent recycle that need to be evaluated at larger scale. At least some of this work will be done at the process development unit now being built at the Georgia Institute of Technology. The most promising directions that need development are:

- the scale-up of high rates and high yield laboratory hydrolysis systems, and
- the development of methods for acid and chemical recycle schemes.

There are three types of approaches that have been employed for enzymatic hydrolysis of cellulosic biomass. These are summarized in figure I-D-3. They all involve some initial size reduction to increase the surface area available for enzymatic attack. In schemes A and B, the incoming cellulosic biomass is split into two streams; one is used to grow organisms that produce cellulolytic enzymes called cellulases, and the other is used to produce sugar.

In scheme A, the cellulases are recovered and then added to a separate enzyme hydrolysis reaction. They hydrolyze both the cellulose and hemicellulose, and the resulting sugar solution is then passed to an ethanol fermentation stage where hexoses are converted by a yeast fermentation to ethanol. Utilization of the pentose requires a separate fermentation. Re-

sidual lignin, which is removed before (by solvents extraction) or after hydrolysis, is used to provide energy for ethanol recovery, Extensive work on this approach has been done at the University of California, Berkeley, and the U.S. Army Natick Laboratories.

In scheme B, the cellulase is not recovered but rather, the whole fermentation broth from cellulase production is added to the cellulosic biomass along with ethanol-producing yeast. The result is a simultaneous cellulose hydrolysis (saccharification) and fermentation. (In the production of ethanol from starch, the starch hydrolyzing enzymes are added at the same time as the yeast for simultaneous saccharification and fermentation.) This technology has been demonstrated by the Gulf Oil Co. After fermentation, the ethanol is recovered and the residual lignin can again be used for energy for distillation. The problem of unused pentose sugar still remains and will require a separate fermentation step.

A third alternative (scheme C, figure I-D-3) shows a simpler approach, namely a direct fermentation on cellulose. This approach has been developed at the Massachusetts Institute of Technology. It utilizes bacteria that will produce cellulase to hydrolyze the cellulose and hemicellulose and ferment both the hexose and pentose sugars to ethanol in a single-stage reactor. The advantage of this approach is a minimal requirement for pretreatment, a combined enzyme production, cellulose hydrolysis and ethanol fermentation, and simultaneous conversion of both pentose and hexose sugars to ethanol. This concept is new and work still needs to be done to increase the ethanol concentration, minimize side product formation, and increase the rate of ethanol production. Again, residual lignin will be used to provide the energy for ethanol distillation.

FERMENTATION OF ETHANOL

An examination of the economics for ethanol production shows that the dominant cost is the process raw material. As seen in table 1-D-2 the feedstock represents 60 to 70 percent of the manufacturing cost. Thus, it is clear that any improvement in substrate utilization efficiency is of substantial benefit. The theoretical yields of ethanol from glucose, sucrose, and starch or cellulose are 0.51, 0.54 and 0.57 gram (g) ethanol/g material, respectively; the differences result from the addition of a molecule of water on hydrolysis. There are several approaches to improve the yield above the typical value of 90 to 95 percent currentl y achieved. These are:

- increase the ratio of ethanol produced per unit weight of cells, e.g., through cell recycle, vacuum fermentation, immobilized cells, or improvement in specific productivity (g ethanol/g cell hr), by increasing the content and/or activity of those enzymes in the pathway to ethanol;
- increase the utilization of other materials in the substrate, e.g., the use of oligosaccharides, especially branched, in starch, and the use of contaminating sugars such as galactose or mannose for hemicellulose; and
- develop a route for the utilization of pentose sugars, especially xylose, present in hemicellulose.

Table I-D-2.—A Comparison of the Distribution of Manufacturing Costs for Several Ethanol Production Processes

Substrate	Molasses	Corn	Grain Sorghum
Cost component (%)			
Capital	9	12	10
Operating	20	26	30
Feedstock	71	62	60
Total	100	100	100
Cost on energy basis ($/MMBtu)	12.5	14.9	12.7
Cost/gal ethanol ($/gal)	1.05	1.25	1.07
Capital investment ($/annual gal)	1.02	1.05	1.75

SOURCE: "Comparative Economic Assessment of Ethanol From Biomass," Mitre Corp., report HCP/ET-2854).

The potential effect of oligosaccharides or contaminating sugar utilization is relatively small, since they represent typically 1 to 3 percent of the total sugar content. However, if cellulosic biomass containing 15 to 25 percent hemicellulose is used, then the impact of pentose conversion to ethanol is great.

Cellulosic biomass is made up primarily of cellulose, hemicellulose (mostly xylan) and lignin. Other components such as protein, ash, fats, etc., typically comprise about 10 percent. The composition of biomass can be expressed in terms of the following equation:

$$\frac{F_{,}. + \cdot \; \cdot + \cdot \; \cdot}{- \cdot \; \cdot} = 10 \qquad [1]$$

where F_C, F_H, , F and F_A are the weight fractions of cellulose, hemicellulose, lignin, and ash, respectively. Assuming that the ash is 10 percent (F_A = 0.1) and that F_C and F_H are the only fermentable components in the biomass, then:

$$F_C = F_H = 0.9 - F_L \qquad (2)$$

The maximum amount of ethanol from one unit of biomass S (Y_E is;

$$Y_{E/C} = Y_{E/H} \cdot = Y_E \qquad (3)$$

Where ; Y_1 and l Y_E are the yield of ethanol for cellulose and hemicellulose, respectively. Equation 2 can be rearranged to relate the fractions of cellulose:

$$F_c = 0.9 - F_L - F_H \quad (4)$$

Substituting this into equation 3 gives:

$$Y_{E/B} + Y_{E/c}(0.9 - F_L - F_H) + Y_{E/H}F_H \quad (5)$$

From equation 5, the effect can be calculated of hemicellulose content and conversion yield on the overall conversion of biomass to ethanol. Assuming a lignin content of 15 percent ($F_L = 0.15$) and using $Y_{E/c} = 0.57$ g/g the following equation is obtained:

$$Y_{E/B} = 0.43 + F_H(Y_{E/H} - 0.57) \quad (6)$$

The theoretical yield value on hemicellulose, $Y_{E/H}$, is not well-defined because so little is known about the biochemistry of anaerobic pentose metabolism. If one mole of ethanol is produced per mole of xylose, the yield is 0.3 g ethanol/g xylose. It two moles of ethanol could be obained, $Y_{E/H}$ would be 0.61; however, neither the mechanism nor the thermodynamics of the conversion is sufficiently well-defined to allow one to expect this value. The maximum observed values are about 0.41 g ethanol/g xylose.[3] The sensitivity of the overall yield to this value is shown in figure I-D-4. The impact of pentose utilization depends on the amount of hemicellulose present. From the value in figure I-D-4 and the observation that 70 percent of the manufacturing cost is the raw material cost, it is possible to estimate the economic benefit of pentose utilization. Equation 7 relates the overall ethanol yield to the manufacturing cost:

$$C_E = \frac{C_B \times 6.6}{Y_{E/B} \quad 0.7} \quad (7)$$

where C_E is the manufacturing cost per gallon of ethanol, C_B is biomass cost (cents/lb), 6.6 is the conversion from pound to gallon of ethanol, and 0.7 is the 70-percent factor for relative biomass cost to ethanol cost. For a biomass costing 2 cents/lb and containing 20 percent hemicellulose, the manufacturing cost is reduced from 59 to 43 cents/gal, when the yield on pentose goes from zero to 0.6.

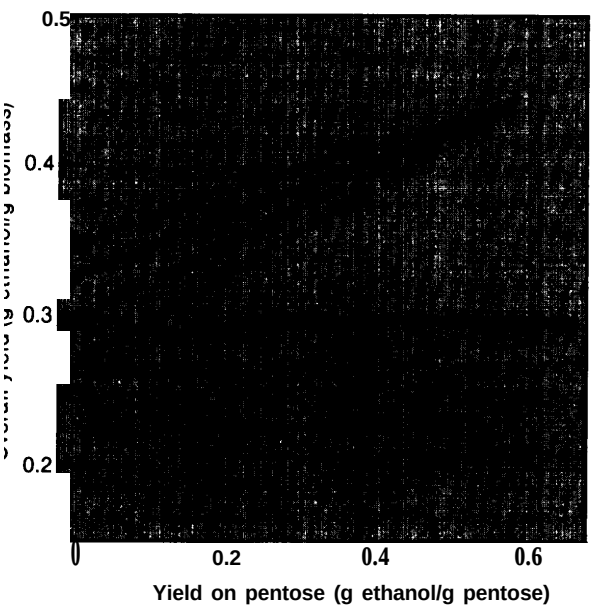
At the present time, there are few organisms that produce more than one mole of ethanol per mole of pentose and none of **the usual alcohol producing yeasts will ferment pentoses to ethanol. Addition or improvement of the ability to use pentose will have a major impact on the economics of ethanol production.**

The second major cost in ethanol production relates to the cost of operation. Typically, 20 to 30 percent of the final manufacturing cost is accounted for by the sum of labor, plant overhead, administration, chemical supplies, and fuel costs. The chemical supplies represent less than 1 cent/gal ethanol and may be neglected. The labor, overhead, and marketing costs vary with plant size, but represent 11 to 7 cents/gal for a 20 to 100 million gal/yr plant, respectively. Any improvement in the reduction of plant size or complexity will reduce this cost; however, the economic impact is small. The major component of the operating cost is the fuel charge for plant operation and for distillation. Plant operations, e.g., mixing, pumping, sterilization, starch gelatinization, biomass grinding, etc., represent about 20 to 30 percent of the energy cost. The remainder is for ethanol distillation and residual solids drying. Considerable effort has been focused on methods to improve the energy efficiency, of distillation to reduce it from the 160)000 Btu/gal required for beverage alcohol. While considerable differences in opinion exist as to the minimum, a reasonable expectation is about 40,000 Btu/gal although current technology requires 69,000 Btu/gal.[4] Forty thousand Btu is about half of the energy content of ethanol per gallon.

A discussion of process improvements relating to ethanol recovery has two components. The first is

[3] D. Wang and Cooney, Massachusetts Institute Technology, unpublished results.

Figure I= D-4.- Effect of Pentose Yieid ($Y_{E/H}$) on Overall Yield of Ethanol from Cellulosic Biomass ($Y_{E/B}$) with Varying Fractions of Hemicellulose (F_H).



SOURCE: Massachusetts Institute of Technology.

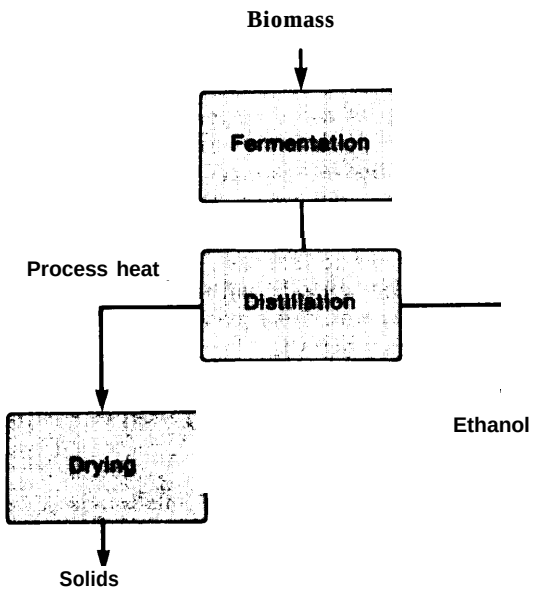
[4] Report of (he Gasohol ady Goup o of the Energy Research Advisory ard D Department of ergy, W Washington, D. C., go Gibbs, and D. DeMoss, "Ethanol Formation in *Pseudomonas /indneri,* " *Arc/l.* phys. 3 478-479 1951.

related to operating costs and the second is related to energy efficiency. If coal is used to provide energy for distillation, and it is valued at $30/ton, with 10,500 Btu/lb or $1.50/million Btu, then the energy cost for distillation (optimistically assuming 40,000 Btu/gal) is $6/gal. If lignin from cellulosic biomass is used as a fuel, the cost is reduced further. On the other hand, if oil at $40/bbl (130,000 Btu/gal and 42 gal/bbl) or $7/million Btu is used, then the energy cost is 28 cents/gal of ethanol.

From a common sense, economic, and political point of view, it does not seem reasonable to utilize liquid fuel to produce liquid fuel from biomass. Therefore, it will be assumed that petroleum will not be used for distillation and that either coal or biomass will be employed.

In order to assess the impact of process improvements on the energy demand, it is necessary to look at an overall material balance. This is summarized in figure 1-D-5. Only a portion of the entering biomass feedstock is fermented to ethanol and there are two product streams, one containing ethanol and the other solids, both must be separated from water. It is important to note that as the ethanol concentration is increased, the energy requirement for both ethanol recovery from the water and for drying will decrease. Therefore, the impact of developing ethanol tolerant micro-organisms is seen as a reduction in energy cost.

Figure I-D-5. - Process Schematic for Material and Energy Balance



SOURCE: Massachusetts Institute of Technology.

The third major cost for ethanol manufacturing is the capital investment, which represents about 4 to 12 percent of the manufacturing cost. The capital investment is determined by the complexity of the processes and the volumetric productivity of ethanol production. Thus, the development of a micro-organism that will require a minimum amount of feedstock pretreatment and will produce ethanol at a higher rate will reduce the net capital investment.

The volumetric productivity (Q_E) for ethanol production is given by:

$$Q_E = q_P X$$

where q_P is the specific productivity expressed in g ethanol per g cell hr, and X is the culture density. Therefore, there are two approaches to obtain high productivity: first, to choose or create an organism with a high specific rate of ethanol production and second, to design a process with high cell density.

The application of genetics can be used to enhance the intracellular enzyme activity of the enzymes used for ethanol production. The resulting increase in q_p, will result in reduced capital investment requirements.

There are four types of ethanol processes based on different organisms; they are:

1. Saccharomyces cerevisiae and related yeast,
2. *Saccharomyces cerevisiae/Trichoderma reesei,*
3. *Zymomonas mobilis,* and
4. *Clostridium thermocellum/thermosaccharolyticum,* or *thermohydrosulfuricum.*

The first is the traditional yeast based process using S. *cerevisiae* to ferment soluble hexose sugar to ethanol. In the second, the substrate range is extended to cellulose by the use of cellulase produced by T. *reesei.* The third approach utilizes Z. *mobilis;* this organism is a particularly fast and high ethanol yielding one. Its range of fermentable substrates, however, is limited to soluble hexose sugars.

In many tropical areas of the Americas, Africa, and Asia, alcoholic beverages prepared from a mixed fermentation of plant steeps are popular. Bacteria from the genus *Zymomonas* are commonly employed. In the early 1950's, the genus *Zymononas* acquired a certain fame among biochemists by the discovery that the anaerobic catabolism of glucose follows the Enter-Doudoroff mechanism.' This was very surprising, since *Zymomonas* was the first example of an anaerobic organism using a pathway mainly in strictly aerobic bateria.[6]

In spite of its extensive use in many parts of the world, its great social implications as an ethanol pro-

[5] M. Gibbs and R. I de Moss, "Ethanol Formation, in I *Psuedomona lindneri Arch.*) *Biochem. Biophys.*, 34:478-47 *1951.*

[6] Swings and J. . DeLey. "The Biolo of f *Zymomona*" Bacteriological Reviews 41: 1-46, 1977.

ducer, and its unique biochemical position, Zymomonas has not been studied extensively. T

The organism most often studied is *Zymomonas mobilis*, which can produce up to 1.9 moles of ethanol per mole of glucose. Recent studies reported from Australia, have established the *Z. mobilis* can ferment high concentrations of glucose rapidly to ethanol in both batch and continuous culture with higher specific glucose uptakes rates for glucose and ethanol production rates than for yeasts currently used in alcohol fermentations in Australian.[89]

For example, several kinetic parameters for a *Z. mobilis* fermentation were compared with *Saccharomyces carlsbergensis*[10] specially selected for its sugar and alcohol tolerance.[11] Both specific ethanol productivity and specific glucose uptake rate are several times greater for *Z. mobilis*. This result is mainly due to lower levels of biomass formation and glucose consumption. The lower biomass produced would seem to be a consequence of the lower energy available for growth with *Zymomonas* than with yeasts—the Enter-Doudoroff pathway producing only 1 mole of adenosine triphosphate (ATP) per mole of glucose, compared to glycolysis with 2 moles ATP per mole glucose. In none of the first three examples can ethanol be produced from pentose sugar.

The fourth approach utilizes a mixed culture of *Clostridia*, which will utilize cellulose and hemicellulose, hexoses, and pentoses for ethanol production.

The application of genetics for improving microbial strains

In the previous sections, the process steps have been identified that are particularly sensitive to the quality of the microbial strains. The following are improvements of microbial characteristics that are either now possible or might be so in the future and that will have an impact on the overall economics of the process. The effect of new genetic techniques requiring future research is similar for all micro-organisms in two ways.

1. Manipulations could be attempted today with less effort and greater chance of success if tools like cell fusion and recombinant DNA (rDNA) techniques were available for all of the microbes of interest.
2. Manipulations require further knowledge in a specific area or the development of an entirely new genetic system in ethanol producing microbes—e.g., there is no genetic system for the thermophilic anaerobic bacteria. Knowledge on how to genetically alter ethanol tolerance of both bacteria and yeast is lacking.

The economics of the fermentation of a substrate into alcohol is primarily controlled by three factors:

1. *Ethanol* yield.—The amount of product produced per unit of substrate determines the major raw materials cost of the fermentation.
2. *Final ethanol concentration.*—The cost of separating the ethanol from the fermentation broth is a function of the ethanol concentration in that broth.
3. *Productivity.*—The amount of ethanol produced per liter of fermenter volume per hour determines the capital cost of the fermentation step, once the type of fermenter and the annual output have been chosen. Productivity is not independent of the final ethanol concentration, and so an optimum compromise between these variables must be chosen.

The impact of genetics on ethanol yield

Most microbes that are chosen for making ethanol already produce nearly the theoretical maximum yield. In these cases little improvement can be made.

The yield may be lower when the microbe has been chosen for its other technical advantages such as ability to degrade cellulose. Lower yield of a microbial end product, like ethanol, can result from the diversion of substrate to cell mass or to an alternative product. Both of these faults can be readily attacked. A number of cell changes (e.g., leaky membranes) can cause the microbe to waste energy, requiring it to metabolize more substrate into alcohol to make the same cell mass. Where the thermodynamics and redox balance of the fermentation allow, unwanted waste products can be eliminated by mutation of the relevant pathways. Only limited work has been done on this type of research with industrially significant bacteria.

The impact of genetics on final ethanol concentration

This is amenable to genetic manipulation, both empirical and planned. An improvement in ethanol tolerance decreased both separation costs and fermenter capital cost (through increased productivity).

When traditional distillation is used, the effect on

Gibbs et al., op. cit.

J. J. Lee, D. J. Tribe, and J. L. Rogers, "Ethanol Production by *Zymomonas mobilis* in Continuous Culture at High Glucose Concentrations," in *Biotechnology Letters* 1:421-426, 1979.

J. L. Rogers, K. J. Wood, and D. J. Tribe, *Biotechnol Lett* 1:165-170, 1979.

Ibid.

Adv. Biochem. Eng. 19 (2), 1976, pp. 1-12

the separation cost of increased ethanol tolerance is smaller once ethanol concentrations have reached approximately 6 percent. However, the importance of increased ethanol concentration to fermenter productivity remains.

It is likely that the most important inhibitory action of ethanol takes place at the cell membrane. Strategies for manipulating the cell membrane composition and properties, and understanding in this area, are increasing rapidly.

Genetics and ethanol tolerance

The study of ethanol tolerance by micro-organisms has been approached using strains with altered genetic makeup. Several kinds of Escherichia *coli* mutants have been isolated having different tolerances to ethyl alcohol.[12] Solvent resistant strains either had larger amounts of total phospholipid (type III) or had an altered phospholipid and membrane-bound protein composition (type 11). On the other hand, mutants with a lesion mapping close to *pss* gene (which codes for phosphotidylserine synthetase) were either solvent sensitive or resistant .13

The physiology of an *E. coli* ethanol resistant mutant has been characterized similarly .14 This strain had pleiotropic growth defects including abnormal cell division and morphology. It also had an altered lac permease that was not due to a mutation in the Y gene. It was concluded that altered membrane composition was responsible for this abnormal behavior.

More recently, ethanol tolerant mutants have been isolated from C. *thermocellum.*[15] Indirect evidence lead to the conclusion that strain S-4 was defective in hydrogenate, since this strain produced lower amounts of acetic acid.[16] A different ethanol resistant isolate of the same bacterium, strain C9, proved to have a lower activation energy for growth than the wild type, a property that has been related to membrane composition.

There are three categories of changes that could influence the fermentation process:

1. Manipulate the existing controls on metabolism. Consider an example. In many organisms the energy level of the cell, expressed through adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) levels, partially controls the rate of glycolysis. A defective cell membrane would provide an energy sink, to keep glycolysis at its maximum rate. Strategies such as this could be attempted now.
2. Increase the amount of each transport and catabolic enzyme in the fermentation pathway. This requires the ability to isolate the genes of interest and to amplify them with in vivo or in vitro recombinant techniques in the microbe of interest. This is not an immediate prospect.
3. Accomplish complete deregulation of the fermentation pathway in the microbe of interest. Essential catabolic enzymes are difficult to manipulate, and this is also not an immediate prospect.

Genetic manipulation of the microbe can influence fermentation processes in other ways as well. These are less important than improvements in yield, final ethanol concentration, and productivity, but they also affect the cost. Examples are:

- type of fermenter used;
- nonsubstrate nutrients;
- strain stability;
- cell separations for byproducts, recycle, or ethanol recovery (i.e., increased size for recovery);
- operating conditions, i.e., higher growth temperatures for yeast and mesophilic bacteria; and
- range and efficiency of substrate utilization (i.e., complete utilization of all sugars).

More detailed examples are:

• ***Type of fermenter.*** —If the organism, whether it be a yeast or a bacterium, can be made to grow under conditions of pH, ethanol concentration, temperature, etc., that preclude contamination, inexpensive lined basins can be used instead of tanks, since steam sterilization of the fermenter is not required. In this case, some operating and capital costs associated with sterilization are avoided as well.

A type of continuous beer fermenter requires growth in the form of fast-settling pellets. In other fermenters, fast-settling particles (such as mycelia) present problems that are best avoided by agglomeration of the cell mass. This type of control over the growth form of micro-organisms is amenable to genetic manipulations.

• ***Nonsubstrate medium costs.*** —In addition to the carbon-energy substrate and water, growing cells must be supplied with other nutrients. Some organisms can make all of their biochemical from quite simple sources of nitrogen, phosphorus, sulfur, magnesium and trace metals. Others require more

12 P. J. Clark and J. P. J. Beard, "Altered Phospholip Composition in Mutants of' *Escherich Coli* i Sensitiv or Resistant to organic Solvents," *Gen. Microbio* 1 13:267-274, *1979.*

13 Ohki and Shibuy, "Membrane g Phospholip Synthesis and t Phon tVD (correlation of *Ecoli* Mutant," *J Bacteril* 132:434-44 1977.

14 Friedland Novick, "Organic Solvon as Probes s fo the o Structu and Functio the Bacterial Membrane: Effects of Ethanol on the Wild Tyl and as Ethan Resistant Muta of' *Escherich Coli,*" *Bacteri* 114:239-24 195

15 D. Wang, 'production of Ethan From Cellulose by *Clostridium Thermocellu*," M Thesis, Department of Nutrition and Food Science, Mass chusot Institute of Technology, 1982

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complex molecules, ready-made, such as amino acids and vitamins.

The more cheaply these nutrient needs can be provided, the better. Whenever an organism can be given genes from another source by applied biotechnology techniques, there is a possibility that complex nutrient requirements can be obviated. However, this requires that all the genes in a given pathway be located in the source and be made to function in the new microbes. The feasibility of this is uncertain, but solutions would decrease the cost of producing ethanol with yeast as well as *clostridia.*

- ***Stain stability.*—Many of the suggested ethanol processes propose to employ continuous culture. Although this offers several advantages over batch culture, it is somewhat vulnerable to deleterious mutations of the microbe used, particularly if the microbe has been extensively altered in ways that make it less competitive.**

These deleterious genetic changes are almost entirely catalysed by biological systems in the microbe. Alteration of these systems, so that the frequency of unwanted genetic changes is decreased, could greatly extend the period of operation that is possible before having to shut down and restart the fermentation. So far, this is a possibility only in microbes that have a highly developed genetics. It may be that strain stabilization of this sort would not be possible in other microbes until their genetics are highly developed.

It is also possible to design strategies using current strain development techniques that might lead to genetically stable strains, but these are unproven.

- **Cell *separations.*** —Many fermentation schemes incorporate cell recycle to boost productivity. This requires that cells be separated from effluent broth. Others need to separate cells from other residue as a byproduct. In addition, some of the low-energy alternatives to distillation, such as adsorption, could require separation of the cells from the broth prior to ethanol recovery.

In these cases, microbes that can be made to flocculate and redisperse, or that can be made to reversibly change their morphology would allow cheap gravity separations (settling or flotation).

- ***Operating conditions.—An* increase in the temperature an organism will tolerate is advantageous for heat removal and in situ ethanol removal schemes. The feasibility of accomplishing this is uncertain.**

The extreme of productivity improvement via cell recycle is an immobilized cell reactor, It is conceivable that cells could be made less prone to degradation under the conditions of immobilization, by modifying sensitive components and degradation systems, and by adding protective systems. This is not at all a near-term possibility.

- ***Range and efficiency* of *substrate utilization.-A*** single-step conversion of a substrate to ethanol is highly desirable. This often requires that the ethanol fermenting organism possess a degradation capability it does not have.

As an example, consider ligno-cellulose. It consists of hexosans, pentosans, and lignin, All of these components should be used. Assume that one cellulase-producing candidate does not use pentoses, while a related noncellulase producing organism does, this is exactly the situation with clostridia. If the second organism can be given the cellulase genes of the first, a microbe better-suited to direct conversion could be created. The pace at which such a manipulation could be developed cannot be predicted with confidence, although this is not necessarily a long-term prospect.

Another obvious area that merits attention is the enhancement of cellulase activity. Classical genetic manipulations, employing mutation and selection or screening, should result in micro-organisms better equipped to degrade cellulose. E.g, it should be possible to isolate strains that are deregulated in cellulase production (hyperproducers) as well as those in which the cellulase is not subject to product inhibition. In addition, it is tempting to think about the possibilities of amplifying cellulase genes by means of DNA technology and cloning. However, this latter approach must await further understanding of the biochemistry and genetics of the cellulase system as well as the development of the appropriate genetic systems in cellulolytic micro-organisms,

Utilization offermentation byproducts

Presently for each gallon of ethanol produced, approximately 14 liters of stillage is formed. [17] If ethanol is mixed with gasoline to make gasohol (10 percent ethanol), the total stillage produced annually in the United States would be in the billions of liters. Surely a problem of this magnitude deserves serious attention. The utilization of stillage or fermentation byproducts could be greatly improved by genetic means in several ways, In actuality, only a rational long-range genetic approach can increase the value of such a fermentation byproduct. Value can be increased in two main ways. The first is to increase the nutritive value of the fermentation byproduct followed by developing economical processing technol-

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W. L. Tyner, "The Potential of Obtaining Energy From Agriculture, Symposium on *Biotechnology The Energy Production and Conservation*, Gaithersberg, Term., 1979.

ogies that stabilize and preserve nutritive value. The second approach is to increase the functionality of the byproducts so that more useful products can be developed.

For this one can envisage clever and novel ways to utilize mutants to increase the value in a manner similar to those described. [18 19 20] Ethanol production is not compatible with producing a valuable byproduct. E.g., a filamentous yeast may be useful for direct texturization or fortification of an animal food but production of ethanol may not be suitable with such an organism. A possible solution to this type of conflict involves the development and engineering of two-stage fermentation processes. In the first stage, ethanol producing organisms are propagated under optimal economic conditions for ethanol production. After the production phase is over, the organisms are then transferred to a second-stage reactor, where desirable phenotypic properties are then expressed. Signals for expression of phenotypic properties can be extrinsic environmental parameters, such as temperature, or levels of oxygen or carbon dioxide, or intrinsic parameters, such as specific nutrient requirements.

Thus the large-scale utilization of fermentation byproducts as feed or other materials will then become more valuable when genetic engineering can decrease processing costs and increase product quality. Most of these types of studies remain to be done. However, the potential for innovative applications is great, but such applications may not result because of the current lack of any Government agency that has a sound program for funding biotechnology research.

Recommendations and areas in which applied genetics should have an impact

There has been little published research done in the United States on the genetic improvement of ethanol production processes with bacteria such as Zymomonas and clostridia, and only limited studies with yeast. In light of previous discussion, the following points have been identified as being the most important and relevant in the application of genetics for improving ethanol-producing processes:

- improvements on ethanol yield;
- increased ethanol tolerance to achieve higher final ethanol concentrations in the fermentation broth;
- increased rates of ethanol production;
- elimination of unwanted products of anaerobic catabolism, that is, direction of catabolism towards ethanol;
- enhanced cellulolytic and/or saccharolytic capabilities to improve rates of conversion of cellulose and/or starch to fermentable sugars;
- incorporation of pentose catabolic capabilities into ethanol producers;
- development of strains capable of hydrolyzing cellulose and starch as well as of producing ethanol from pentoses and hexoses;
- improved temperature stability of micro-organisms and/or their enzymes; and
- improved harvesting properties of cellular biomass produced during fermentation.

J. [illegible] J. Bouchard, Lee, J. DeAngeli, Y. Miyasaki, [illegible] and R. V. Tamminbaum, "Applications of Temperature-Sensitive Mutants for Single-Cell Protein Production," in *Proceedings of U.S./U.S.S.R. Conference on Mechanisms and Kinetics of Uptake and Utilization of substrates in Processes for the Production of Substances by Microbiological Means*, p. MOSCOW-PUSHCHINO p. 362, June 4-11, 1977. [illegible] 283-330-T.

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