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CONVERSION OF BAGASSE CELLULOSE INTO ETHANOL

**PREPARED FOR THE UNITED STATES
DEPARTMENT OF ENERGY
Under Cooperative Agreement
No. DE-FC36-96G010164.A000**

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Abstract

The study conducted by Arkenol for the Department of Energy (DOE), entitled "Conversion of Bagasse Cellulose into Ethanol" was performed under contract number DE-FC36-96G010164.A000. It was designed to test the conversion of feedstocks such as sugar cane bagasse, sorghum, napier grass and rice straw into fermentable sugars, and then ferment these sugars using natural yeasts and genetically engineered *Zymomonis mobilis* bacteria (ZM). The study did convert various cellulosic feedstocks into fermentable sugars utilizing the patented Arkenol "*Concentrated Acid Hydrolysis Process*" and equipment at the Arkenol Technology Center in Orange, California. The sugars produced using this process were in the concentration range of 12-15%, much higher than the sugar concentrations the genetically engineered ZM bacteria had been developed for. As a result, while the ZM bacteria fermented the produced sugars without initial inhibition, the completion of high sugar concentration fermentations was slower and at lower yield than predicted by the National Renewable Energy Laboratory (NREL). Natural yeasts performed as expected by Arkenol, similar to the results obtained over the last four years of testing. Overall, at sugar concentrations in the 10-13% range, yeast produced 85-90% theoretical ethanol yields and ZM bacteria produced 82-87% theoretical yields in 96 hour fermentations. Additional commercialization work revealed the ability to centrifugally separate and recycle the ZM bacteria after fermentation, slight additional benefits from mixed culture ZM bacteria fermentations, and successful utilization of defined media for ZM bacteria fermentation nutrients in lieu of natural media.

Acknowledgement

Arkenol gratefully acknowledges the matching monetary contribution from the Department of Energy which made this study possible. We also wish to thank the engineers and scientists at the National Renewable Energy Laboratory for their assistance in providing and advising us on the care, maintenance and use of their genetically engineered *Zymomonis mobilis* bacteria strains ZM1 and ZM2 described in this report.

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Section I

D.O.E Program Objectives

A. Background

An unsolicited proposal entitled "*Conversion of Bagasse Cellulose into Ethanol*" was submitted by Arkenol, Inc. to the Department of Energy (D.O.E.) on July 1, 1996. The proposal consisted of three phases aimed towards proving process commerciality, successful financing and construction of a project in the State of Hawaii.

The objective of Phase One, the only phase for which shared funding was requested, was to convert a variety of existing and potential Hawaiian biomass feedstocks into fermentable sugars using the patented *Arkenol Concentrated Acid Hydrolysis Technology*¹ process and then test fermentation methods of converting those sugars into ethanol.

Phase One was broken into three separate programs for execution and projected to be eight months in duration. The programs were identified as :

1. The Feedstock Test Program encompasses the provision of feedstocks, the decrystallization and hydrolysis of cellulosic components and the testing of the patented National Renewable Energy Laboratory (NREL) *Zymomonis mobilis* bacteria on the resulting fermentable sugars.
2. The Experimental Growing Program was proposed to identify and test the commercial potential of growing selected biomass materials in Hawaii to feed potential future biomass conversion to ethanol plants.
3. The Liaison Program with the State of Hawaii Department of Business Economic Development and Tourism (DBEDT) was proposed to identify, draft and support public questions concerning potential regulations supporting a new ethanol industry.

D.O.E. elected to cofund Phase One of the proposal effective August 1, 1996 and identify it #DE-FC36-96G010164.A000.

¹*Method of Producing Sugars using Strong Acid Hydrolysis of Cellulosic and Hemicellulosic Materials*, U.S. Patent#'s 5,562,777, 5,580,389, 5,597,714, 5,620,877, and others pending.

Section II

The Feedstock Evaluation Program

A. Program Goals

The most significant allotment of time and resources was dedicated to the Feedstock Evaluation Program. The Feedstock Evaluation Program itself was broken into Four main areas:

- 1) Feedstocks, preferably from the Hawaiian Islands already, would be sourced, prepared for shipment, received at the Arkenol Technology Center in the City of Orange, California and tested for composition.
- 2) Biomass samples would be converted to fermentable sugars using Pilot Plant equipment and the *Arkenol concentrated acid hydrolysis technology* (the "Technology"). The results would be used to verify the performance of these samples against the Arkenol Kinetic Model.
- 3) Fermentable sugars from the chemically sterilized hydrolysate would be used to test fermentation performance of NREL's genetically engineered *Zymomonis mobilis* (ZM1) bacteria (CP4/ZB5) in a factorial design experiment varying temperature, culture density and total mixed sugar concentration.
- 4) Fermentation Commercial Optimization was added to the test program in June 1997 to test recycling capabilities of the bacteria in the Arkenol process, performance of ZM1 on a defined rather than natural nutrient media, and potential for improved performance using mixed cultures of ZM1 and ZM2 in series or parallel fermentations.

B. Feedstocks

The proposed feedstocks for consideration included sugar cane bagasse, Hawaiian Napier grass, sweet sorghum and California rice straw as a control. DOE later requested that we examine spruce wood chips since it was relevant to another program they were investigating at the time.

Arkenol had rice straw on site when the study started so the testing was started with this material so that hydrolysate could be developed to start the fermentation testing. California rice straw is typically dried standing in the fields and then harvested in bales. Rice straw received and stored at the Arkenol Technology Center has seldom exceeded 12% moisture and degrades very slowly (2-3% per year C+H loss) when stored indoors.

The program was started in August 1996 and was too late to consider planting sorghum, even in Hawaii. Therefore, another source of sorghum was identified. A crop from the summer had been left standing and had not been harvested. The Texas A&M University, Agricultural Research and Extension Center in Weslaco, Texas was able to help us out by cutting and drying the sorghum in their greenhouse.

The bulk of the testing was destined to be performed on sugar cane bagasse. While the harvest

season for the sugar cane was also ending, one grower on the island of Maui still had fresh bagasse. The problem however was twofold, first it was reported that the bagasse we were getting was from one of only two mills which were even planning on reopening in Hawaii, second, the process of air drying was going to be complicated by a tropical storm hovering in the islands. Arkenol determined that the only way to recover this feedstock was to air freight the moist bagasse to Los Angeles, immediately pick it up and transport it to the Arkenol Technology Center, and finally air dry the material in Orange County, California. The received bagasse had a moisture content of 47.5% wt., down from approximately 60% mill discharge and still had most of the 1.6% residual sugar present. Normally bagasse will contain as much as 3% residual sugar if not processed for optimal extraction, but leave the mill at <50% moisture.

Hawaiian Napier grass, used as a cattle feed, was the last feedstock sourced. The material we requested was from a field after grazing, since we felt this would be closer to the actual feedstock available at the lowest cost. This material suffered the tropical storm drying problems similar to the bagasse. However, a longer drying period, fresh chipping and more efficient turning regime produced a 13.8% wt. moisture shipped product.

C. Materials and Methods - Feedstock Characterization

Arkenol tests feedstocks both for the initial sugar conversion potential in calculating yields and for setting the critical variables in the Arkenol concentrated acid hydrolysis process.

Some testing reported for biomass samples determines the amount of cellulose and hemicellulose by decrystallizing and hydrolyzing the sample using their particular process technique. These methods fail to recognize a percentage of cellulose and hemicellulose unconverted or unrecoverable in any of the various processes available today. The tests identified below, we believe, more accurately reflect the true potential composition of the biomass sample in terms of the definition of cellulose and hemicellulose rather than terms related to a specific hydrolysis process.

This program required testing for the following composition constituents:

Cellulose plus Hemicellulose	Determined by a modified USP (United States Pharmacopoeia) method of dichromate oxidation, in which a sample is oxidized with 0.5 N potassium dichromate and back titrated with 0.1 N ferrous ammonium sulfate. Alpha cellulose is used as a control.
Hemicellulose	Hemicellulose is extracted (dissolved) from the same dry sample as above with 17.5% sodium hydroxide, corrected for the amount of ash present (test derived from the definition of hemicellulose). Alpha cellulose is used as a negative control, since this is insoluble in caustic.
Ash	AOAC (Association of Official Analytical Chemists) test procedure heats the sample of known weight to 650-700 °C for ½ hour and weigh sample every ½ hour thereafter until the weight remains constant.
Silica	Residual ash sample from above test is extracted with 36% hydrochloric acid. In this modified AOAC procedure, silica is the remainder, which is then dissolved in hydrofluoric acid as a

confirmation that the remainder is truly silica.

Inorganics

This has been defined by Arkenol as the "other" portion of ash which is not silica and is determined by difference of the above two tests.

Protein

Referred to as the AOAC "Kjeldahl" test, this procedure digests the sample in concentrated sulfuric acid to produce ammonia which is collected in a boric acid solution and then back titrated with ferrous ammonium sulfate.

Sugar (Residual)

The sample is extracted with hot water and a sample is run in the High Performance Liquid Chromatograph (HPLC) using an amino column and refractive index detector.

Moisture

A standard USP procedure for determining the moisture content was used whereby a sample is heated to 105 °C for at least 5 hours and then weighed every hour until the weight remains constant.

Lignin

Lignin may be defined by changing the polymer to phenols and measuring the phenol content. However, this test underestimates lignin by a large extent due to the existence of polyphenols. Arkenol believes that lignin should be defined as the difference in the sample weight after subtracting moisture, cellulose, hemicellulose and ash, also protein and residual sugar if these are present in the sample. This conforms to the material which will be produced in any hydrolysis process.

D. Results - Feedstock Determination

Results of the Arkenol Feedstock testing are reported in Tables II-1 through II-3 as Follows:

Hawaiian sugar cane bagasse and Sacramento rice straw - Table II-1

Hawaiian Napier grass and Texas sorghum - Table II-2

DOE spruce wood and historical Arkenol California pine - Table II-3

Table II-1

Hawaiian Sugar Cane Bagasse

Sacramento Rice Straw

Component	% As-Rcv'd	%-Dry	%As-Rcv'd	%Dry
Moisture	47.60		10.50	
Cellulosics	40.90	78.00	68.00	76.00
Cellulose	26.10	49.90	45.65	51.00
HemiCell	14.80	28.10	22.37	25.00
Ash	3.10	6.00	11.19	12.50
Silica	0.10	0.20	7.16	8.00
Inorganics	3.00	5.80	4.03	4.50
Protein	N/A	N/A		
Resd Sugar	1.60	3.00		
Lignin/inerts	6.80	13.00	10.29	11.50

Table II-2

Hawaiian Napier Grass

Texas Sorghum

Component	% As-Rcv'd	%-Dry	%As-Rcv'd	%Dry
Moisture	13.80		16.20	
Cellulosics	59.10	68.50	65.50	78.20
Cellulose	33.60	38.90	32.80	39.10
HemiCell	25.50	29.60	32.70	39.10
Ash	5.90	6.90	6.00	7.20
Silica	0.10	0.10	0.10	0.10
Inorganics	5.90	6.90	6.00	7.20
Protein	9.90	11.50		
Resd Sugar	N/A	N/A		
Lignin/inerts	11.30	13.10	12.20	14.60

Table II-3

NREL
Spruce Wood
California
Pine

Component	% As-Rcv'd	%-Dry	%As-Rcv'd	%Dry
Moisture	58.40		25.50	
Cellulosics	33.60	80.80	52.60	70.70
Cellulose	25.70	61.90		
HemiCell	7.90	18.90		
Ash	0.10	0.20	0.88	1.10
Silica	0	0		
Inorganics	0.10	0.20		
Protein	N/A	N/A		
Resd Sugar	N/A	N/A		
Lignin/inerts	7.90	19.00	21.02	28.20

E. Hydrolysate Production in the Arkenol Technology Center

The next phase of biomass testing involves the conversion of cellulose and hemicellulose from the samples into fermentable sugars. There are several processes for this conversion. However, only the Arkenol process produces higher concentration mixed sugars without producing degradation products. The following is a brief description of the Arkenol Concentrated Acid Hydrolysis Process:

Figure II-4

Arkenol Concentrated Acid Hydrolysis Process

Mixed Sugars Converted to Ethanol by Fermentation

(1) Arkenol Concentrated Acid Hydrolysis of Biomass-to-Ethanol Process Description

Figure II-4 on the previous page represents a typical ARKENOL acid hydrolysis of biomass to ethanol process flow diagram. The discussion below narrates this flow diagram:

1. **Feedstock Preparation;** Biomass is either reclaimed from a storage pile or loaded directly onto the feedstock grinder feed conveyor. The feedstock is ground in either a tub-grinder or hammer-mill depending on its milling characteristics (ie. split or break along longitudinal axis). The grinding specification also varies with the specific biomass in that particle size is smaller for materials which absorb acid slower ranging from 10-25mm in length. If the biomass moisture is above 10-15 %, a biomass dryer is then used to remove the moisture down to that level. Dry particles are pneumatically transported to a feed bin above the first decrystalyzer/hydrolyzer mixer.
2. **Decrystalization;** Concentrated acid is carefully mixed into the prepared feedstock material to break down the crystalline cellulose structure without damaging the amorphous cellulose and hemicellulose also present. Patented pressure and temperature conditions ($< 110^{\circ}\text{C}$, atmospheric pressure) in combination with controlled mixing dynamics produces a paste like material ready for hydrolyzation.
3. **Hydrolysis;** At the conclusion of decrystalization, the temperature and pressure conditions are changed and water is added to provide easily controlled conditions for hydrolysis. The hydrolysis process breaks up the sugar polymer chains into component sugar molecules. For rice straw, approximately 50% of the hemicellulose polymer chains are polymers of C_5 or pentose sugars. These sugars degrade quickly under certain conditions. The remainder of the polymers present are polymers of C_6 or glucose sugars which are much more stable. Based upon the feedstock characterization, a time is selected for completing the first hydrolysis to recover the most pentose sugars. After the first hydrolysis, the material is separated into a liquid acid and sugar fraction and remaining cellulose fraction. The liquid fraction is cooled and the degradation of sugars essentially stops. The remaining cellulose fraction is then re-hydrolyzed in a second step to recover the remaining glucose sugars. The solid fraction remaining after the final separation contains lignin, inerts and unreactable cellulose and hemicellulose. This fraction, after washing and neutralization may be used as a solid fuel, soil amendment or other peat replacement.
4. **Acid/Sugar Separation;** Industrial scale ion chromatography has been widely used for the last twenty years, primarily in the sugar separation and purification industry. The same principal that allows chemists to determine the composition of samples by separating their component molecules in laboratory chromatographs can be used to separate process streams. The commercial unit utilized by Arkenol consists of 10 stages of chromatographic separation units operating in a continuous feed application.

The resulting streams of acid and sugar have purities and recoveries in the mid to high 90% range.

5. **Acid/Sugar Stream Final Processing;** The acid/sugar streams from the chromatographic separator each contain 1-2% of the other streams components. The acid is concentrated in a multistage evaporator to return it to its initial concentration for reuse. Any sugar or organic molecules present in this process are reduced through dehydration to carbon which will end up being filtered into the lignin cake. The sugar stream is neutralized and pH adjusted by the addition of lime. Any acid or inorganics in the sugar stream end up with (CaSO₄) as hydroxides in a solid cake. This can subsequently be centrifugally separated or filtered.
6. **Fermentation or Chemical Processing;** The final sugar stream may be used in any number of fermentations to produce ethanol, a wide variety of chemicals, chemical intermediaries, or converted directly into sugar derivative chemicals. For fermentation, nutrients are added and the acidity (pH) is adjusted to match the fermentation conditions desired for the specific product desired. Since 90% of yeast cake commonly used for fermentation is recycled in Arkenol's ethanol fermentations, only a small yeast growth train is required to insure a vigorous metabolic organism. Typical 72-96 hour ethanol fermentations are predicted for the anticipated mixed sugar feed material at Arkenol yeast culture loadings.
7. **Ethanol Distillation & Dehydration;** If ethanol is the product of choice, further processing is required to convert the beer produced in fermentation to a useable product. Fuel grade of ethanol is a standard in that it represents the largest market. Typically 40-50 stages of distillation are required to produce a 95% ethanol stream from a 6-10% ethanol beer. An industrial alcohol specification would add another side stripper column and storage tank for fusel oils (higher alcohols). Dehydration is typically accomplished using a vapor phase molecular sieve with hot product vapor regeneration and distillation reflux condensation.

(2) The Arkenol Technology Center

The Arkenol Technology Center is comprised of offices, laboratories and a Pilot Plant which uses semiworks scale vendor equipment capable of full commercial scale-up to demonstrate the Arkenol process and evaluate feedstocks for customers of full size plants.

The Arkenol Technology Center depicted by Figure II-5 serves both Arkenol, Inc., the concentrated acid hydrolysis process licensor, and Applied Power Concepts, Inc. (APC), a scientific investigation, consulting and laboratory services company under contract to Arkenol. Both companies share the same facilities in a synergistic arrangement whereby APC provides scientific support and Pilot Plant staffing for Arkenol.

The Arkenol Technology Center, located at 1738 North Neville Street, Orange, California 92865, is housed in a 15,000 square foot tilt-up concrete warehouse building. Offices, meeting rooms, bathrooms and administrative equipment occupy approximately 1,600 square feet and serve approximately six degreed scientific staff (chemist, biologists, technicians) and 2 senior scientists. Another 3,400 square feet is occupied by the laboratories which include both wet chemistry setups, analytical instruments, electronic servicing facilities and biology laboratories. The Pilot Plant area occupies the remaining 10,000 square feet and serves as the main utilities support as well as equipment unit operations testing area.

Laboratory equipment consists of analytical instruments and wet chemistry setups. The wet chemistry laboratory is staffed and supplied to run a variety of distillation, vacuum distillation, filtering, gas and liquid chromatography, simple or complex organic or inorganic reactions under vacuum, atmospheric or intermediate pressure conditions. Approximately four separate areas are available currently and space exists for several more.

The analytical laboratory includes equipment and instruments to perform a variety of measurements in liquid, solid or gas phase. Viscometers, pH, Dissolved Oxygen, heat capacity, filter test units, enthalpy, phase separation, surface area (BET) measurement, precision scales and wet chemistry titration setups augment the sensitive analytical instruments listed below:

- ▶ FTIR
- ▶ Ion Chromatograph
- ▶ Liquid Chromatograph with automated sample injection unit
- ▶ 3 x Gas Chromatograph
- ▶ 2 x Infrared Spectrometers
- ▶ 2 x UV - visible Spectrometers
- ▶ Spectral Radiometer

The Pilot Plant semi-works equipment are arranged so that each piece of equipment may be run as a unique unit operation and interconnected so that products may be measured, sampled or sent to the next unit operation for combined material balance evaluation. This arrangement allows for scale equipment to be optimized for performance individually and then interconnected to perform in conjunction with other downstream equipment.

Vendor equipment used for the unit operation evaluation of performance can eventually be scaled to full commercial size from the data collected and evaluated by Arkenol and the equipment supplier. This usually results in better testing coordination on actual anticipated

feed materials with the added advantage of vendor participation in scale-up and commercial equipment performance guarantees. Specific equipment available for testing includes:

- (1) 5-Hp Hammermill with 2 square foot feed chute and screen sizes of 1/4, 3/8, 1/2, 3/4 and 1 inches. A set of manual sieve trays provides the capability to determine feed and ground particle size distribution. Motor current measurements allow for energy consumption projection.
- (2) 75-gallon Jaygo (Sumix) orbital screw variable speed paste mixer with 150 °C plastic coating is capable of variable rate mixing with either heating or cooling in the jacket, direct steam sparging in the vessel, low pressure or vacuum operation. Temperature (internal and jacket), pressure (positive and vacuum), motor current, speed and interconnected computer mass balance tracking and operation analysis are just some of the tools currently available to allow mixing analysis and process control capabilities.
- (3) 30-gallon Pfaudler anchor blade type glass lined variable speed paste mixer is capable of variable rate mixing with either heating or cooling in the jacket, direct steam sparging in the vessel, low pressure or vacuum operation. Temperature (internal and jacket), pressure (positive and vacuum), motor current, speed and interconnected computer mass balance tracking and operation analysis are just some of the tools currently available to allow mixing analysis and process control capabilities.
- (4) 20-gallon Pfaudler glass lined round T-bar blade paste mixer is capable of fixed rate mixing with either heating or cooling in the jacket 150 psig pressure operation. Temperature (internal and jacket), pressure, motor current, and interconnected computer mass balance tracking and operation analysis are just some of the tools currently available to allow high pressure and temperature mixing analysis and process control capabilities.
- (5) 1.5 GPM Moyno positive displacement screw type variable speed pump with pressure transducers and metering bin is capable of measuring the pumping rate of different high viscosity fluids.
- (6) Eimco 20 square foot (5 plates) vertical pressure leaf filter with variable pressure bladders, air and intermediate fluid rinse capability, 125 psig pressure and PVDF construction including valves. Step by step sampling and rinse fluid volume measurement allow for mass balance monitoring of filter efficiency.
- (7) 6 foot height, 35 gallon chromatographic pulse column separator with computer controlled sequence valves to partition separated solutions into two or more categories using either anion or cation chromatographic separation resin.

- (8) 2.5 liter IWT commercial pseudo-moving bed chromatographic separation unit with computer control, variable speed pumps and weigh scales for feed and elution fluid. Unit is capable of operation with cation or anion resin and will separate species with commercial scale-up guaranty from manufacturer.
- (9) 200 gallon single stage forced circulation vacuum evaporator with graphite exchanger and circulating pump. Computer control varies batch cycles against evaporation curve measuring pressure, temperature and weight of condensate.
- (10) 5 x 110 gallon sealed polyethylene fermenters with nutrient addition, variable sterile air diffusers, pumped circulation and copper sulfate vent traps.
- (11) Pressure submicron filter sterilizer for cold sterilization of neutral media allows storage and shipment of fermentable liquids under sterile conditions.
- (12) 20 gallon 20 inch diameter by 3 foot deep live steam autoclave with pressure and temperature control for sterilization or low temperature reactions.
- (13) 50-gallon Pfaudler fixed speed paste mixer in esterification arrangement is capable of fixed rate mixing with heating in the jacket, vacuum evacuation, chilled water condenser and 30 gallon product collector. Temperature (internal and jacket), pressure (positive and vacuum), motor current, and interconnected computer mass balance tracking and operation analysis are just some of the tools currently available to allow esterification analysis and process control capabilities.
- (14) 2 x 4 liter Autoclave Engineers hydrogenators with 600°C heating and 3,000 psig pressure capabilities, product temperature control, high pressure sampling points, variable speed mixing and computer pressure control all within a concrete block enclosure.

Arkenol Technology Center

Figure 11-5

(3) Arkenol Process Pilot Plant Equipment Utilization

The decrystallization/hydrolysis step can be demonstrated in either of two pieces of equipment. The larger orbital screw mixer unit handles batches from 30-100 lbs. of material per batch. A smaller anchor blade paste mixer runs samples of 5-15 lbs. per batch. The larger mixer is also connected to a positive displacement pump and plate and frame filter press. This allows for better processing in larger batches, but requires much larger sample sizes.

Final separation of the acid and sugar solution was accomplished in a large pulse column resin separator using chromatographic resin. Pulses of feed were alternated with water elutions to effect a chromatographic separation. Product pulses are shifted to different vessels depending on the time through the column. Very good separations are possible with this equipment, however, larger than normal elution water requirements dilute the products beyond what is expected for commercial equipment. A commercial pilot chromatographic separator was also used, but batch loading is much lower on this equipment and the amount of hydrolysate needed for fermentation was relatively large.

This study utilized both sizes of equipment, with bagasse and rice straw being converted in the larger batches and the remainder in small batches.

F. Small Decrystalyzer/Hydrolyzer Testing

Test results for the small (5 gallon) Decrystalyzer/Hydrolyzer are shown in Table II-6. A description of the terms used follows:

Date	This is the date a particular test was run.
Max Sugar Time	This is the time elapsed from the time hydrolysis temperature is reached until the time maximum sugar concentration is achieved.
2nd Order Curve Time	This is the predicted time for maximum sugar concentration from a second order curve fit of the data. The time predicted integrates the actual time @ temperature during the heating period going from the decrystallization temperature to the hydrolysis temperature.
Peak Sugar %	The peak sugar percentage is measured in the actual hydrolysate leaving the decrystalyzer/hydrolyzer. If it were measured at the discharge of the filter press, after separation

	from the cake, it would be as much as 30% higher depending on the difficulty of washing the cake.
Average Decrystalyzer Temperature	This is the temperature, in degrees centigrade, at which the biomass decrystalyzation occurs under preset conditions of particle size, acid concentration and mixing regime.
Average Hydrolysis Temperature	This is the temperature, in degrees centigrade, at which the biomass hydrolysis occurs under preset conditions of particle size, acid concentration and mixing regime.
Time to Hydrolysis Temperature	This is the time it take the physical equipment to elevate from the decrystalyzation temperature to the hydrolysis temperature. The hydrolysis reaction proceeds under less than ideal temperature so this must be taken into account when running the test.
Ratio of Glucose to Xylose	While the ratio of cellulose to hemicellulose has been determined for the various samples tested, the actual ratio of glucose to xylose has not been determined. This is due to hemicellulose being a variable combination of glucose and xylose sugars in polymeric form which varies from biomass to Obiomass. The ratio of C ₆ to C ₅ sugars is very important because it affects the conversion time and efficiency for different fermentation products.

Small Decrystalyzer/Hydrolyzer Run Analysis

Napier Grass Date	Max Sugar Time	2nd Order Curve Time	Peak Sugar %	Avg Decryst Temp	Avg Hydr Temp	Time to Hydr Temp	Ratio G:X
3/11/97	20	35	11.62	35	97	15	1.6:1
3/18/97	10	10	14.53	35	92	10	1.21:1
3/20/97	35	35	10.74	35	95	15	1.6:1
4/8/97	28	22	12.31	39	92	20	1.05:1
4/10/97	42	15	8.92	35	90	15	0.7:1
4/15/97	35	35	14.18	34	91	15	1.51:1
4/21/97	30	30	8.55	38	91	15	0.64:1
Wood Chips							
3/21/97	20	40	14.54	36	96	30	5.7:1
3/26/97	20-30	40	15.82	37	96	30	1:0
Sorghum							
4/1/97	30	40	15.82	35	95	15	1.11:1
4/3/97	14	20	11.35	36	96	25	0.72:1
Bagasse							
1/10/97	40	35	8.36	30	100	16	4.35:1
1/27/97	10	0	16.4	37	98	22	2:1
1/29/97	16	12	13.98	32	88	24	1.8:1
4/24/97	15	10	13.66	34	90	10	1.52:1

Table 11-6

G. Arkenol Kinetic Model Verification

The thermally activated, acid catalyzed conversion of cellulose and hemicellulose to glucose and xylose is represented by the simultaneous solution of four equations:

1. The forward conversion of cellulose to glucose.
2. The forward conversion of hemicellulose to glucose and xylose.
3. The degradation of glucose to organic acids and carbon.
4. The degradation of xylose to organic acids and carbon.

The formation and degradation equations can be described by differential equations of the form:

$$\frac{d[G]}{dt} = k_1[C] - k_2[G]$$

Where:

[C] = Concentration of Cellulose, or Hemicellulose

[G] = Concentration of Glucose, or Mixed Sugars

k_1 = Rate constant for formation

k_2 = Rate constant for degradation

All of the rate constant equations have the Arrhenius rate equation form:

$$k_1(A,T) = p_1 A^{x_1} e^{-\frac{E_1}{R(T+460)}}$$

Tens of runs have been plotted and averaged to display the actual glucose and xylose formation correlation to calculated and predicted values. Figures II-7A and II-7B represent the glucose and xylose curves respectively.

H. Fermentation of Arkenol Process Produced Hydrolysate

Fermentation of the Arkenol Process generated hydrolysate is the last phase of the

Feedstock Analysis portion of the overall program. Natural microorganisms have been grown and tested by Arkenol on the chemically sterilized hydrolysate, however, a new metabolically engineered form of *Zymomonis mobilis* (ZM1) bacteria (CP4/ZB5) developed by NREL reportedly showed promise of improved yields in shorter fermentation times.

NREL's previous experience on ZM1 indicated the capability to metabolize both glucose and xylose mixed sugars in concentrations less than 8%, and higher concentrations of the individual sugars separately.

Samples of the ZM1 inoculum were provided by NREL under a license agreement for testing by Arkenol at the Arkenol Technology Center. Procedures provided by NREL indicated the plasmid in the ZM1 bacteria contained tetracycline resistance. Therefore, 10 mg/L of tetracycline was added to all ZM1 nutrient media to insure that only the modified bacteria would survive. The media commonly used by NREL (RM Media) for growing the ZM1 is a natural media in that it contains 10 g/L of yeast extract, 2 g/L of KH_2PO_4 and sugar at either 30 g/L for growth or up to 180 g/L for fermentation. Fermentations are carried out anaerobically, at temperatures in the range of 30-35 °C and pH of 5.5 or slightly higher.

Figure II-7A

Glucose Table

	p1g	x1g	E1g	p2g	x2g	E2g
avg	7.306E+19	1.78	36800	1.681E+15	0.555	27000

Figure II-7B

Xylose Table

	p1x	x1x	E1x	p2x	x2x	E2x
avg	1.136E+21	1.78	37986	9.069E+19	0.555	35000

The inoculum was grown until the cell count as measured by microscope was 10^8 CFU's per ml. or higher. Fermentations were carried out with a 5-10% by volume seed rate of the grown inoculum. Yeast (*P. stipitis*, NREL-Y-7124), used as a control, was grown to a roughly equivalent culture density of 10-20 g/L on Arkenol defined media at the same temperature but pH of 2.5-4.5. The yeast fermentation was carried out under microaerophilic conditions of approximately 1-9 ppm dissolved oxygen (DO).

Tests were begun with pure sugars to verify the procedures used and immediate repression of the ZM1 was noted at even low (8%) sugar concentrations. It was discovered that in the process of sterilizing the pure sugar media in an autoclave, the 120-130 °C temperature caused slight xylose degradation. Even the presence of these slight degradation products inhibited the fermentation using the ZM1 bacteria. When the sugars were sterilized separately, the fermentations reproduced those reported by NREL. The yeast control did not show any sensitivity to the autoclave order or mixtures.

The program began with temperature (30-35 °C) and ZM1 culture density (10^6 - 10^8 cells/ml) testing which did not exhibit remarkable differences. A temperature of 32°C and 10^8 culture density was standardized for the remaining sugar concentration testing.

Sugar concentrations were initially varied from 10-15% starting mixed sugars. These were naturally diluted somewhat when the inoculum was added, but remained at 8-12% at a minimum. The sugar composition was roughly 80% glucose and 20% xylose produced largely from sugar cane bagasse hydrolysate and supplemented with pure sugars. Results indicated that ZM1 fermentations universally started metabolizing both glucose and xylose sugars, but stopped utilizing the xylose sugars when the ethanol concentration exceeded 2-3%. Glucose fermentations continued until the glucose was fully utilized, but the xylose sugar utilization stalled.

Testing for the cause of this xylose inhibition proceeded with the following plan and results:

- 1) ZM1 was tested with 10% pure xylose with little improvement. It was postulated that the presence of a small amount of glucose or glucose oxidase enzyme may be required for improved efficiency.
- 2) ZM1 was next tested with 9% xylose and 1% glucose with a small improvement noticeable.
- 3) ZM1 was finally tested with 6% total sugars with good results and near total xylose utilization, but the xylose fermentation was only completed after 48 hours.
- 4) Chemically sterilized hydrolysate was sent to NREL for testing. Their laboratory analysis indicated a lack of inhibitors, and fermentation testing

duplicated Arkenol results.

NREL indicated that due to the anticipated use of ZM1 on a lower concentration sugar substrate, tests on mixed sugars had not exceeded 6-8% total sugars. They also indicated that they had found similar inhibition to xylose fermentation in the presence of glucan sugars with other similar bacteria. They indicated that they had other strains of ZM which may perform better, but that we may be further frustrated in trying to push the ZM1 strain.

Yeast fermentations on mixed sugars and high total sugar concentrations did not produce the same stalled xylose utilization as the ZM1 exhibited. However, the following observations were confirmed from earlier studies:

- 1) Yeast fermentations with concentrations above 10 g/L were difficult to complete due to the limiting oxygen uptake possible in 2 L flasks with sterile stoppers using ambient air diffusion only. Alternate pumped air systems in the range of 0.1 to 0.5 mg/L of dissolved oxygen are needed.
- 2) Fermentations performed with air, and oxygen, improved results significantly. A series of two sample (start/finish) fermentations were run with oxygen to verify the importance of microaerophilic fermentation conditions with the yeast. The results are shown in Tables II-8 A and B. The results of runs 7 & 8 in Table II-8A show reasonable yields for a totally anaerobic fermentation where instead of microaerophilically metabolizing the xylose with yeast, ZM-2 were added in mixed culture. Table II-8B repeat the results found in Table II-8A plus two larger (9L) continuously stirred fermenter results. Overall, average conversions above 90% were seen for microaerophilic conditions with near total xylose utilization in 96 hours. These conditions dropped to the mid 70% level with lesser xylose use when too much air was introduced.
- 3) Yeast fermentations metabolized the glucan sugars in preference to the xylose sugars. When glucan sugars dropped to approximately 50% of the total sugars, xylose sugar utilization picked up. Overall xylose sugar utilization was not completed in 96 hours (as projected, with this culture density loading), however, full utilization occurs if left to an additional 12-36 hours.
- 4) Starting sugar concentrations and resulting ethanol concentrations did not appear to affect yeast fermentations in the ranges studied (6-13.7% mixed sugars, 2.1-5.6 g/100ml ethanol). Previous testing done by Arkenol has proved ethanol tolerance of 9% by weight ethanol with the same cultures.

Testing of fermentation samples also resulted in additional information. The original program anticipated the utilization of FTIR spectroscopy to determine both the sugar and ethanol concentrations in a fast and cost effective means utilizing the Applied Power Concepts

(APC) patented² FTIR method for analysis. Difficulty arose with the ability to identify additional chemicals in the mixture. The APC method requires that components being measured are known and calibrated as standards. The initial calibration assumed that glucose, xylose, xylitol and ethanol would be present in a defined medium. Components not expected included glycerol, yeast extract and lactic acid. While additional standards testing, defined media or centrifugation may have overcome these problems, this was not possible in the current test program.

As a result of the problems with FTIR, liquid chromatography was used which enabled us to identify when unexpected chemicals were present by the appearance of new peaks. This was seldom the case, but did indicate the presence of lactic acid when fermentations were contaminated and glycerol when high residual calcium was present as a result of improper hydrolysate neutralization.

Graphical representations of the ZM1 and yeast fermentations are provided as figures II-9 through II-24. The depiction of the results as represented are described as follows:

Time	Time that actual samples were taken after the start of the fermentation.
Sugar Weight %-	Grams of sugars, either glucose, xylose or combined per Liter.
Ethanol Measured	Total ethanol measured minus initial amount attributable to culture inoculum.
Ethanol Predicted	Theoretical ethanol predicted from the amount of sugar utilized 0.51 g/g.
Ethanol Yield	Ethanol measured divided by the total ethanol possible (initial sugar times 0.51).

²Method for Monitoring and Controlling a Chemical Process, U.S. Patent # 5,262,961.

Table 8A

Runs 1-6 were <i>Pichia stipitis</i> with oxygen at approximately 0.5 mg/L										
26-Sep							predicted	actual	ethanol	ethanol
1	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.16	2.73	11.89	1.93				
	Finish	96	0.00	0.50	0.50	7.24	5.81	5.31	0.91	0.88
26-Sep							predicted	actual	ethanol	ethanol
2	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.07	2.65	11.72	1.91				
	Finish	96	0.00	0.15	0.15	7.38	5.90	5.47	0.93	0.92
26-Sep							predicted	actual	ethanol	ethanol
3	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	8.32	2.96	11.28	1.84				
	Finish	96	0.00	0.10	0.10	7.37	5.70	5.53	0.97	0.96
26-Sep							predicted	actual	ethanol	ethanol
4	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	10.03	2.69	12.72	1.46				
	Finish	96	0.00	0.10	0.10	7.27	6.44	5.81	0.90	0.90
26-Sep							predicted	actual	ethanol	ethanol
5	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.97	2.30	12.27	1.57				
	Finish	96	0.00	0.20	0.20	7.13	6.16	5.56	0.90	0.89
26-Sep							predicted	actual	ethanol	ethanol
6	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.93	2.50	12.43	1.60				
	Finish	96	0.00	0.50	0.50	7.13	6.08	5.53	0.91	0.87
Runs 7-8 were totally anaerobic and were mixed cultures of yeast and <i>Zymomonas mobilis</i> ZM-2										
26-Sep							predicted	actual	ethanol	ethanol
7	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.66	2.00	11.66	2.45				
	Finish	96	0.00	0.10	0.10	7.09	5.90	4.64	0.79	0.78
26-Sep							predicted	actual	ethanol	ethanol
8	hour	glucose	xylose	total	ethanol	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.13	2.34	11.47	2.50				
	Finish	96	0.50	0.20	0.70	6.67	5.49	4.17	0.76	0.71

Table 8B Page 1

Runs 7-8 were totally anaerobic and were mixed cultures of yeast and Zymomonas mobilis ZM-2										
2-Oct							predicted	actual	ethanol	ethanol
7	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	6.90	2.50	9.40	2.84				
	Finish	96	0.00	0.10	0.10	7.32	4.74	4.48	0.94	0.93
2-Oct							predicted	actual	ethanol	ethanol
8	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	9.80	2.22	12.02	3.22				
	Finish	96	0.00	1.10	1.10	7.17	5.57	3.95	0.71	0.64
Regular runs - it looks like we got too much air in these and grew cells to some extent rather than made all ethanol.										
13-Oct							predicted	actual	ethanol	ethanol
1	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	11.59	2.41	14.00	1.13				
	Finish	96	0.50	1.20	1.70	6.03	6.27	4.90	0.78	0.69
13-Oct							predicted	actual	ethanol	ethanol
2	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	10.88	2.57	13.45	1.46				
	Finish	96	0.20	1.50	1.70	6.04	5.99	4.58	0.76	0.67
13-Oct							predicted	actual	ethanol	ethanol
3	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	10.57	2.43	13.00	1.07				
	Finish	96	0.00	0.70	0.70	6.01	6.27	4.94	0.79	0.75
13-Oct							predicted	actual	ethanol	ethanol
4	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	12.07	2.30	14.37	0.67				
	Finish	96	0.20	1.10	1.30	5.72	6.67	5.05	0.76	0.69
13-Oct							predicted	actual	ethanol	ethanol
5	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	11.48	2.01	13.49	1.16				
	Finish	96	0.20	0.50	0.70	5.90	6.52	4.74	0.73	0.69
13-Oct							predicted	actual	ethanol	ethanol
6	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	12.00	2.50	14.50	1.05				
	Finish	96	0.10	0.50	0.60	6.51	7.09	5.46	0.77	0.74
Runs 7-8 were totally anaerobic and were mixed cultures of yeast and Zymomonas mobilis ZM-2										
13-Oct							predicted	actual	ethanol	ethanol
7	hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion	
	Start	0	9.66	2.00	11.66	2.45				
	Finish	96	0.00	0.10	0.10	7.09	5.90	4.64	0.79	0.78
13-Oct							predicted	actual	ethanol	ethanol

Table 8B Page 2

8		hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.13	2.34	11.47	2.50				
	Finish	96	0.50	0.20	0.70	6.67	5.49	4.17	0.76	0.71
The following are 9-liter fermenter runs										
13-Oct							predicted	actual	ethanol	ethanol
9-L1		hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion
	Start	0	8.77	2.03	10.80	0.67				
	Finish	96	0.00	0.00	0.00	6.08	5.51	5.41	0.98	0.98
13-Oct							predicted	actual	ethanol	ethanol
9-L2		hour	glucose	xylose	total	ethanol	ethanol	ethanol	yield	conversion
	Start	0	9.35	1.75	11.10	1.07				
	Finish	96	0.20	0.80	1.00	5.00	5.15	3.93	0.76	0.69
9-L1 is <i>Pichia stipitis</i> and 9-L2 is <i>Candida shehetae</i> (at not quite 10 g/L)										

FIGURE II-9

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-10

Yeast Fermentation 30C, 10% sugar, min cell density

FIGURE II-11

Yeast Fermentation 30C, 10% sugar, min cell density

FIGURE II-12

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-13

FIGURE II-14

FIGURE II-15

FIGURE II-16

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-17

ZM Fermentation 30C, 10% sugar, min cell density

FIGURE II-18

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-19

ZM Fermentation 30C, 10% sugar, min cell density

FIGURE II-20

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-21

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-22

ZM Fermentation

30C, 10% sugar, min cell density

FIGURE II-23

ZM Fermentation 30C, 10% sugar, min cell density

FIGURE II-24

I. Fermentation Commercial Optimization

Arkenol proposed, and received approval (May 21, 1997) to proceed on, several additional study sidelines which may improve the commercial utility of the NREL ZM organism for biomass sugars produced using the *Arkenol Concentrated Acid Hydrolysis* Process. These are:

- ▶ Series fermentation with either ZM1 followed by ZM1 or new culture identified ZM2 may improve the utilization of xylose after the conversion of glucose sugars.
- ▶ The ability to centrifuge and recycle microorganisms from the clear sugar stream in the Arkenol process allows for achieving higher fermentation concentrations or reduced culture growth trains. This may be especially helpful in organisms which are not readily adaptable to product sales of protein in animal feeds.
- ▶ Substituting a defined growth media for an undefined one, especially where the provision of yeast extract or corn derivatives is not readily available or expensive, which could save up to \$3,000,000 per year for a 12 MMGPY facility.

(1) Series and Parallel Fermentations with Pure and Mixed Strain Cultures

NREL suggested another strain of the *Zymomonis mobilis* culture (39676/PZB5) which they developed and tested for its ability to metabolize xylose sugars in the absence of glucose sugars. This strain is reportedly effective up to 10% xylose concentration but limited to a maximum of 1% mixed glucose sugars.

Fermentations run with ZM1 followed by fresh ZM1 culture when the glucose level dropped and the xylose conversion seemed to stall (at 48 hours) appeared to have virtually no improvement to the overall conversion of the mixed sugars as shown in Figures II-25, & 26.

Initial fermentations run with ZM1 followed by ZM2 at the same time period resulted in an improved conversion of xylose sugars, see Figures II-27 & 28. But, the conversion of xylose did not improve the overall yield of ethanol. Furthermore, lactic acid and other possible contamination coproducts were determined by laboratory analysis not to account for the difference. Glycerine was detected and is believed to be the byproduct accounting for the missing sugar mass. Possible calcium pathway enhancement due to residual gypsum content in the neutralized hydrolysate is the expected cause.

A third test was run in which ZM1 was followed by a mixed culture of ZM1 and ZM2 as shown in Figures II-29 & II-30. Although the low overall yield and discrepancy between the utilization of sugar and production of ethanol still exist, Figure II-29 illustrates that this

discrepancy did not worsen with the addition of mixed culture and onversion of sugars proceeded.

An interesting feature of the ZM1 and ZM2 is that when under stress, the bacteria tend to coagulate or clump together. This can normally be observed in medium used in the laboratory since the organisms fall to the bottom of the fermenter. When the organisms clump they also produce more lactic acid than ethanol.

Fermentation testing was interrupted during this period of testing due to the receipt and use of new nutrient medium. As already explained, the undefined or complex medium prescribed by NREL utilizes a yeast extract. Suppliers of this natural yeast extract can use several different sources of yeast extract material all with different solubilities. The original yeast extract supplied to Arkenol was a highly soluble material suitable for optical density measurements. The second batch of material, sent under the same order number (Sigma Y0375) , was a lower solubility material which produces enough insoluble solids that the bacteria coagulated on it and dramatically changed the fermentation results.

Testing was resumed with the utilization of new and more soluble yeast extract. A ZM1 followed by a ZM2 fermentation showed some small improvement over the sole ZM1 fermentation as illustrated in figures II-31 & 32.

An even better fermentation was noted with the addition of both ZM1 and ZM2 to the original ZM1 fermentation as shown in figure II-33 & 34. However, overall conversion of the mixed sugars to ethanol are still only in the 72-75% range. This is still below the natural yeast cultures used by Arkenol. However, the mixed ZM cultures tested convert more sugars in the same time period, only not all of the conversion is directed towards ethanol.

Potentially, a better system could be devised and evaluated in which a natural bacteria reduces the glucose sugars and a genetically engineered organism is added either at the same time or after the glucose sugars are metabolized.

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32 C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32C, Augmented Culture

ZM-1 Fermentation 32 C, Augmented Culture

(2) Centrifugal Removal and Recycling of Organisms

Current fermentations in the corn ethanol industry use prepackaged Red Star yeast or grow them in a yeast growth train. Cheese and other fermentations using a variety of organisms must use a growth train to replenish once through utilization. Only the pharmaceutical and molasses industries try to recapture and recycle their organisms in fermentations.

Growing organisms in small aerated fermenters consumes product sugars and converts them into organism mass. Sometimes, these organisms are collected, sterilized and sold as part of an animal feed product and the partial cost can be recovered (\$150-\$500/dry ton). More often however, this cost cannot be recovered and is an operating cost. The Arkenol process uses clear soluble sugar streams in its fermentations and can therefore separate organisms from this stream while retaining their fermentive vitality.

Yeast are generally 3-10 μm , whereas bacteria fall into the size range of 0.1-1 μm . Along with size concerns, and roughly relative to its size, is the stress susceptibility of the organism and its cellular membrane walls. Arkenol has tested and found its yeast compatible with centrifugal separation and recycle. However, bacteria have not been tested in a similar fashion.

Arkenol used a conventional laboratory centrifuge capable of 4,300 RPM and scalable to commercial equipment separations for the test. Sterilized 100 ml centrifuge tubes were used, and the live cultures transferred under a sterile hood. The centrifuge was operated until the culture density at the bottom of the tube was visibly increased. The approximate density at the bottom of the tube was similar to yeast cultures (150 gm/l), but the relative volumes were much less.

The centrifuged culture was reintroduced into growth media and stirred slightly to distribute the bacteria. There appeared to be no undue coagulation of the organisms and growth along with fermentation appeared to proceed normally. There was no specific bacteria count or examination of the culture to determine a percentage of dead organisms, nor was there a specific record of the fermentation yield. However, Arkenol considers the test successful and bacteria recycle viable. It is important to note however, that the bacteria growth rate is much greater than the yeast growth rate due to the method of cellular reproduction, and this somewhat reduces the necessity of culture recycle.

(3) Substitution of a Defined Growth Media for a Natural One

Complex media is generally considered one which incorporates naturally grown or sterilized organism matter which while nutritious, is inherently variable from batch to batch. Natural media constituents such as corn extract, yeast extract or other crop residuals are also more difficult to supply, store and keep, as well as being more expensive relative to chemicals in a defined media.

Defined media is a mixture of chemicals which approximates the nutrients in a natural media, or may even surpass natural media if organisms have special needs. Defined media can be easier to source, store in dry form and typically has a longer shelf life. The chemicals can be competitively priced for the most part and cost approximately one tenth of the equivalent natural media.

Arkenol tried several mixtures of defined media before settling on the composition below:

- Urea or NH_4NO_3 for nitrogen
- Macronutrients consisting of:
 - NH_4SO_4
 - KH_2PO_4
 - MgSO_4
 - NaCl
- Micronutrients including:
 - $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$
 - $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 - $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
 - $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$
 - KI
 - $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 - myo-Inositol
 - Calcium Pantothenate
 - Nicotinic Acid
 - Pyridoxine-HCl
 - Thiamine-HCl
 - p-aminobenzoic acid
 - Riboflavin
 - Biotin
 - Folic Acid

The defined media testing compared ZM with undefined media versus ZM with defined media. The remaining conditions were selected at 32 °C, 6% overall sugars with 2.3% xylose. Defined media which promoted fermentations that approached the natural media results were retained and improved. Results of these fermentations is shown in figures II-35 and 36

Although the ZM bacteria has demonstrated its sensitivity to environmental conditions throughout the testing program, and defined media results never quite surpassed the natural media yields obtained, it is felt that with a longer conditioning period the ZM fermentations on defined media will be more commercially reproducible than those on natural media for the reasons stated in this section.

ZM1 Defined Media Fermentation

32C, 6.3% sugar, 2.3% xylose

ZM2 Defined Media Fermentation

32C, 5.9% sugar, 2.2% xylose

Section III

Experimental Growing Program

The proposed experimental growing program consisted of three main elements:

1. Identify and locate existing growers of preferred cellulosic materials.
2. Identify farmers willing to grow cellulosic crops approved for use in the State of Hawaii.
3. Procure and prepare for shipment, cellulosic crops for testing at the Arkenol Technology Center.

Arkenol enlisted the aid of Energy Resources, a local Hawaiian group interested in promoting new agricultural enterprises in Hawaii. Energy Resources was tasked with the above work scope and sugarcane bagasse, sweet sorghum and napier grass were identified as the crops of interest.

In addition, Energy Resources was tasked with preparing an estimate of the costs required for large scale planting, cultivation and harvesting of biomass materials in Hawaii.

The overall program was started later in the year than anticipated, and the availability of crops was severely limited. In fact, only sugarcane bagasse and napier grass were found suitable for our needs.

Sugarcane bagasse is normally harvested 8-11 months of the year in Hawaii. However, due to the ailing sugarcane industry, many of the mills (approximately 23 of 26) have closed, never to reopen. One of the mills was able to provide Energy Resources with approximately 1,000 pounds of bagasse but were unable to dry it themselves. Energy Resources spread the bagasse for air drying, but unseasonable rains forced them to airship the material to California before reaching the 20% moisture limit maximum imposed by Arkenol. The material was further air dried by Arkenol, and only minimal fermentation of the residual sugar was experienced.

Energy Resources next contacted Hawaiian Gardens, a subsidiary of Orchard-Marine Company, who grow napier grass on the main island. They were able to supply 400 pounds of 30% moisture napier grass which had been foraged already by cattle. It was felt that this material better represented the type of crop residue from napier grass which would be available to biomass plants. Attempts to air dry the material in Hawaii were again unsuccessful, but due to the lower residual sugar in the initial biomass, there was little fermentation damage by the time it was air shipped and dried in California.

Attempts to grow speciality crops, like sorghum, were stopped due to the late growing

season and the required timing of the overall program. Still intent on growing sorghum, Arkenol found that a stand of it was still available through the Texas Agricultural Experiment Station of the Texas A&M University. Mr. John Drawe, Farm Research Services Manager, generously cut, and dried the sorghum in their greenhouse, shipping approximately 100 pounds of the material to the Arkenol Technology Center.

No additional attempts were made to grow specialty crops in Hawaii for this program.

Section IV

New Regulatory Support

The original D.O.E. program proposed that draft regulations and a supporting base of economics, ethanol education materials and public information be developed to nurture a developing industry of biomass to ethanol in Hawaii. This program would be coordinated with the State Department of Business, Economic Development and Tourism (DBEDT) to test and implement the necessary structure where a new industry could exist. The purpose of this effort was to participate and provide industry-based coordinated input and support in this process in order to ensure that the adopted regulations would fulfill its intended purpose of creating a meaningful ethanol market to foster the development and vitality of the industry in Hawaii. Specific tasks would include:

1. Literature Review - In preparation for the development of Hawaiian blending regulations, the regulations and experience of States with comparable requirements would be assessed.
2. Draft Regulations - The project team would draft a proposed set of regulations and exchange and edit DBEDT generated drafts and comments. Assistance would also be provided in briefings and responding to the Department and Administrative reviews necessary to achieve overall State government approval.
3. Public Hearings and Testimony - Testimony of local public, industry and scientific community members will be drafted and presented at each of the anticipated public hearings.
4. Public Education - The project team would work with DBEDT to develop and disseminate materials to accurately educate the public on the production and use of ethanol blended gasoline.
5. Hearing Review and Responses - The project team will work with DBEDT to develop appropriate responses to concerns or recommendations which arise during the hearing process. Activities will include follow-up communications, redrafting of the regulations and public reply development.
6. Vendor Implementation - In order to address the concerns of gasoline vendors, DBEDT and Arkenol would work with the vendor associations and individual vendors to conduct necessary economic analyses, amend the regulations to accommodate valid concerns, and support vendor efforts at compliance.

Due to the changing nature of the Hawaiian agricultural industry recently, support for the development of a local ethanol industry fueled by either crop or biomass conversion

ethanol has temporarily evaporated. Arkenol's local consultant and prime subcontractor for this portion of the work, Mr. Brian Nakamura, indicated in part:

"Pursuant to our conversations, I have made inquiries as to the State of Hawaii's plans to implement the ethanol blending mandates contained in State law.

Ms. Maria Tome of the State of Hawaii Department of Business, Economic Development and Tourism (DBEDT) is the State Official responsible on the staff level for the development and adoption of the regulations necessary to implement the blending mandate. I have spoken extensively with Ms. Tome regarding the State's plans.

In short, DBEDT has decided to defer the promulgation of the regulations because there are no actual or prospective producers of local ethanol. In the absence, of a foreseeable local supplier, the purposes and policies underlying the blending requirement would be undermined in that the market is likely to be completely occupied by foreign suppliers, or there will not be adequate supplies to satisfy the State's mandates.

While DBEDT recognizes that the absence of a blending requirement may serve as a disincentive to the development of local suppliers, it believes that it would be irresponsible to proceed at this time. Consequently, little or nothing would be gained by our proceeding with the drafting or education portions of our proposed State legislative program...."

Arkenol proposed to DOE in March 1997 and it was approved by DOE in May 1997, that this portion of the overall program be curtailed and that the proposed monies be redirected to additional studies on the main topic of *Zymomonas mobilis* commercial fermentations.