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ABSTRACT

The studies of biological delignification have focused on the adaptation of the lignocellulosic thermophilic mold Chrysosporium pruinorum to growth on maple wood fibers. The addition of trace elements and thiamine hydrochloride to the C. pruinorum growth medium has been found to stimulate culture growth by a factor of about two. The nutritional salt tolerances of C. pruinorum have been determined in order to facilitate the design and operation of a bench top biological delignification bioreactor. Nutrient concentrations below of about 2.5 times that required to support C. pruinorum growth have no significant deleterious inhibitory effects and are anticipated to be adequate to support growth and delignification in a bioreactor. Studies directed at developing a satisfactory mutation protocol for producing a C. pruinorum mutant with enhanced enzymatic activity are continuing. Experiments are underway to confirm the optimum concentration of the Oxgall and Rose Bengal hyphal inhibitors which are required to yield single colonies at a given inoculum size. Flask studies of the growth and delignification of C. pruinorum on maple fibers have been initiated.

Work on the design and construction of a bench-top high solids biological delignification bioreactor has been initiated. The bioreactor will consist of a 3 feet by 6 inch I.D. screw conveyor with cut and folded flights housed in a tubular trough. The unit will be equipped to vary the nutrient feed, air, and moisture concentration and distribution. Test and calibration experiments will be performed using 20% avicel and sawdust slurried.

The mixed culture microbiological studies have focused mainly on nutritional growth requirements and rates of cellulose digestion and ethanol production. In

small test tube mixed culture (sporocytophaga (US) + thermophilic bacillus (NW)) fermentations, the rate of cellulose degradation was found to proceed at a very high volumetric efficiency, 2.4 g/l-hr. The yield of ethanol from the mixed culture fermentation of cellulose varied between 24 and 50% of theoretical. The higher yields were obtained in the presence of insoluble calcium carbonate added to retard the rate of pH decline and to increase the CO₂ tension. A mutation procedure for the thermophilic bacillus NW has been developed in order to select strains which produce higher yields of ethanol.

Bench-top fermentations at the 1 liter scale have been performed to verify, under controlled pH, agitation and dissolved oxygen conditions, the results achieved in small scale test tube experiments. The specific growth rate of thermophilic bacillus NW on glucose was calculated to be 0.59 hr⁻¹. In mixed culture fermentations of amorphous and microcrystalline cellulose the specific rate of substrate depletion was calculated to be 0.087 hr⁻¹ and 0.0346 hr⁻¹, respectively. Ethanol production in these fermentor runs was slower than the rates of acetic acid production, which persisted after ethanol production ceased. In the fermentation of microcrystalline cellulose, 2,3 butanediol was produced in addition to ethanol and acetic acid.

BIODELIGNIFICATION

INTRODUCTION

Biological delignification of a lignocellulosic substrate requires that sufficient lignin be solubilized or disassociated from cellulose to make the latter readily susceptible to enzymatic saccharification. We are pursuing two alternative approaches to accomplishing this objective with Chrysosporium pruinsum which do not result in excessive soluble sugar consumption. The most direct approach already demonstrated in cattle manure fibers (50% cellulose, 25% lignin) involves a temperature shift to arrest sugar metabolism while continuing enzymatic lignin degradation. The alternative approach is to select a glucose-, cellobiose-mutant which will not utilize cellulose-derived sugars for growth. In the latter case, growth would be at the expense of lignin and hemicellulose monomers. The work reported in this section reviews the experimental progress in adapting C. pruinsum to growth on wood, in determining the nutritional requirements and salt tolerances of C. pruinsum, and in developing a satisfactory mutation protocol.

C. PRUINOSUM GROWTH ON WOOD

Conditions previously found to support good growth of C. pruinsum (M-10) on washed cattle manure fibers were employed to examine its growth on Ponderosa Pine sawdust (see Second Quarterly Report). Dried sterilized sawdust (~200 mg) was placed on a membrane filter which was layered on a 200 ml bed of supplemented mineral (SM) agar medium. The membrane was used to facilitate and sample handling. The center of the sawdust was inoculated with a spore suspension of C. pruinsum. Despite numerous attempts, we were unable to observe growth of C. pruinsum on Ponderosa Pine.

Additions of yeast extract and/or peptone to the medium, each at a concentration of 0.1 and 0.5%, resulted in good growth of M-10 on the agar surface but not on the sawdust. Similarly, Ponderosa Pine which was ball-milled for 18 hours in a porcelain jar also failed to support the growth of M-10. To test the possibility that Ponderosa Pine contained C. pruinosum growth inhibitors, a manure fiber/pine fiber gradient plate was prepared and inoculated with M-10 (Figure 1).

Petri Dish Containing SM Agar Medium

FIGURE 1 Fiber Gradient Plate

After the first seven days, C. pruinosum growth was observed on the manure fiber and only on the agar medium to the right of the manure fiber. These results were interpreted to indicate that a diffusible substance in the pine sawdust was interfering with the growth of M-10. Several attempts at solvent-extraction of this substance proved unsuccessful. Ten grams of pine sawdust fibers were placed in 100 ml acetone and agitated at room temperature overnight. The acetone was decanted and replaced with 100 ml of absolute ethanol and agitated for an additional nine hours at room temperature. The ethanol was decanted and the residual fibers were rinsed four times with water, filtered, washed again with 1 liter of distilled water, and dried at 60°C overnight. Even after this rigorous washing

procedure, M-10 failed to grow on these solvent-extracted fibers.

Since the inhibitor appeared to diffuse in agar, we examined distilled water extracts of the pine sawdust for growth inhibition. Pine sawdust wash water was used as a partial diluent in preparing SM broth. When inoculated with M-10, C. pruinosa grew as it typically does in the broth, which suggests that if the pine does contain an inhibitor, it is not readily soluble in water.

Several unsuccessful attempts were also made to use a manure fiber infusion and a water soluble extract to stimulate growth on pine sawdust fibers. In one case, 3 ml of filtered aqueous extract, prepared by agitating 5 g of manure fibers with 100 ml of distilled water, was added to 200 ml of the SM agar medium. In the second case, 2 ml of sterile manure fiber extract was added directly to 200 mg of pine fibers on an SM agar plate. To prepare an infusion, 1.8 g of manure fibers in 1800 ml of distilled water was heated with agitation at 75°C for 14 hours and at 100°C for 30 minutes. The suspension was filtered and combined with 200 ml of 10 x SM to give 2 liters of SM agar medium. No growth on pine was obtained using either the aqueous manure fiber extract or the infusion. Neither a large inoculum nor the use of a vegetative cell inoculum showed any growth on pine sawdust, and inocula grown in the presence of cellulose as opposed to dextrose behaved similarly.

The branch from an untreated growing pine tree was stripped of the bark, ground into small fibers, and examined as described above. Since this pine fiber would also not support the growth of M-10, we excluded the possibility that a preservative or pesticide was causing the inhibition.

Various other woods were also examined, e.g. ash, maple, and birch. Growth as judged by the appearance of white filamentous hyphae was observed on maple and

birch, but only after four weeks on ash. Growth on maple is not as rapid as on manure fiber, seven days vs three days, respectively, but appears sufficient to permit the studies to go forward. Growth optimization can be performed in the future.

In the interests of integrating these studies with other work tasks, we have elected to discontinue further work on pine sawdust and to concentrate on maple. C. prunosum strains adapted to growth on pine could no doubt be developed but not without impeding other aspects of the proposed research.

NUTRITIONAL STUDIES

The minimum nutritional requirements of C. prunosum (M-10) for growth were previously determined (see Second Quarterly Report) using 1% glucose and a supplemented mineral medium (SM). However, supplying mineral nutrients (mainly nitrogen) to a bioreactor operating on a high solid to liquid ratio (greater than 10% solids) in amounts adequate to support growth and delignification could pose a problem if the required amount of mineral be greater than its solubility in water or if the high salt concentrations inhibit growth. To resolve the issue, we examined the growth characteristics of M-10 in 1% glucose in the presence of excess nutrient salts. Growth inhibition occurs above concentrations of about 2.5 times the minimum nutritional requirement level (see Second Quarterly and Bimonthly Report).

Further work on the nutritional salt tolerances of C. prunosum (M-10) demonstrated that the major supplemented mineral (SM) constituents (KH_2PO_4 , Na_2HPO_4 , and $(\text{NH}_4)_2\text{SO}_4$) are responsible for the observed growth inhibition above the 2.5 excess level (Table I). The concentration of the carbon source (1.0% glucose) was kept constant while various ingredients were added in five-fold

excess concentration with remaining SM components constant at 1x. Dry weight cellular mass yields after three days incubation at 38°C in shake flasks were used as the measure of growth inhibition.

TABLE I C. Pruinosum Nutritional Salt Tolerance

<u>SM Component(s) at 5x</u>	<u>Dry Weight (mg)</u>
$\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	8.4
$(\text{NH}_4)_2\text{SO}_4$	3.6
$(\text{NH}_4)_2\text{SO}_4 + \text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	3.6
Trace Elements	24.2
Thiamine-HCl	22.3
Control (all 1x)	15.7

The inorganic nitrogen source $(\text{NH}_4)_2\text{SO}_4$ appears to have the most deleterious effect at the 5x level. Experiments in progress which compare the effect of added yeast extract and/or peptone on M-10 growth on maple suggest that the latter is more effective. Taken together, these results indicate that substitution of part of the inorganic nitrogen with an organic nitrogen source may reduce the observed growth inhibition. The strong stimulatory effect of both the trace elements and thiamine-HCl is noteworthy and encouraging.

Additional experiments are planned to optimize the growth stimulation attained at the higher concentrations of trace elements and thiamine-HCl. Various combinations of $(\text{NH}_4)_2\text{SO}_4$ and organic nitrogen will also be optimized for maximum cell mass yield.

If one assumes a 20 to 30% sawdust fiber feed concentration in the biodelignification reactor and also that no more than 25% of the available sugar (50% of sawdust) will be consumed for growth and delignification, then the total amount of sugar utilized will be on the order of 2.5 g/100 ml of feed (20 grams fiber x 0.5 cellulose x 0.25 = 2.5 g). The 1x level of nutrient concentration is adequate to support M-10 growth on glucose. Since the minimum required nutrient concentration is consistent with maximum expected sugar utilization, we may anticipate that even a two-fold excess nutrient feed concentration may be employed in the bioreactor without significant effect on the growth of M-10.

MUTATION PROTOCOL

Development work on an effective mutation protocol for C. pruinosum has been initiated in anticipation of the desirability of isolating M-10 mutants with enhanced enzyme production or activity and/or which are unable to utilize cellulose-derived soluble sugars. The objective of the current experiments is to select plating conditions which reproducibly yield 50 - 100 colonies per plate. Oxgall and Rose Bengal, which are known hyphal inhibitors, are being investigated for their ability to restrict the spreading plate growth of C. pruinosum.

Adjusting the inoculum level and the concentration of one or both of these agents has proved difficult. At a given inoculum size, reproducible plate growth is difficult to achieve with either inhibitor. In order to obtain clearing zones and discrete colonies, we have investigated several experimental parameters; agar/cellulose monolayers vs bi-layers; microcrystalline, attrited microcrystalline, and acid-swollen cellulose substrates; various Oxgall and Rose Bengal concentrations; and inoculum size. Zones of clearance, due to the presence of cellulase, on monolayer plates using

5% attrited microcrystalline cellulose as the carbon source have been clearly produced with both inhibitory agents. Phosfon D, which enhances cellulase detection, was used once but requires further work. At high inhibitor concentrations, ($> 0.5\%$ Oxgall and $> 5.0 \text{ mg\%}$ Rose Bengal) no M-10 growth was observed. At lower inhibitor concentrations, variation of the inoculum size from 10^{-1} to 10^{-6} dilutions of a standardized inoculum resulted in, respectively, the appearance of excessive colony plate counts or single nondiscrete colony formation with no hyphal inhibition.

The following procedure was used to standardize the inoculum of C. pruinosa. A PDA plate (Potato Dextrose Agar) was inoculated in the form of a spread plate with C. pruinosa, incubated at 38°C for eight to ten days. The spores were harvested from the plate with 10 ml sterile distilled water by rubbing the surface gently with a glass spreading rod. The 10 ml of spore concentrate was vortexed in a tube containing sterile glass beads (4 mm) to break up any clumps. The spore concentrate usually read 1% LT @ 525 nm on a B & L Spectronic 20. A 10^{-1} dilution was always adjusted to read 45-50% LT; this inoculum was considered standardized for experiments. One tenth ml of the spore concentrate was used to inoculate another PDA plate for future inoculum.

At this point, it would appear that Oxgall should be used at 0.025% or 0.05% and Rose Bengal at 2.5 mg% or 5.0 mg%, while the optimum inoculum concentration has to be confirmed.

GROWTH AND DELIGNIFICATION

Previous attempts to reproducibly grow M-10 in high solid moist environments were successful only on petri dishes which contained an agar mineral medium overlaid with lignocellulosic fibers supported on a porous membrane to facilitate

the upward diffusion of nutrients and quantitative recovery of cell mass and substrate. We have recently succeeded in culturing C. pruinosa in moist flasks containing only SM and maple sawdust fibers. These encouraging results indicate that we can now scale our experiments up from petri dish to flasks and undertake the quantitative evaluation of cellulose accessibility vs lignin removal as a function of various growth and elevated temperature shift conditions for optimal lignin solubilization.

BIOLOGICAL REACTOR

Solid State Bioreactor Design and Construction

The proposed all biological ethanol-from-wood fermentation process relies heavily upon the effectiveness of the first stage biological pretreatment unit operation to solubilize sufficient lignin to make the residual cellulose readily available for enzymatic saccharification. In the design and construction of a high solids or solid state bioreactor, the most crucial design parameters are the nutrient, air, and moisture distribution systems. Previous studies with C. pruinosum have demonstrated that the ability of this organism to grow and solubilize lignin is particularly dependent upon the moisture content of the environment and the nutrient supply rate. In view of these considerations and the need to handle a concentrated slurry feed, we elected to employ a screw conveyor with cut flights on the shaft for the continuous biological pretreatment unit operation (Figure 2). The conveyor cut flights will provide slow substrate mixing, while the mist spray and pressurized air inlet controls distribute and maintain optimum moisture and oxygen levels. The C. pruinosum nutrient salt tolerance studies reported in the first section of this report indicate that nutrient concentrations two times higher than required for growth can be added to the bioreactor without significant deleterious effect on growth. Accordingly, we are proceeding with the construction of a 3 foot by 6 inch ID screw conveyor with cut and folded flights housed in a tubular trough. Test and calibration experiments will be performed using 20% avicel and sawdust slurries. The initial experiments will be conducted using a 1.5-fold excess nutrient concentration. The residence or retention time will be controlled by the shaft rotation speed. Further

adjustments in the bioreactor design will be made on the basis of results achieved in the flask studies of *C. pruinosa* growth and delignification on maple sawdust.

FIGURE 2: Screw Conveyor with Cut Flights on the Shaft for Continuous High Solids Biological Pretreatment

MIXED CULTURE MICROBIOLOGY

The mixed culture fermentation of cellulose to ethanol is conducted by employing a thermophilic sporocytophaga for rapidly saccharifying cellulose to soluble sugars which are readily converted by a compatible thermophilic bacillus co-culture into ethanol. Mixed culture compatibility is favored primarily by the dependence of each culture upon growth factors supplied by the other and by the more rapid growth of the bacillus. Differences in the response of each culture to initial and altered pH, temperature, oxygen tension, and agitation rate will be used to control the growth rate of both cultures and the rates of saccharification and ethanol production during the fermentation.

Mixed-culture cellulose fermentation studies are proceeding on schedule; however, defining the growth requirements and product yields of sporocytophaga have proven more difficult than originally anticipated. It now appears that sporocytophaga requires for growth some product derivable from the bacillus co-culture that is not found in common yeast extract, peptone, or casein preparations. The growth of sporocytophaga in mixed culture is always preceded by the initial development of modest bacillus growth; however, additions of sonicated or heat-killed bacilli are ineffective in initiating the growth of sporocytophaga. While we have not yet determined what function the bacillus performs in initiating the growth of sporocytophaga, it is very probable that the former supplies a specific nucleic acid or peptide or helps control the oxygen tension. The first possibility arises from the fact that mixed-culture growth proceeds quite well on a medium containing only mineral salts and cellulose as the only source of carbon and energy. The second possibility is based upon the mixed-culture growth characteristics

initial growth of the bacillus at the meniscus followed by growth of sporocytophaga at the bottom of the test tube). The effect of added specific growth factors and O_2 tension in the growth of sporocytophaga will be investigated in the next quarter.

CELLULOSE UTILIZATION AND ETHANOL PRODUCTION

Previous studies of mixed-culture fermentation of cellulose in test tubes showed some variability in ethanol yields. The thermophilic bacilli also displayed some variability in ethanol production when grown on soluble sugars. Controlling experimental parameters during test tube fermentation is admittedly difficult; however, the information gained is valuable in determining appropriate conditions for bench-top fermentations and in minimizing the number of required fermentor runs. Because of the complex interactions in mixed culture growth, it was decided to examine the effect of added yeast extract and solid calcium carbonate (primarily for pH control) on ethanol yields of cultures growing on cellulose, glucose, and cellobiose.

EXPERIMENTAL

In the first series of experiments, 10 ml of previously described mineral broth plus 0.25% yeast extract and a strip of Whatman filter paper (40 ± 2.5 mg) were inoculated with 0.2 ml of the sporocytophaga US and one of the two most promising thermophilic bacillus cultures NW and ZBB2. The mixed cultures were grown at $55^\circ C$ without shaking. After the filter paper disappeared, the total culture was assayed for residual carbohydrates (anthrone) and for ethanol (ethanol oxidase procedure). All samples were run in duplicate. The results are presented in Table II.

In a second series of experiments, we examined the rate of cellulose utilization

TABLE II

Ethanol Yield from Mixed Cultures
US and NW and ZBB2 Cellulose Fermentation

<u>Mixed Culture</u>	<u>Cellulose Added (mg)</u>	<u>Cellulose Found* (mg)</u>	<u>Cellulose Utilized* (mg)</u>	<u>Ethanol Found* (mg)</u>	<u>%** Yield</u>
US+NW-CaCO ₃	40	AV 9.36	30.64	1.30	9.2
+CaCO ₃	40	13.60	26.40	6.85	56.4
US+ZBB2-CaCO ₃	40	24.70	15.30	1.75	24.0
+CaCO ₃	40	9.45	30.55	6.30	44.8

* average of duplicate runs

** % yield = $\frac{\text{mg ethanol}}{0.46 \times \text{cellulose utilized}}$

0.46 = maximum yield of ethanol from glucose by yeast fermentation

and ethanol production of a mixed culture growing on avicel in the presence of excess calcium carbonate. The carbonate serves as an insoluble buffer and carbon dioxide source which may be required to stimulate the growth of US.

The mixed culture US + NW was grown in mineral broth plus 0.25% yeast extract in Roux bottles and test tubes as previously described. Avicel (90 μ from FMC) was used as a source of microcrystalline cellulose. Calcium carbonate powder was sterilized separately or in water suspension and added to the mineral broth after sterilization. Heat sterilization of calcium carbonate in the presence of phosphate buffer promotes the production of calcium phosphate and potassium carbonate with a final pH greater than 10.0.

Roux bottles were inoculated with a 50% inoculum of a rapidly growing mixed culture from another Roux bottle. After growth for 5-20 hours, 8 ml aliquots were transferred to test tubes so that growth and avicel loss could be monitored. The test tubes were mixed at hourly intervals and samples taken at the time indicated. The remaining cellulose was determined gravimetrically after rupture of the cells by treatment with 8% formic acid for 20 hours at 55°C. The cellulose was collected on 0.45 micron Millipore filter, washed with water, and dried at 55°C to constant weight. The supernatants from the centrifuged test tubes were assayed for ethanol using the ethanol oxidase method. The results are presented in Table III and plotted in Figure 3.

The influence of added excess calcium carbonate was also examined on the two bacillus cultures growing on varying concentrations of glucose and cellobiose. Test tubes containing 10 ml of mineral broth plus 0.25% yeast extract and 0.5 or 1.0% glucose or cellobiose with and without 100 mg of excess solid calcium carbonate were inoculated with the thermophilic bacilli cultures NW and ZBB2. The cultures

TABLE III

Cellulose Utilization and Ethanol Production
by Mixed Culture US + NW

<u>Time Hours</u>	<u>Cellulose Found g/l</u>	<u>Ethanol Found* mg/l</u>
0	20.4	0
1.0	18.0	29
2.0	16.8	104
3.5	13.9	181
5.0	11.6	327
6.5	-----	341
8.0	8.3	641
10.5	8.0	768
12.0	-----	787

*Average of duplicate runs

were grown at 55°C for 23 hours with occasional shaking. Turbidity measurements taken at 17, 19, and 23 hours provided a measure of when culture growth stopped. The initial pH of the broth was 7.4. After growth, the culture was centrifuged. The supernatants were assayed for ethanol and residual sugar concentrations. The results are presented in Table IV.

The effect of calcium carbonate and yeast extract additions on the fermentation products produced by the thermophilic bacillus ZBB2 growing on cellobiose was also examined in test tube cultures. The culture was grown in 10 ml of mineral broth, previously described, which contained 1% cellobiose. Calcium carbonate (100 mg) was added to one set of test tubes for pH control. The cultures were grown at 55°C for two days with occasional hand shaking and assayed for ethanol, lactic, and acetic acids by gas chromatography. The results are given in Table V.

RESULTS AND DISCUSSION

Sporocytophaga had previously been shown (see Second Quarterly Report) to grow very poorly at a pH below 7.0 and in the presence of phosphate buffer concentrations which exceeded 0.06 molar. Since high buffer salt concentrations and low pH arising from the accumulation of acid end-products limit growth and cellulose utilization in test tubes, solid calcium carbonate additions offered a means of retarding the pH drop without adding excess soluble buffer. The mixed culture cellulose fermentation results presented in Table II indicate that carbonate addition substantially enhanced the cellulose utilization and ethanol production of the mixed culture US + ZBB2. With the mixed culture US + NW, only the yield of ethanol was enhanced.

Figure 3 graphically presents the data given in Table III on the rate of mixed culture cellulose utilization and ethanol production. After eight hours, very

TABLE IV

Ethanol Production from Glucose (G)
and Cellobiose (B) by Thermophilic Bacillus

CaCO_3 (mg)	Sugar		Ethanol Found (mg)	% Yield
	Added (mg)	Found (mg)		
0	5 G	0.84	.56	40.7
0	10 G	4.22	.44	24.4
0	5 B	2.01	.60	30.6
0	10 B	4.92	.56	21.1
200	5G	0.24	1.00	43.9
200	10G	3.61	1.24	42.2
200	5B	0.14	1.00	32.9
200	10B	0.77	1.40	32.9

TABLE V

Fermentation Products of ZBB2 Grown on Cellobiose (1%)

CaCO_3 (mg)	Yeast Extract	Product Concentration (mg/ml)		
		Ethanol	Acetic Acid	Lactic Acid
0	0.25%	0.63	0.99	0
0	0.50%	0.63	0.99	0.26
200	0.25%	0.74	2.60	0.48
200	0.50%	1.37	1.61	0.98

little ethanol was produced. In spite of the presence of calcium carbonate and hourly mixing, there was a gradual decline in pH. It is possible, therefore, that the low pH or end-product formation inhibited further ethanol production after eight hours. The unexpected low yield of ethanol compared to those found earlier is difficult to explain. Experiments are planned to investigate the possibility that ethanol is oxidized to acetic acid in the latter stage of fermentation.

Cellulose utilization, on the other hand, proceeded at a very high rate (specific rate 0.12 hr^{-1}) amounting to a volumetric efficiency of 2.4 g/l-hr .

The effect of calcium carbonate additions to the thermophilic bacilli NW and ZBB2 growing on cellobiose and glucose is small at 0.5% sugar but large at sugar concentrations of 1%. These results are most easily interpreted as indicating that at the higher sugar concentrations, the pH drop or metabolite end-product formation inhibits growth and ethanol production earlier than that at the lower substrate concentration.

The relative proportions of ethanol, acetic, and lactic acids produced by the bacillus ZBB2 growing on cellobiose vary with the concentration of added yeast extract broth in the presence or absence of added calcium carbonate (Table V). A material balance was not possible because CO_2 and other possible products, such as hydrogen and butyric acid, were not determined. However, no odor of butyric acid was noticed. Under the best conditions examined to date, ZBB2 produces about 1 mole of acid for each mole of ethanol.

The observed rates of cellulose utilization, while rate limiting, can probably be improved by pH, and dissolved oxygen and temperature optimization, which are not subject to control in test tube experiments. The observed variable yields of ethanol probably arise in part due to variations in experimental parameters which

are difficult to control in test tube fermentations. In addition to retarding pH decline, calcium carbonate may stimulate growth by providing an increased CO₂ tension. Attaining ethanol yields beyond those observed will require the development of mutant strains with reduced ability to produce acidic end-products.

BENCH-TOP FERMENTATION

Bench top fermentations at the 1-liter scale are employed as a means of verifying, under controlled pH, temperature agitation and dissolved oxygen conditions the results achieved in small scale test tube experiments. In this section, we review the results achieved when a mixed culture of sporocytophaga (US) and the thermophilic bacillus (NW) are grown on microcrystalline and amorphous cellulose and compared to a fermentor run with NW grown on glucose.

EXPERIMENTAL

The fermentations were conducted in 1-liter flasks at a constant pH of 7.4 and a temperature of 55°C. The desired amount of substrate was suspended in 800 ml of basal medium and sterilized in the fermentation flask for 15 minutes at 125°C. In the case of glucose, the substrate was sterilized separately. The fermentor was changed with a 20% inoculum which was grown in the same medium as in the fermentor. The pH was maintained at 7.4 by the continuous addition of 3N. NaOH solution, by means of a peristaltic pump, regulated by an automatic pH controller. Anaerobic conditions were maintained by continuously passing nitrogen gas above the medium. As the fermentation progressed, aliquots were withdrawn aseptically and analyzed for cellulose, protein, soluble sugar, ethanol, and volatile acids.

MATERIALS

Amorphous cellulose was prepared according to Walseth.⁽¹⁾ Microcrystalline cellulose (PH 105, 19 µ) was dissolved in cold 0.85% H_3PO_4 and then reprecipitated in cold water. The precipitated swollen cellulose was continuously washed with

¹C. Walseth, TAPPI, 35, 228 (1952)

distilled water until the wash water pH was neutral. The swollen cellulose was resuspended in distilled water and stored in the refrigerator. The swollen cellulose was used directly in the fermentor runs without drying.

Growth Medium Composition per 1000 ml:

$(\text{NH}_4)_2\text{SO}_4$	5.0g
NaCl	1.0g
KH_2PO_4	0.9g
K_2HPO_4	10.4g
Yeast Extract	2.0g

Trace Metals:

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g
FeSO_4	20.0 mg
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	20.0 mg
CaCl_2	20.0 mg
ZnSO_4	8.0 mg
Versenol	0.2g

In the case of swollen cellulose, the concentration of cellulose was 6g/liter of distilled water.

RESULTS AND DISCUSSION

Growth of NW on Glucose: The kinetic profile of NW growth, glucose uptake, and ethanol and acetic acid production are shown in Figure 4. The fermentor was inoculated with a 1% suspension of an 18 hour old culture. The long lag time was probably due to the use of a stationary phase inoculum. The specific growth rate was calculated to be 0.59 hr^{-1} (generation time 70 minutes) based upon turbidity

GROWTH OF THERMOPHILIC BACILLI ON 1% GLUCOSE

AT pH 7.4 • 55°C

FIGURE 4

measurements at 615 mμ. Ethanol and acetic acid were the major metabolites produced during the fermentation. The production of ethanol and acetic acid paralleled the increase in cell mass. The rate of ethanol production was slower than the rate of acetic acid production and reached a maximum value at the same time as maximum cell density was attained. However, acetic acid production was found to increase even after the cessation of culture growth.

Mixed Culture Fermentation on Amorphous Cellulose: The specific substrate depletion rate of US-NW growing on amorphous cellulose (Figure 5) was calculated to be 0.087 hr^{-1} . Soluble sugar accumulation did not occur during the fermentation and its relative concentration remained small. As with the NW glucose fermentation, ethanol and acetic acids were the primary metabolic products. Again, acetic acid production was faster than ethanol production and the former continued after ethanol production ceased. The yield of ethanol was about 24% of theoretical.

Mixed Culture Growth on Microcrystalline Cellulose: The specific substrate depletion rate of US-NW growing on microcrystalline cellulose (Figure 6) was calculated to be 0.0346 hr^{-1} . No soluble sugar accumulation occurred during fermentation, as observed in the two previous fermentor runs. Acetic acid production was faster and persisted longer than ethanol production. However, 2,3 butanediol was found to be an additional metabolite, occurring in yields and rates of production which were comparable to ethanol. Ethanol appeared earlier than 2,3 butanediol; however, the observed yield of ethanol was below that (14-19%) found during growth on swollen cellulose. Extracellular cellulase was observed in the broth; however, for meaningful assessments, the substrate-bound activity (which was not measured in the fermentor run) must also be taken into account.

MIXED CULTURE FERMENTATION OF AMORPHOUS

CELLULOSE (SWOLLEN CELLULOSE) AT

pH 7.4 • 55°C

FIGURE 5

MIXED CULTURE FERMENTATION OF MICROCRYSTALLINE CELLULOSE

AT pH 7.4 • 55°C

Figure 6

FERMENTATION BROTH GAS LIQUID CHROMATOGRAM

FIGURE 7

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TEMP1      115
TIME1      5.3
RATE       14.00
TEMP2      10
TIME2      7.3
INJ TEMP   250  250
FID TEMP   300  300
AUX TEMP   300  300
OVEN MAX   300
  
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CHT SPD     5.50
ATTN 21     10
FID SGNL    A
SLP SENS     1.50
AREA REJ    100
FLOW A       89
FLOW B       34
OPTN        0
  
```

The ethanol production and cell growth from NW growing on glucose fell off even when substantial glucose was still present. Since glucose starvation is unlikely under these conditions, the most likely interpretation is either ethanol or acetic acid inhibition. Since acetic acid production persisted after ethanol production stopped, NW may in the vegetative state convert ethanol to acetic acid for supplying maintenance energy. However, growth factor depletion normally supplied by US in mixed-culture growth cannot be entirely ruled out. Mixed-culture growth on amorphous cellulose displayed similar ethanol yields; however, the substrate depletion rate was lower, and the ratio of ethanol to acetic acid fell from 0.9 to 0.6 when compared to the NW glucose fermentation. Unlike the latter case, glucose levels relative to cell mass were low when log phase cell growth stopped, which suggests that glucose starvation arrested growth. Mixed-culture growth on microcrystalline cellulose displayed still lower yields of ethanol, and the ethanol to acetic acid ratio dropped to 0.3. Soluble sugar concentration remained negligible throughout the fermentation. The appearance of 2,3 butanediol (Figure 7) toward the latter stage of fermentation was unexpected; however, since the fermentation time is considerably longer on microcrystalline cellulose, either a change in culture physiology leading to a different mix of products or a contaminant could explain the appearance of this product. The change in metabolic pathway with culture age is the more likely explanation if the 2,3 butanediol pathway is more energy conserving. Ethanol, acetic acid, and 2,3 butanediol account for about 85% of the cellulose utilized assuming the production of 50% CO_2 . If lactic acid is produced as observed in the test tube experiments, its concentration in the 1-liter fermentor runs is negligible.

SUMMARY AND FUTURE WORK

The observed ethanol yield and rate of production vary with the carbon substrate as does the molar ratio of ethanol and acetic acid. The relatively slow rate of microcrystalline cellulose degradation appears limited by the accessibility of this substrate to enzymatic saccharification, which may in turn lead to an alteration in the culture physiology and to the production of 2,3 butanediol, at the expense of ethanol. The formation of acetic acid from ethanol by resting bacilli cells may also be occurring in the later stage of fermentation. Ethanol yields in relatively uncontrolled test tube fermentations, are higher than observed in 1-liter pH controlled fermentations suggesting that other parameters such as agitation, dissolved oxygen, and CO_2 tension may also influence the yield of ethanol.

In order to understand and improve the ethanol yield in bench top fermentation, we plan to study the continuous fermentation of thermophilic bacilli growing on soluble sugar at different pH and glucose concentrations. These studies will help clarify the biochemical reactions occurring during the various stages of culture growth.

A semi-continuous mixed culture fermentation of microcrystalline cellulose will be studied as a function of pH, dissolved oxygen, and CO_2 concentration and stirring rate. In addition to delineating the effect of these variables upon ethanol production and cellulose degradation, these studies should pinpoint the conditions which lead to 2,3 butanediol production.

GENETIC IMPROVEMENT OF THERMOPHILIC BACILLUS ETHANOL PRODUCTION

While the observed rate of ethanol production by thermophilic bacilli appears superior to conventional yeast ethanol fermentation rates, the ethanol yields are not comparable. The co-production of acetic acid by the thermophilic bacillus is currently limiting the attainable ethanol yield to about 50% of that achieved by commercial yeast homofermentors. We have initiated a mutation project design to isolate a thermophilic bacillus which converts glucose and cellobiose into ethanol and carbon dioxide. For our initial experiments, we have chosen N-methyl N¹nitro N-nitrogenoguanidine (MNNG) as the mutagenic agent.

MATERIALS AND METHODS

The thermophilic bacillus NW was grown for 16 hours at 55°C in mineral broth containing 0.5% yeast extract and 0.5% cellobiose. Test tubes were inoculated with 0.1 ml of a 1/100 dilution of a 24 hour culture grown on the same medium. The cells were centrifuged, washed with one volume of M/15 phosphate buffer and resuspended in an equal volume of the same buffer. The mutagenic agent MNNG was added to test tubes containing 10 ml of a suspension of NW cells in concentrations of 100, 250, 500, 750, and 1000 µg per test tube. The tubes were incubated at 55°C for 30 minutes with shaking. After incubation, the tubes were diluted and streaked on mineral agar plates containing 0.5% yeast extract and 0.5% cellobiose.

RESULTS AND DISCUSSION

Figure 8 shows the killing curve obtained with the mutagenic agent MNNG. From these results, we concluded that a dose of 65 mg/l for 30 minutes at ^{55°C} is adequate

SURVIVAL CURVE OF THERMOPHILIC BACILLUS NW

FIGURE 8

to obtain a 99% kill. This dose will be employed in the future mutation studies.

FUTURE WORK

The initial screen for mutants with higher ethanol production will be made on the basis of decreased acid production. Indicator plates containing phenol red will be used to distinguish those mutant colonies with modest or negligible acid production. Such colonies will then be transferred to a mineral broth medium containing yeast extract and cellobiose and analyzed for ethanol yields.