DEPARTMENTAL PAPER 58

INHIBITION OF *Cellulomonas* sp. BY HEAT-TREATED SUGARCANE BAGASSE AND RICE STRAW

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ABSTRACT

High-temperature/high-pressure (400 psi for 20 to 90 seconds) treatment of sugarcane bagasse and rice straw produces substances inhibitory to the growth of *Cellulomonas*. These growth-inhibiting substances were found to be pH-dependent; that is, the lower the pH, the greater the inhibition. Addition of 4 percent NaOH or NH_4OH can reduce the production at high-heat treatment. The chemicals K_2HPO_4, Na_2HPO_4, $KHCO_3$, and $NaHCO_3$ were found to promote the growth of *Cellulomonas* by neutralizing the inhibition.

Key words: sugarcane bagasse, rice straw, *Cellulomonas*, growth inhibitors, high-heat treatment.

ACKNOWLEDGMENTS

We wish to thank our colleagues at the Western Regional Research Laboratory, Albany, California, for the sample of high-pressure-treated bagasse.

We are also indebted to Steven E. Olbrich and Elgin B. Hundtoft of the College of Tropical Agriculture and Human Resources, University of Hawaii, for assistance in the preparation of samples.

Thanks also to the Hawaiian Sugar Planters' Association for making samples of sugarcane bagasse available to us.

This project was supported by USDA-CSRS Grant No. 116-15-12.

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Chia-hsiung Chi[1] and T. O. M. Nakayama[2]

INTRODUCTION

Sugarcane bagasse, cane trash, and rice straw are low-value, high-bulk agricultural wastes that have been the subject of numerous attempts at utilization. One such attempt is their utilization as animal feed, either as a carbohydrate source or as substrate upon which to grow microorganisms (*8*), which then increases the protein content of the feed. The intractible nature of these residues in their native form requires that they be pretreated in various ways to increase their digestibility (*8, 10*).

An *in vitro* method of assaying the results of pretreatment was to determine the disappearance of insoluble dry matter after treatment with cellulolytic and proteolytic enzymes, which can then be expressed as "total solubles after enzymes" (TSAE) (*9*). To measure the effects of different heat treatments on bagasse and rice straw, a method approximating that of ruminants using an artificial rumen for measuring dry matter digestibility was used (*15*). Bagasse responds dramatically when treated with high-pressure steam at 400 psig for short periods and the *in vitro* digestibility is increased from 15 to 55 percent; however, attempts to use such treated material directly as animal feed have been disappointing, as there was very little weight gain when either bagasse or rice straw comprised 50 percent of the ration (*4, 7, 12*). Attempts to use this bagasse as substrate upon which to grow organisms led to the suspicion that substances inhibitory to microorganisms were present (*16*). Such a situation would explain the poor animal showing as well as the poor growth under certain conditions in the laboratory. The work reported here is an attempt to characterize some aspects of the inhibition upon a strain of *Cellulomonas.*

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MATERIALS AND METHODS

THE MICROORGANISM

The cellulose-utilizing bacterium *Cellulomonas* sp. ATCC 2/399, which was isolated from the soil of sugarcane fields by Han and Srinivasan (*11*), was used as the testing organism. It was cultured on a trypticase soy agar (TSA) (BBL) slant at 37°C for 24 hours and then stored at 5°C until used.

THE MEDIA

When tests were conducted, *Cellulomonas* was transferred to trypticase soy broth (TSB) and incubated at 37°C for 24 hours. This culture was kept at 5°C until used. The TSB (Difco) has the following composition: tryptone, 1.7 percent; soytone, 0.3 percent; dextrose, 0.25 percent; sodium chloride, 0.5 percent; and dipotassium phosphate, 0.25 percent.

MICROBIOLOGICAL ASSAYS

Methods to assess antimicrobial substances present in foods were investigated (*1, 2, 3, 5, 6, 13, 14, 17, 19*). Two of these methods were applied in this research.

Inhibition zone method. Neeman et al. (*14*) and Vedamuthu et al. (*19*) used a disc-assay technique to demonstrate the presence of inhibition. Their technique was modified by pressing a 2-g sample into a circular disc. The pellets were placed on the TSA plate inoculated with *Cellulomonas*. The inoculated plate was then incubated at 37°C for 24 hours. The width of the inhibition zone around the pellets, in millimeters, was used as an index of inhibition.

Turbidity method. The method used by Rosen et al. (*17*) and Teuber (*18*) was also applied in this research. Inhibition of growth was measured by observing changes in absorbance. Treated or nontreated sugarcane bagasse and rice straw were soaked in cold water statically for 24 hours, then filtered through Whatman No. 2 filter paper. The filtrate was autoclaved. A definite amount of TSB, sterilized cold water extract of sample, and *Cellulomonas* culture was measured and incubated at 37°C for 24 hours. Amount of growth was measured photometrically at 600 nm (Spectronic 20 colorimeter, Bausch and Lomb, Inc.), and the results were expressed as a percentage of inhibition. The relationship of cell concentration to absorbance was established as follows: a weighed amount of *Cellulomonas* cells that had been washed twice was resuspended in 10 ml cold water extract; absorbance at 600 nm was then used to measure the cell concentration on the series of half dilutions.

Five milliliters TSB were added to varying amounts (1 to 5 ml) of water extract of heated (400 psi, 60 seconds) rice straw. Sterile distilled water was added to bring the volume to 10 ml. The control tube contained 5 ml TSB and 5 ml sterile distilled water. The tubes were inoculated with 0.5

ml of the same concentration of *Cellulomonas* and incubated at 37°C for a total of 48 hours. Measurements were made at 24 and 48 hours. Percentage of inhibition was calculated from the absorbance at 600 nm.

In order to determine what effect the length of the cold water extraction had on the inhibition, samples of heated (400 psi, 60 seconds) rice straw were soaked statically in distilled water for 24, 48, and 72 hours. The inhibitive action of the water extracts was then determined as before.

TYPE OF INHIBITION

In order to learn the nature of the inhibitions, tubes containing constant amounts of TSB and water extract of heated rice straw (400 psi, for 60 seconds) were inoculated with various dilutions of *Cellulomonas* sp. The tubes were then incubated at 37°C for various periods, and their cell concentrations were determined by absorbance at 600 nm. The amount of absorbance was used to determine the type of inhibition.

Rosen et al. (*17*) reported that the inhibition of *Bacillus megaterium* by a trimethylamine oxide-associated browning reaction product was expressed primarily as an increase in the lag phase of growth. Attempts were made to compare the growth curve of *Cellulomonas* sp. with and without inhibitors in the medium. To the culture tube without inhibitors were added 5 ml TSB, 5 ml sterilized distilled water, and 0.5 ml *Cellulomonas* culture. The tube with inhibitors received 5 ml TSB, 2 ml water extract of heated (400 psi, 60 seconds) rice straw (which was obtained from 0.025 g sample per milliliter of water extract), 3 ml sterilized distilled water, and 0.5 ml *Cellulomonas* culture. Samples were incubated at 37°C. Growth measurements by absorbance at 600 nm were taken at 2-hour intervals for 24 hours.

COUNTERACTING THE INHIBITION BY COMPONENTS OF TSB

Morris and Williams (*12*) reported that inhibition of the growth of *Lactobacillus bulgaricus* by purine deoxyribonucleotides can be reversed by liver extract, thymidylic acid, and purine ribonucleotides. Calcium, iron, and magnesium were used by Ashton and Busta (*1*) to overcome the inhibition of *Bacillus stearothermophilus* by milk components. Similarly, various components of the TSB were added to determine whether they could counteract the inhibition.

EFFECTS OF CHEMICALS IN COUNTERACTING THE INHIBITION

Since it was known that K_2HPO_4 could counteract the inhibition, various chemicals were tried to find out if they could reverse the inhibition. A sample using 0.2 g heated rice straw (400 psi, 60 seconds) and 0.05 g of the chemical was mixed and pressed into a pellet, then placed on a TSA plate inoculated with *Cellulomonas* sp. Sterile distilled water (0.05 ml) was added to the pellet to facilitate diffusion of the chemical. The plates were incubated at 37°C for 24 hours and observed for production of inhibition. If the chemical did counteract the inhibition, no clear zone around the pellet would be observed.

FURTHER OBSERVATION ON CHEMICAL COUNTERACTION OF INHIBITION BY THE TURBIDITY METHOD

The effectiveness of those chemicals that counteract the inhibition was further examined by the turbidity method. $KHCO_3$, $NaHCO_3$, K_2HPO_4, and Na_2HPO_4 were added individually into each tube to test their effects in counteracting the inhibition. Growth was measured by absorbance after a 24-hour incubation at 37°C. The pH (Digicord pH meter) was measured before and after incubation.

OPTIMUM pH RANGE OF *Cellulomonas*

In order to clarify the relationship of the pH changes to inhibition, the optimum pH range for the growth of *Cellulomonas* sp. was determined. Various concentrations of sodium phosphate buffer at pH 7.0 were used to determine the optimum concentration as measured by growth. After it was found that 0.1 *M* gave the best results, various pHs of the broth were prepared by adding a dehydrated TSB medium to the buffer. Because of the presence of buffering components (K_2HPO_4, tryptone, and soytone) in the TSB medium, the final pH of the prepared TSB was determined after it was autoclaved. Each tube containing 10 ml of the particular pH of TSB and 0.5 ml of culture was incubated at 37°C for 24 hours. Growth was measured by absorbance at 600 nm.

CHANGES OF pH WITH TIME

After determining that the buffer could counteract the inhibition and knowing the optimum pH range of *Cellulomonas,* attempts were made to ascertain any difference in pH under the two conditions; that is, if the pH in the inhibited medium differed from that in the control during certain growth periods. The pH was taken at the 8th, 16th, and 20th hours of the growth curve.

COMPARISON OF AMOUNTS OF INHIBITION PRODUCED BY DIFFERENT PRETREATMENTS

Samples treated were tested for the amount of inhibition produced using turbidity methods. Sugarcane field trash, treated at high temperature and pressure with and without NH_4OH added, was used. Procedures for measurement were the same as those described previously in the section on inhibition by turbidity.

RESULTS AND DISCUSSION

There was no inhibition zone observed in the untreated sugarcane bagasse and rice straw. On the other hand, an

Table 1. Inhibition on TSA plate

Sample	Treatment	Width of inhibition zone[a] (mm)
Bagasse	Without heat treatment	–[b]
Bagasse	400 psi for 45 seconds before rotary dryer	4.25
Bagasse	400 psi for 45 seconds after rotary dryer	3.50
Rice straw	400 psi for 90 seconds	4.75
Rice straw	400 psi for 20 seconds	2.12
Rice straw	4% NaOH, 400 psi for 20 seconds	–

[a]Average width of inhibition zone after incubating the 0.2-g pellet at 37°C for 24 hours.
[b]No inhibition.

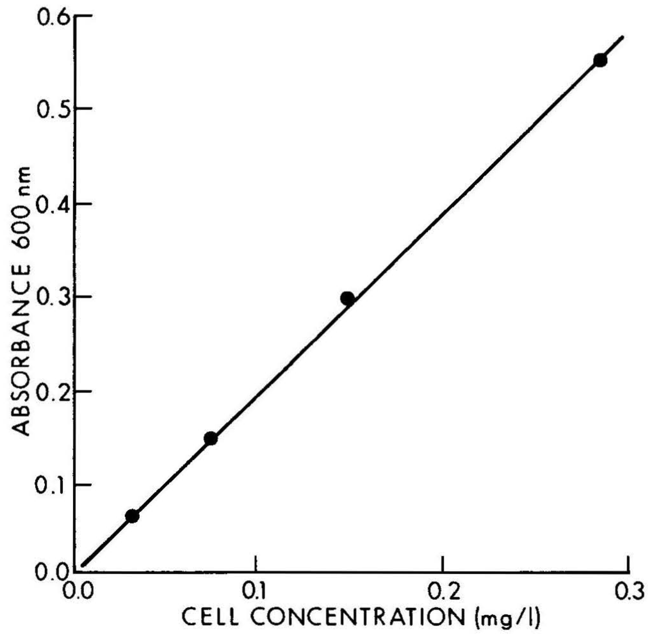

Figure 1. Cell concentration vs absorbance.

inhibition zone was obtained from the high-pressure, high-temperature (400 psi) heated samples, unless 4 percent NaOH was added during the heat treatment (Table 1). The turbidity method also showed that high-pressure treatment without NaOH will produce inhibition (Table 2). The presence of inhibitors may be used to explain the findings reported by Han and Callihan (*10*) that high-pressure cooking of bagasse alone had little or no effect on microbial growth unless pretreated with NaOH or NH_3.

It was found that a brown color present in the cold water extract did not interfere with the linear relationship of cell concentration to absorbance measurement (Figure 1). Thus, the turbidity method conformed to Beer's Law; hence, any increase in absorbance indicates that the cell concentration has increased. Figure 2 shows that the concentration of inhibiting substances present in a sample could be quantified by its proportional relationship with the percent of inhibition. It also indicates that after 24 hours there was no significant increase in cell growth. Furthermore, for convenience of operation, a static cold water extraction of 24 hours was found to be sufficient to extract most of the inhibiting substances (Figure 3).

Even though the cell concentration was diluted to only a few cells, microorganisms could still increase their concen-

Table 2. Inhibition of growth by turbidity measurement

Sample[a]	Increased absorbance at 600 nm	Inhibition (%)[b]
Control (without water extract)	0.43	
Bagasse without heat treatment	0.43	0
Bagasse, 400 psi for 45 seconds after rotary dryer	0.30	30
Rice straw without heat treatment	0.43	0
Rice straw, 400 psi for 60 seconds	0.25	42
Rice straw, 4% NaOH, 400 psi for 20 seconds	0.43	0

[a]Contents in tube: 5 ml trypticase soy broth, 3 ml sterilized distilled water, 0.5 ml *Cellulomonas* culture, and 2 ml sterilized cold water extract obtained from 0.025 g sample per ml of water extract. (This concentration is used throughout this research.)

$$^{b}\% \text{ inhibition} = \frac{(\text{increased absorbance of control} - \text{increased absorbance of sample}) \times 100}{\text{increased absorbance of control}}$$

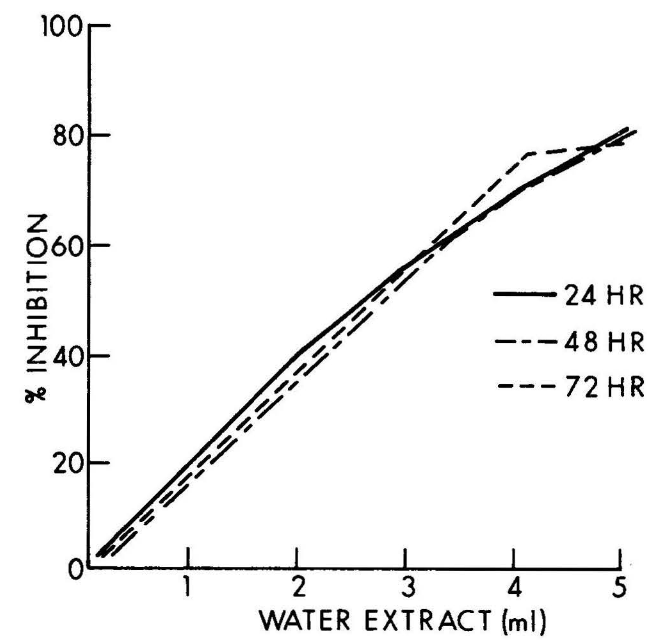

Figure 2. Effects of concentration of cold water extract and length of incubation upon inhibition.

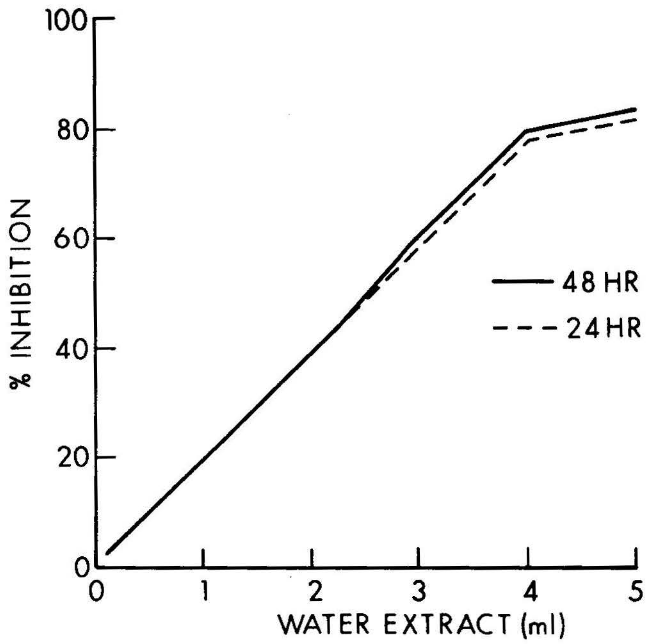

Figure 3. Effects of length of cold water extraction on inhibition.

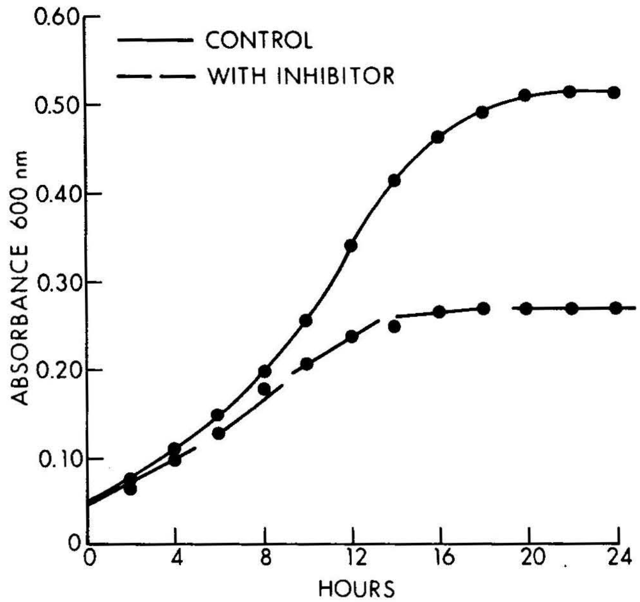

Figure 4. Effects of inhibition on the growth curve of *Cellulomonas*.

tration to the same level after 72 hours if the amount of TSB remained the same (Table 3). This means that the inhibition is a bacteriostatic type, rather than bacteriocidal. Extrapolation to the rumen would mean that the microorganisms, although not killed, could not reproduce normally. Therefore, cellulolytic activity would not be produced continuously for adequate digestion of the feed. This appears to explain the disappointing results of the animal tests.

The two growth curves in Figure 4 show that as growth proceeded there was a gradual decrease in its rate in the tube containing the water extract of heated rice straw. Also, the control has a later stationary phase than the water extract. These facts indicate that there are inhibiting substances in the water extract that increase the inhibition to approximately 45 percent. These results confirm the earlier experiments (see Figures 2 and 3).

As shown in Table 4, the inhibitory action can be neutralized by the addition of either peptone or K_2HPO_4 to the media. Because of the complex composition of peptone, only K_2HPO_4 and related salts were used in later investigations of the mechanism neutralizing the inhibition. Of the various related chemicals used, only K_2HPO_4, $KHCO_3$, and $NaHCO_3$ showed neutralization of the inhibition zone, as shown in Table 5. It was further confirmed by turbidity measurements that Na_2HPO_4, K_2HPO_4, $NaHCO_3$, and $KHCO_3$ could counteract inhibition (Table 6). These chemicals could also promote the growth of

Table 3. Bacteriostatic action of heated rice straw (400 psi, 60 sec)

Increased absorbance at 600 nm	Dilution of microorganisms											
	Control[a]	0[b]	1[c]	2	3	4	5[d]	6[e]	7[f]	8	9	10
Increased absorbance at 24 hr	0.47	0.22	0.23	0.18	0.13	0.07	0.02	0	0	0	0	0
Increased absorbance at 48 hr	0.49	0.24	0.27	0.27	0.27	0.27	0.26	0.24	0.19	0	0	0
Increased absorbance at 72 hr	0.49	0.24	0.27	0.27	0.27	0.27	0.26	0.25	0.24	0	0	0
Total % inhibition at 72 hr		47	40	40	40	40	42	44	47	0	0	0

[a]Tube contains 5 ml TSB + 5 ml sterilized distilled water + 0.5 ml original concentration of *Cellulomonas* culture.
[b]Tube contains 5 ml TSB + 3 ml sterilized distilled water + 2 ml sterilized water extract of heated rice straw + 0.5 ml original concentration of *Cellulomonas* culture.
[c]Tube contains 5 ml TSB + 3 ml sterilized distilled water + 2 ml sterilized water extract of heated rice straw + 0.5 ml 1/10 dilution of original concentration of *Cellulomonas* culture. The number 1 refers to 1/10 dilution of the original concentration.
[d]Average plate count in the 10^6 dilution is 53.5.
[e]Average plate count in the 10^7 dilution is 7.5.
[f]Average plate count in the 10^8 dilution is 0.5.

Table 4. Effect of components of trypticase soy broth (TSB) in counteracting the inhibition

	5 ml TSB 5 ml water 0.5 ml culture	5 ml TSB 2 ml extract[a] 3 ml water 0.5 ml culture	5 ml TSB 2 ml extract 2 ml water 1 ml peptone (17%) 0.5 ml culture	5 ml TSB 2 ml extract 2 ml water 1 ml glucose (3%) 0.5 ml culture	5 ml TSB 2 ml extract 2 ml water 1 ml NaCl (5%) 0.5 ml culture	5 ml TSB 2 ml extract 2 ml water 1 ml K_2HPO_4 (2.5%) 0.5 ml culture
Increased absorbance after 24 hr	0.49	0.27	0.42	0.27	0.32	0.48
Percent inhibition		45	14	45	35	2

[a]Water extract from heated rice straw (400 psi, 60 sec).

Table 5. Effect of chemicals in counteracting the inhibition

Chemicals	Control[a]	NaH_2PO_4	K_2HPO_4	KH_2PO_4	$KHCO_3$	$CaCl_2$	KCl	$NaHCO_3$	$Ca(H_2PO_4)_2$
Inhibition[b]	+	+	-	+	-	+	+	-	+

[a]No chemical added to 0.2 g heated rice straw pellet.
[b]+ means inhibition produced; - means no inhibition produced.

Cellulomonas; and all have buffering capacity. Thus, it was suspected that the inhibition is correlated with a change in pH.

In enriched TSB media with 0.1 *M* sodium phosphate buffer, a typical pH growth curve for *Cellulomonas* was obtained with optimum growth at pH 6.2 to 6.8 and decreasing growth at the acidic and basic sides of the curve (Figure 5).

Han and Srinivasan (*11*) reported a broader optimal pH range for *Cellulomonas* of 6 to 8. The difference may be due to the fact that they used a chemically defined medium and incubated at 30°C.

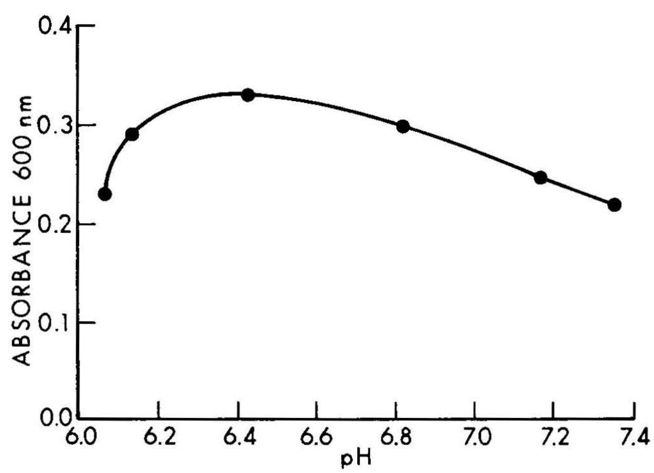

Figure 5. Optimum pH of *Cellulomonas* on TSB with 0.1*M* sodium phosphate buffer.

Although there was no difference in pH between control and inhibitors at the 16th and 20th hours, there was better growth in the former (Table 7). This may be used to explain that the inhibition is not solely due to the change of pH below the range in which *Cellulomonas* is able to grow. Therefore, it is postulated that the inhibition is pH-dependent—that is, the pH can cause modification of the structure of the inhibitors. The lower the pH, the more effective is the inhibition.

It was found that the higher the temperature and pressure of the heat treatment, the greater the amount of inhibitory products (Table 8). The results also indicated that NH_4OH can to a certain degree prevent the production of inhibition. With suitable controls of temperature and pressure and the addition of NH_4OH, the inhibition can probably be minimized. Moreover, NH_4OH can be used as a nitrogen source in the feed (*7*). Identification of these chemical inhibitors remains to be done.

CONCLUSION

High-temperature, high-pressure (400 psi for 20 to 90 seconds) treatment of sugarcane bagasse and rice straw produces substances inhibitory to the growth of *Cellulomonas.* These growth-inhibiting substances were found to be pH-dependent; that is, the lower the pH, the greater the inhibition. Addition of 4 percent NaOH or NH_4OH can reduce the production of inhibitors at high-heat treatment.

Table 6. Effect of buffering agents in counteracting the inhibition of heated rice straw (400 psi, 60 sec)

	5 ml TSB 5 ml water 0.5 ml culture	5 ml TSB 2 ml extract 3 ml water 0.5 ml culture	5 ml TSB 2 ml extract 3 ml 0.1 *M* $KHCO_3$ 0.5 ml culture	5 ml TSB 2 ml extract 3 ml 0.1 *M* $NaHCO_3$ 0.5 ml culture	5 ml TSB 2 ml extract 3 ml 0.1 *M* K_2HPO_4 0.5 ml culture	5 ml TSB 2 ml extract 3 ml 0.1 *M* Na_2HPO_4 0.5 ml culture
Increased absorbance after 24 hr	0.41	0.24	0.57	0.63	0.61	0.64
Original pH	7.13	7.03	7.62	7.62	7.53	7.54
pH after 24 hr	5.40	5.41	6.87	6.86	6.84	6.76

Table 7. Changes of pH with time

	Hours		
	8	16	20
5 ml TSB + 5 ml H_2O + 0.5 ml *Cellulomonas*	6.86	5.87	5.58
5 ml TSB + 2 ml extract + 3 ml H_2O + 0.5 ml *Cellulomonas*	6.44	5.84	5.59

Table 8. Effect of ammonium hydroxide (NH_4OH) on production of inhibitory substances from sugarcane residues[a]

Pressure (psi)	NH_4OH (%)	Time (sec)	Inhibition observed (%)
400	0	60	47
400	1.75	60	32
400	0	30	37
400	1.75	30	27
400	0	10	23
400	1.75	10	23
300	0	60	30
300	1.75	60	21
300	0	30	23
300	1.75	30	17
300	0	10	21
300	1.75	10	19

[a]Includes leafy trash.

The application of low pressure (temperature) with NH_4OH to reduce the formation of inhibition may be the best choice for the pretreatment of sugarcane bagasse and rice straw because NH_4OH can also be used as a nitrogen source.

The chemicals K_2HPO_4, Na_2HPO_4, $KHCO_3$, and $NaHCO_3$ can neutralize the inhibition and thus promote the growth of *Cellulomonas.*

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Departmental Paper 58—December 1979 (1.5M)