Effect of Pretreatment of Cellulosic Wastes on the Production of Cellulase Enzymes by *Trichoderma Reesei*

E. A. KASSIM, *Microbiology Chemical Laboratory, National Research Center, Dokki, Cairo, Egypt,* I. M. GHAZI, *Microbiology Department, Faculty of Agriculture Fayum, Cairo University, Cairo, Egypt,* and Z. A. NAGIEB, *Cellulose and paper Laboratory, National Research Center, Dokki, Cairo, Egypt*

Synopsis

The cellulosic wastes, bagasse and sawdust, were treated with different acidified solvents; chloroform, dioxan, ethanol, ethylacetate, dimethyl sulfoxide and dimethyl formamide; for increasing periods up to 36 h. The treated materials were delignified, dried and introduced in the fermentation media as inducing carbon source for *Trichoderma reesei*. The untreated materials and pure cellulose powder were used individually in control and reference media, respectively. The fermentation media containing bagasse treated for 8 h in chloroform or dioxan gave a promising cellulase activity, nearly the same as that from the reference medium. The untreated bagasse gave a markedly low cellulase activity.

The media with treated sawdust gave a relatively higher enzyme activity but far below the reference medium. The sawdust treatment with the solvents mentioned resulted in a very mild effect because of its high content of admixtures, lighin and hemicellulose bonding cellulose.

INTRODUCTION

Many studies were carried out to find a more economic application for the native cellulosic wastes which represent more than 60% of the agricultural wastes. The majority of these materials are used as animal feed or burned as a source of energy. It is well known that purified cellulose could be easily saccharified by the commercial cellulase enzymes. However, the native cellulose in agricultural wastes contains lignin and hemicellulose as admixtures strongly bounded with cellulose and impair its enzymatic decomposition. Sasaki[1] mentioned that removal of lignin is necessary to increase the effeciency of enzymatic hydrolysis. More than 90% of delignified cellulose can be quickly saccharified by the Cx-glucanase only.

The cellulosic materials could be used as a source of carbon in the fermentation media for the production of different compounds. The presence of cellulose in the medium was found to be essential for higher production of cellulase enzyme.[2-4] The use of native cellulosic wastes as carbon source in the fermentation medium will affect the microbial growth rate and cellulase production. Sasaki[1] reported that bioconversion of cellulose depends on the degree of crystallinity and the admixtures bounding cellulose. The removal of bounding materials and degradation of crystalline structure will increase the bioconversion of cellulose.

Journal of Applied Polymer Science, Vol. 31, 823–829 (1986)
 CCC 0021-8995/86/030823-07$04.00

Several investigators[1,5-7] mentioned different treatments for native cellulose with acids, alkali, and solvents to make it more usable for effecient saccharification or as inducing carbon source in the fermentation medium for cellulase production.

In Egypt, millions of tons of bagasse become waste yearly from the sugar cane industry. The present study was planed to find an effecient and more economic use of such wastes by mild and simple chemical treatments as well as the application of treated materials as inducing carbon source for higher production of cellulase enzyme by *Trichoderma reesei*.

MATERIALS AND METHODS

—All chemicals used were BDH and solvents were purified according to Vogel.[8]

—Bagasse was obtained from El-Hawamdia Factory for sugar and distillates. Sawdust of hard and soft wood were supplied from a local workshop.

—The fungal strain *Trichoderma reesei* QM 9414 was used in the fermentation experiments.

—Chemical treatments: 50 g of grounded (40 mesh) of each cellulosic material were attained for different periods; 4, 8, 12, 24, and 36 h for each of the solvents; chloroform, dioxane, ethylacetate, ethanol, $(CH_3)_2SO_3$, and dimethyl formamide. The solvents were acidified by 2% HCl. After elapse of each period, treated materials were washed with distilled water, delignified, dried, and weighted. The resulting holocellulose from each treatment was individually used as inducable carbon source in the fermentation medium at the rate of 1%.

—Lignin extraction and estimation after treatment with solvents were carried out by the method of Halse et al.[9]

—Ash content was determined by the method of the A.O.A.C.[10]

—Holocellulose, which consists of cellulose and hemicellulose, was estimated by the method of Wise et al.[11]

—Removal of lignin was achieved in 1% NaOH solution at 80°C for 3 h.

—Fermentation process: The basal fermentation medium of Mandels and Reese[12] was used. The medium contained: KH_2PO_4, 0.2%; $(NH_4)_2SO_4$, 0.14%; urea, 0.03%; $MgSO_4 \cdot 7H_2O$, 0.03%; $CaCl_2$, 0.03%; proteose peptone, 0.1%; and traces of $FeSO_4 \cdot 7H_2O$; $ZnCl_2$; $MnSO_4 \cdot H_2O$; $CoCl_2 \cdot 6H_2O$; $CuSO_4 \cdot 5H_2O$.

The carbon source in the control medium was 1% cellulose powder. In treatments the cellulose powder was substituted with the holocellulose resulted from chemical pretreatments. The medium was then adjusted to pH 5.0. Five millilters of a 48 h shake culture were aseptically transfered to each of 250 mL Erlenmeyer flasks containing 50 mL sterilized medium and incubated on a rotary shaker (200 rpm) at 30°C.

The cellulase activity was determined at 72, 96, 120, 144, 168, and 196 h fermentation periods by the method of Levinson and Reese.[13]

RESULTS AND DISCUSSION

Chemical Treatments

The bagasse was treated with different solvents: chloroform, dioxan, ethylacetate, dimethyl sulfoxide, dimethyl formamide, and ethanol; individually

for increasing periods. The samples of treated bagasse were analyzed for the determination of lignin, cellulose, hemicellulose and ash contents. The data presented in Table I show that:

—A part of bagasse always dissolved in any of the solvents used and increased when the attaindance period was prolonged.

—The solvents differ from each other in their capability of dissolving a more or less portion of bagasse content.

—A continuous decline in all contents; lignin, holocellulose, and ash, occurred as a result of the treatment with the solvents. That indicates the presence of a soluble fraction from each constituent, and its solubility differs with respect to the solvent and period of contact.

—With regard to the cellulose content, there was a general trend in all treatments: a decrease after 4 h, then a slight increase after 8 h followed by gradual decline till the end of the treatment (36 h). This may be due to the action of the solvents on the admixtures and the partial degradation of hydrogen bonding in the crystalline cellulose and a part of it was converted to the amorphous form.

—The highest loss in cellulose content was achieved by chloroform and dioxan respectively.

Table II presents the chemical analysis of treated sawdust; from soft and hard wood; with different solvents for 24 h. The data show that sawdust was markedly less effected with the solvents than the bagasse. This may be due to its high content of lignin and hemicellulose masking the cellulose. As a result, the loss in cellulose content was generally very low in comparison with that which occurred with bagasse.

TABLE I:
Chemical Analysis of Bagasse Treated with Solvents

Solvent	Time (h)	Lignin (%)	Cellulose (%)	Loss in solvent (%)
Untreated bagasse		20.3	50.3	—
Dioxan	4	19.8	47.8	4.0
	8	19.4	48.4	4.8
	36	18.7	46.6	8.5
Chloroform	4	19.1	46.0	6.1
	8	18.3	47.0	6.6
	36	16.9	45.9	11.1
Dimethyl sulfoxide	4	20.0	49.0	2.0
	8	19.8	49.3	2.8
	36	18.9	47.9	9
Dimethyl formamide	4	20.1	48.3	2.7
	8	19.7	48.8	3.2
	36	19.0	47.8	5.6
Ethylacetate	4	20.1	48.3	2.9
	8	19.6	48.5	3.5
	36	18.8	47.9	6.4
Ethanol	4	20.1	47.6	3.5
	8	19.8	48.0	4.0
	36	19.1	46.9	7.0

TABLE II
Chemical Analysis of Sawdust from Soft and Hard Wood Treated with Solvents for 24 h

Treatment	Lignin (%)[a]		Cellulose (%)[a]		Loss (%)[a]	
	S	H	S	H	S	H
Sawdust (untreated)	35.6	35.7	15.1	16.4	—	—
Dioxan	34.9	34.7	14.9	15.8	3.3	2.6
Chloroform	34.8	33.4	14.6	15.6	2.8	4.5
Dimethyl sulfoxide	35.2	34.3	14.8	16.2	2.4	2.0
Dimethyl formamide	34.3	35.3	14.9	15.9	2.4	1.7
Ethylacetate	35.1	35.4	14.9	16.0	2.0	0.6
Ethanol	35.2	34.9	15.0	16.0	1.9	0.8

[a] S = sawdust from soft wood; H = sawdust from hard wood.

The treatments of chloroform and dioxan gave a relative higher percent of dissolved contents, which was mostly of hemicellulose. With the high content of admixtures and the hard crystalline form of cellulose in the sawdust, the direct treatment with the solvents did not give a satisfactory results with regard to the degradation of the hydrogen bonds in the utilizable crystalline cellulose.

Microbiological

In all fermentation experiments two control media were used: one containing the untreated material for comparison with treatments and the second containing 1% of pure cellulose powder as standard.

The resulted holocellulose from the treatment of bagasse with chloroform for 4, 8, 12, and 24 h were introduced individually in the fermentation medium. The cellulase activity obtained from the treatments are presented in Table III. The data show that there is a positive relation between the mycelial growth and cellulase production. The standard medium with pure

TABLE III
Effect of Pretreatment Period of Bagasse with Chloroform on Cellulase Production by *T. reesei*

Treatment period (h)	U/mL cellulase after fermentn. (h)						Growth density
	72	96	120	144	168	196	
Cellulose powder(control)	3.25	5.25	8.0	10.25	14.5	16.75	++++
Untreated bagasse	0.5	0.75	1.5	2.25	3.5	4.75	+
4 h	1.75	3.5	5.0	7.0	8.75	10.5	++
8 h	2.75	4.75	6.25	8.5	10.25	12.75	+++
12 h	2.75	4.25	6.0	8.0	9.5	11.5	++
24 h	2.5	4.25	6.0	7.5	8.75	10.75	++

cellulose powder gave the highest growth and cellulase activity. That indicates that the microbe utilized the pure cellulose more effecient than the native and treated bagasse. That could be attributed to the presence of lignin in the untreated material and the relatively higher crystallinity of cellulose in treated bagasse than the pure cellulose powder. This conclusion is in agreement with that mentioned by Sasaki.[1]

Among the treated bagasse, the medium containing 1% of the holocellulose from the 8 h treatment gave the highest cellulase production. With the increase of exposure period to chloroform, the resulting material gave a gradual decrease in the cellulase production. Ryu and Mandels[5] reported that the chemical treatments destruct the hydrogen bonding of cellulose crystalline form and then convert to the easily hydrolyzed amorphous form. It seems that after prolonged treatment with chloroform over 8 h the hydrogen bonds are gradually reformed again; such cellulose with increasing crystallinity was hardly bioconverted and utilized by the fermentation microbe.

The bagasse samples treated with different acidified solvents for 8 h and delignified were introduced individually in the fermentation medium as inducing carbon source. Table IV presents the units of cellulase produced during 196 h fermentation. It is clear from these data that bagasse treated with dioxan gave the highest cellulase activity and slightly more than the standard cellulose powder; followed by the treatments of chloroform, ethylacetate, dimethyl formamide, dimethyl sulfoxide, and ethanol, respectively. The untreated bagasse gave a markedly lower cellulase activity.

The differences in cellulase activity may be due to the variation in the amounts of utilizable amorphous cellulose in the fermentation medium of each treatment. This variation could be attributed to the capability of each solvent to distruct the hydrogen bonding of the crystalline form of cellulose.

The sawdust samples of soft and hard wood were treated with different solvents, delignified, and used in the fermentation media. Table V presents the cellulase units produced in the medium containing the treated sawdust

TABLE IV
Effect of Treated Bagasse on the Production of Cellulase by *Trichoderma reesei*

Treatment with acidified solvent	U/mL cellulase after fermentn. (h)						Growth density
	72	96	120	144	168	196	
Cellulose powder (control)	3.25	5.25	8.0	10.5	14.5	16.75	++++
Untreated bagasse	0.5	0.75	1.25	2.0	3.25	4.75	++
Dioxan	5.25	8.0	10.0	12.75	13.5	17.75	+++
Chloroform	2.7	4.5	6.2	10.5	14.5	16.0	+++
Dimethyl sulfoxide	2.0	3.75	5.0	7.75	9.25	10.75	+++
Dimethyl formamide	3.0	4.25	6.0	8.25	10.0	12.5	+++
Ethylacetate	2.75	4.0	6.25	8.75	11.25	13.75	+++
Ethanol	0.75	1.0	1.75	3.0	5.25	7.5	++

TABLE V
Effect of Treated Sawdust from Soft and Hard Wood on the Production of Cellulase by *Trichoderma reesei*

Treatment	U/mL cellulase after fermentn. (h)						Growth density[a]	
	72		120		196			
	S	H	S	H	S	H	S	H
Cellulose powder(control)	4.8		10.5		17.5		++++	
Untreated sawdust	0.25	0.00	0.75	0.1	1.0	0.25	+	+
Dioxan	0.50	0.25	1.0	0.75	2.5	1.0	++	+
Chloroform	0.25	0.1	0.75	0.25	1.75	1.0	++	+
Dimethyl sulfoxide	0.25	0.0	0.75	0.2	2.25	0.75	++	+
Dimethyl formamide	0.25	0.0	1.0	0.3	2.25	0.75	++	+
Ethylacetate	0.5	0.25	0.75	0.5	2.0	0.75	++	+
Ethanol	0.25	0.12	0.75	0.3	1.75	0.4	+	+

[a] S = sawdust from soft wood; H = sawdust from hard wood.

of soft and hard wood, respectively. The results show that sawdust, in general, was hardly utilized as carbon source by *T.reesie* even after treatment with solvents, which indicates a very high degree of crystallinity. The treated materials gave a slight increase in cellulase production and the sawdust of soft wood gave a relatively higher activity than that of hard wood. The effect of pretreatment with solvents seems to differ from the known effect of steam explosion.

With regard to the effect of different solvents, it was clear that sawdust treated with dioxan and chloroform gave the relatively highest production of cellulase. The ethanol treatments gave the lowest enzyme activity. With the very low activity obtained with sawdust, there was no clear differences between the treatments.

From the results of this work we can conclude that bagasse was a markedly promising cellulosic waste to be used as inducing carbon source in the fermentation medium for the production of cellulase enzyme by *T.reesei* when pretreated with dioxan or chloroform. The advantages of this method are: Better use of the local huge amounts of bagasse, simple technique, avoidance of the corrosion problems when concentrated acids are used for the degradation of native cellulosic materials, and the recovery of solvents after treatment will improve the economics of the process.

References

1. T. Sasaki, *Jarq*, **16**(2), 144 (1982).
2. E. T. Reese and H. S. Levinson, *Physiol. Plantarum*, **5**, 345 (1952).
3. M. Mandels and E. T. Reese, *J. Bacteriol.*, **79**, 816 (1960).
4. M. Mandels, *Biotechnology Bioengineering Symposium No. 5*, Wiley, New York, 1975, p.81.
5. D. D. Y. Ryu and M. Mandels, *Enzyme Microbiol. Technol.*, **2**, 91 (1980).

6. E. A. Johnson, M. Sakajoh, G. Halliwell, A. Madia, and A. Demain, *Appl. Env. Microbiol,* **43,** 1125 (1982).

7. E. A. Kassim, *J. Coll. Sci., King Saud Univ.,* **14**(1), 85 (1983).

8. I. A. Vogel, *A Textbook of Practical Organic Chemistry,* Longmans, Green, London, 1956, p. 175.

9. O. M. Halse, In *Wood Chemistry,* Reinhold, New York, 1952, Vol. 1, p. 163.

10. A.O.A.C., Official Methods of Analysis Assoc., Offic. Agric. Chem., Washington, D.C., 1965.

11. L. E. Wise, M. Murphy, and A. A. D. Addieco, *J. Paper Trade,* **122**(2), 35 (1946).

12. M. Mandels and E. T. Reese, *J. Bacteriol.,* **73,** 269 (1957).

13. H. S. Levinson and E. T. Reese, *J. Gen. Physiol.,* **33,** 601 (1950).

Received January 15, 1985
Accepted June 25, 1985