Acremonium sp. w-398 によるバガスセルロースの分解(農芸化学科)

Degradation of Bagasse Cellulose by *Acremonium* sp. W-398†

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I INTRODUCTION

Agricultural-cellulosic wastes are nowadays being considered as resources. Studies are currently being made on conversion of the wastes by microbial and enzymatic degradation to usable products (such as single-cell protein or sugar syrups) which may provide animal feed and energy sources.[1, 5, 7, 9] Many studies have been done to determine the possibilities of utilizing the wastes by growing microorganisms on the cellulosic materials as substrates with or without the addition of other nutrients.[10, 16, 17]

Sugar-cane bagasse, one of the big cellulosic-agricultural wastes, is rich in cellulose and hemicellulose, and is therefore a potential source of carbon for the growth of microorganisms. Traditionally, bagasse has been used mainy as fuel in sugar cane factories. However, extensive research has been conducted in the past few years on bagasse as a cellulosic raw material for the production of single-cell protein and other bioproducts.[6, 11, 14, 18, 19, 20]
Native bagasse is quite resistant to microbial and enzymatic degradation, as well as other cellulosic materials. Thus native bagasse requires pretreatment by physical or chemical means for enhanced utilization by microorganisms. In our previous report,[21, 22] it was suggested that alkali treatment could significantly increase the susceptibility of bagasse to microbial or enzymatic attack.

The present paper reports the isolation of the fungus *Acremonium* sp. W-398 capable of growing on bagasse and the effectiveness of alkali treatment on the degradability of bagasse cellulose by this fungus.

II MATERIALS AND METHODS

Bagasse: The bagasse used was obtained from a local sugar mill as a waste residue left after extraction of the juice from sugar cane.
Fresh bagasse from the sugar mill was dried in a forced air oven at 70℃ and the dried bagasse was ground through a 40-mesh screen in a Whiley mill. The ground bagasse is called

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untreated bagasse.

Chemical analysis of the bagasse showed 45.5% cellulose, 22.0% hemicellulose, 19.8% lignin, 1.8% ash and 8.2% water on a dry basis.

Alkali treatment of bagasse: Untreated bagasse was treated with NaOH solution (in a mixture of 1 g/20 ml liquid) at 120℃ and the solution was neutralized to pH 6.0 with 1 N HCl. The treated solid was recorvered by filtration through Toyo No. 2 filter paper, washed throughly with distilled water, and dried in a forced-air oven at 70℃ over night. The washed solid and the recorvered treatment-liquor are called alkali-treated bagasse and alkali-extract, respectively.

Media: The isolation medium consisted of mineral salts solution contained 0.2% $(NH_4)_2SO_4$, 0.2%K_2HPO_4, 0.05%KCl, and 0.01%$MgSO_4$ $7H_2O$. The pH of the medium was adjusted to pH 7.0. The medium used for the flask cultures was similar, except that it contained alkali-treated bagasse and alkali-extract instead of untreated bagasse.

Isolation of bagasse-utilizing microorganisms: Samples of soil obtained from sugar-cane fields were used as inocula. About 0.1 g of each of the soils was used to inoculate 5 ml of the isolation medium with untreated bagasse in 25 ml test tubes, and incubated for 3 to 5 days at 30℃ on a reciprocal shaker. The tube showing good growth in the medium was selected, and 0.1 ml of the tube content was taken to inoculate a new test tube with fresh medium. The incubation was repeated for enrichment and isolation of bagasse-utilizing microorganisms. The cultures which better utilized the bagasse were selected and picked to agar solid media. The purity of the isolated culture was confirmed by microscopic examination and by colony morphology on agar plate. The strain isolated was maintained on the potato-glucose agar slant that contained 0.5% potato extract, 0.5% glucose and 1.8% agar.

Culture conditions: The strain isolated was grown in a liquid medium containing mineral salts solution and alkali-treated bagasse with or without alkali-extract. The cultures were carried out in 500ml-shake flask using 100 ml of working volume of the medium. Flask cultures were inoculated with the culture broth (5.0ml) of the strain which had been cultivated on a medium containing alkali-extract as the sole carbon source. The flasks were incubated on a reciprocal shaker at 30℃. After cultivation, fugal mycelium and bagasse residue were harvested by centrifugation, washed with distilled water, dried at 70℃ overnight, and weighed. The dry matter of fungal mycelium with bagasse residue was ground finely for analyses.

Analyses: Total nitrogen of the dry matter was determined by a micro-Kjeldahal method on duplicate samples, and the factor 6.25 was used to caliculate crude protein. The degree of growth was expressed as the total crude protein (mg) calculated from the percentage of protein content of the dry matter. Cellulose was determined by the method of Updegraff.[24] Hemicellulose of the culture broth was estimated by the measurement of total pentose with the orcinol reaction.[2]

III RESULTS

1. Bagasse-utilizing microorganism

After a series of selective cultivation on the medium containing untreated bagasse as the sole carbon source as described in the Materials and Methods, a bagasse-utilizing microorganism was obtained. The isolated strain was a mold of Fungi imperfecti with septate mycelium. The mycelium was prostrate and slender. The hyphae were loose and cobwebby. The mold was identified as a member of the genus *Acremonium* by its morphological characteristcs[3] and named *Acremonium* sp. w-398. Fig. 1 shows a microscopic photograph of

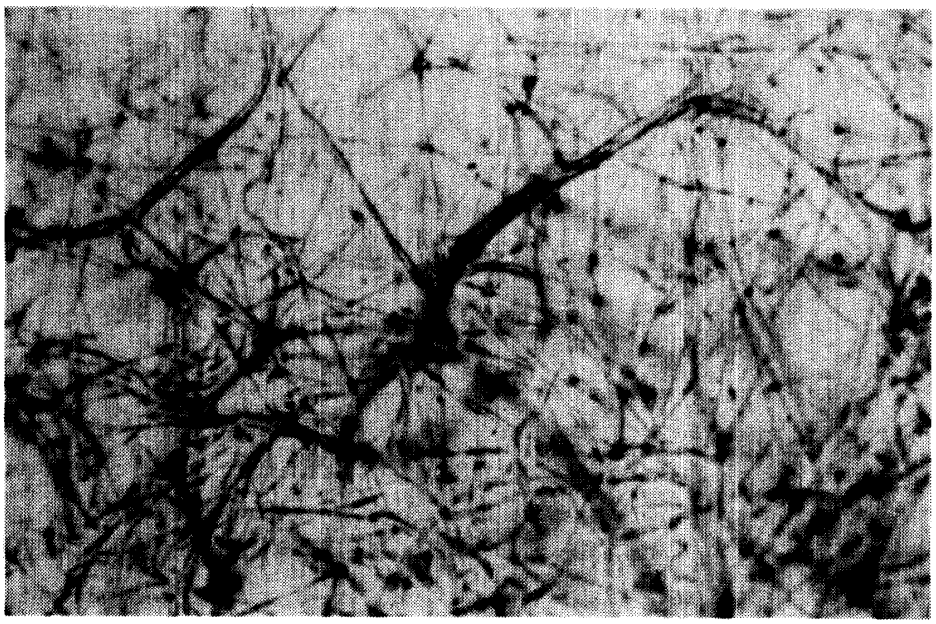

Fig. 1 Photograph of *Acremonium* sp. w-398

the strain grown on the mineral salts solution containing alkali extract from alkali treatment of bagasse as the carbon source. This organism was used for further study.

2. Alkali treatment for growth and bagasse-cellulose degradation

Untreated bagasse (1.5g) was treated with various concentrations of NaOH solution at 120 ℃ for 20 min, and the solutions of the combined alkali-treated bagasse (solid) and alkali-extract (liquor) were used for growth substrate as a source of carbon. Fig. 2 shows the growth and bagasse-cellulose degradation by the isolate, *Acremonium* sp. w-398. When bagasse was not treated with NaOH solution, the strain could not grow. The alkali treatment increased the growth and cellulose degradation. When bagasse was treated with 0.1 to 0.5% NaOH solution, both the growth and cellulose degradation increased in proportion

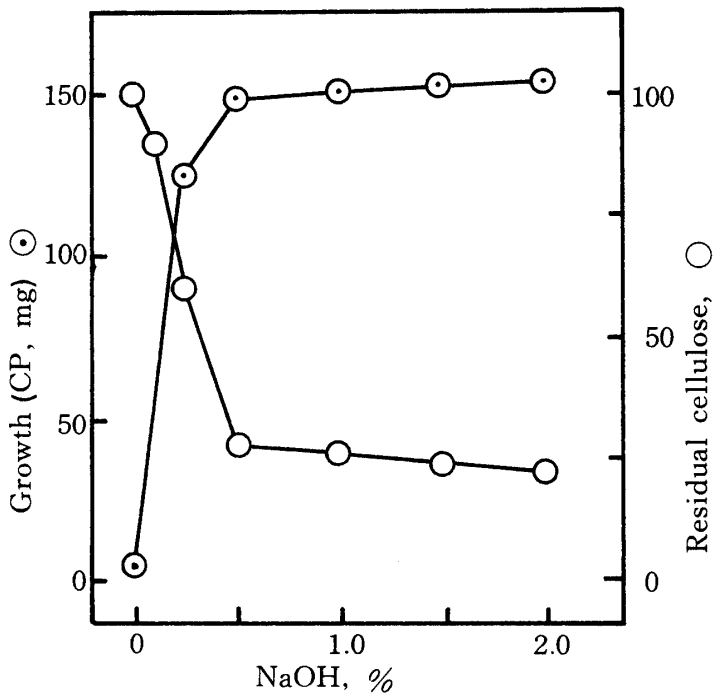

Fig. 2 Effect of alkali treatment of bagasse on the growth of *Acremonium* sp. W-398 and bagasse-cellulose degradation

Bagasse (1.5g) was treated with various concentrations of NaOH solution (1 : 2, bagasse : liquid) at 120℃ for 20 min, and the combined alkali-treated bagasse (solid) and alkali-extract (liquid) was used as a sole carbon source. The strain was grown in shake flasks for 72 hr. Samples(containing mycelium and residual bagasse combined) were harvested, washed, dried, weighed to give the total solids. The dry solids were analyzed for percentage of crude protein (CP) and cellulose. Other conditions are described in the Materials and Methods.

to the increased alkali concentration. When the alkali concentration was increased to 2.0%, the cellulose degradation did not increase further. Therefore, there appears to be a limit to the increase in the cellulose degradation through the alkali treatment.

3. Alkali-treatment temperature for growth and bagasse-cellulose degradation

Untreated bagasse (1.5g) was treated with 0.5% NaOH solution at 30, 60, 100 and 120℃ for 20 min and the solutions of the combined alkali-treated bagasse and alkali-extract were used for growth substrate. The growth and bagasse-cellulose degradation by *Acremonium* sp. w-398 are shown in Fig. 3. An increase in the growth and the cellulose degradation was observed with increasing the temperature. However, both the growth and cellulose degradation could not be enhanced further when bagasse was treated at 150 and 120℃.

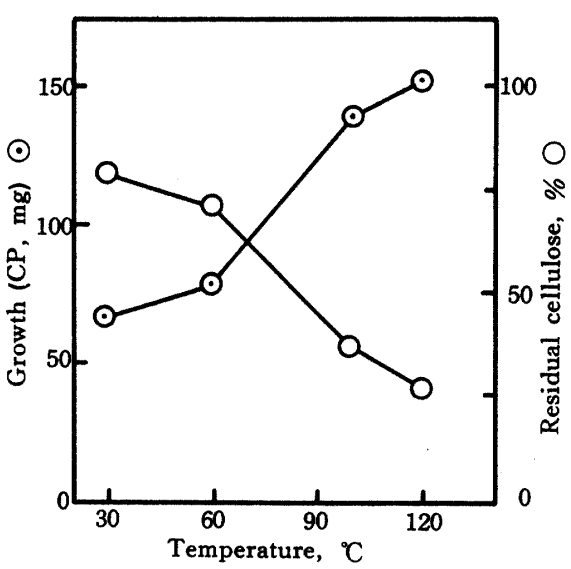

Fig. 3 Effect of alkali-treatment temperature of bagasse on the growth of *Acremonium* sp. w-398 and bagasse-cellulose degradation

Bagasse (1.5g) was treated with 0.5% NaOH solution at 30, 60, 100 and 120 ℃ for 20 min. The strain was grown on the combined alkali-treated bagasse and alkali extract for 72. hr. Other conditions are the same as Fig. 2.

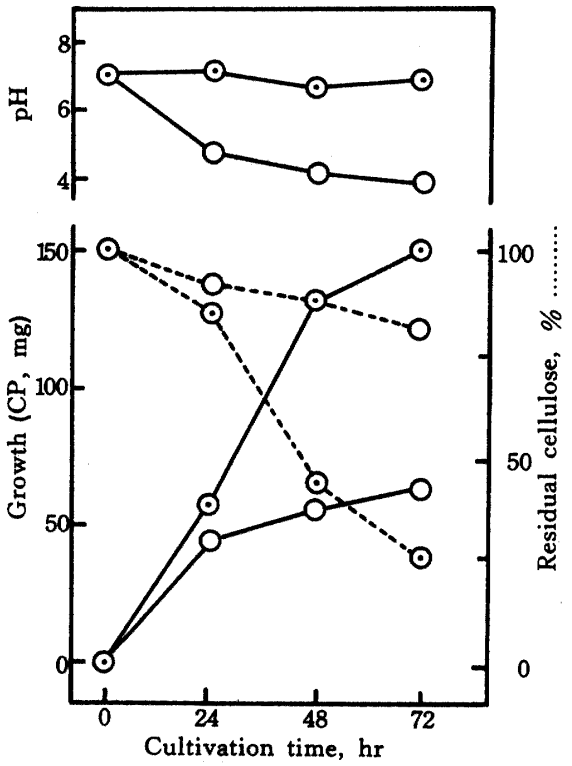

Fig. 4 Growth of *Acremonium* sp. w-398 on alkali-treated bagasse with or without alkali extract and bagasse-cellulose degradation

Bagasse (1.5g) was treated with 0.5% NaOH solution at 120℃ for 20 min. The strain was grown on alkali-treated bagasse with (⊙) or without (○) alkali extract. Other conditions are the same as Fig. 2.

4. Growth on alkali-treated bagasse with or without alkali extract, and bagasse-cellulose degradation

Ground bagasse (1.5g) was treated with 0.5% NaOH solution at 120 ℃ for 20 min and the alkali-treated bagasse (solid) with or without alkali-extract (liquid) was used for growth substrate separately. The growth of the isolate, *Acremonium* sp. w-398, and bagasse-cellulose degradation are given in Fig.4. The strain grew well in the medium of alkali-treated bagasse with alkali-extract and the bagasse cellulose was degraded effectively. However, when the strain was cultured in the medium of alkali-treated bagasse without alkali-extract, the growth was very low and the bagasse cellulose could be hardly degraded. The pH of the culture broth of the

medium of alkali-treated bagasse without alkali-extract dropped from 7.0 to 4.0 with cultivation time. No pH change was observed in the medium of the combined alkali-treated bagasse and alkali-extract.

5. Growth on alkali-treatment liquor

Ground bagasse (1.5g) was treated with 0.5% NaOH solution at 120℃ for 20 min, and the recovered treatment liquor, alkali-extract, was used for growth substrate as the sole source of carbon. The isolate, *Acremonium* sp. W-398, was grown in the medium containing the alkali-extract with mineral salts solution, Fig. 5 shows the growth and the utilization of hemicelluloses in the culture broth. The growth occurred within the first 24 hr of the cultivation. The hemicelluloses extracted from bagasse with NaOH solution were utilized by the strain. The pH of the culture broth increased gradually during cultivation. However, the degree of the growth in the medium of alkali extract was lower than that of the growth in the medium of the combined alkatreated bagasse and alkali-extract.

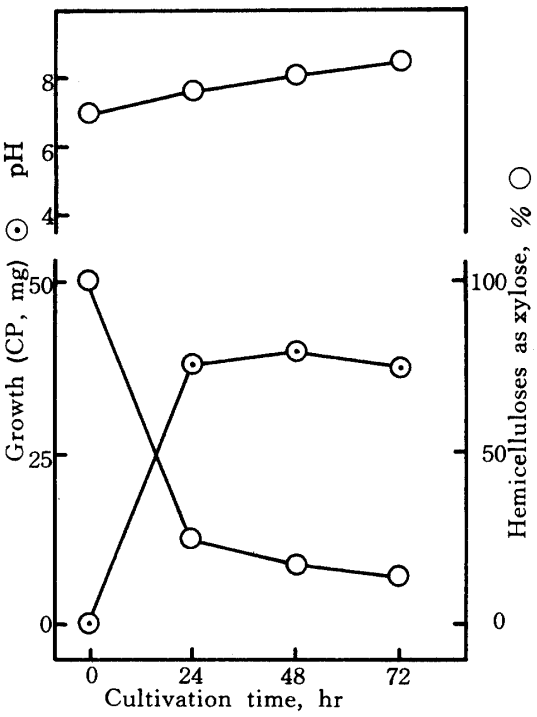

Fig. 5 Growth of *Acremonium* sp. W-398 on alkali extract of bagasse

Bagasse (1.5g) was treated with 0.5% NaOH solution at 120℃ for 20 min. The strain was grown on the alkali extract. Samples containing mycelium were harvested, and the dry solids were analyzed for percent of crude protein. The content of hemicelluloses as xylose in the culture broth was also analyzed.

6. Nitrogen source for growth and bagasse-cellulose dagradation

The isolate, *Acremonium* sp. W-398, was cultivated in the medium containing various nitrogen sources, such as ammonium sulfate, ammonium chloride, sodium nitrate, and urea. The strain showed good growth in the medium of ammonium sulfate containing the combined alkali-treated bagasse and alkali-extract treated with 0.5% NaOH solution at 120℃ for 20 min. The growth increased with increasing ammonium sulfate concentration as shown in Fig. 6. The maximum growth and bagasse-cellulose degradation were obtained at a concentration of 0.2%.

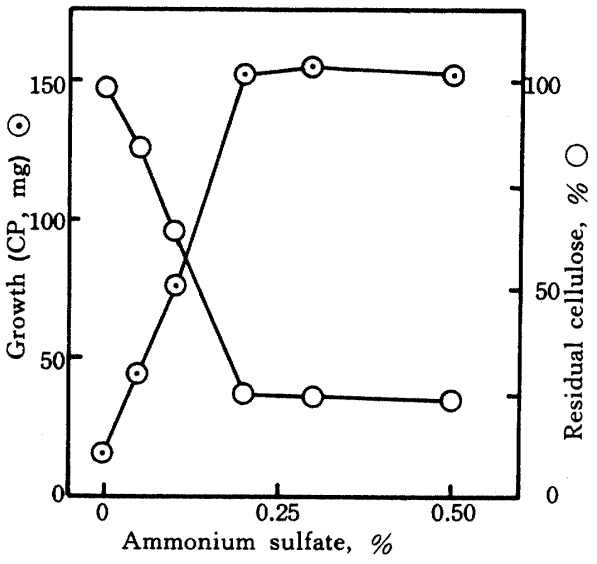

Fig.6 Effect of ammonium sulfate in the medium on the growth of *Acremonium* sp. w-398 and bagasse-cellulose degradation.

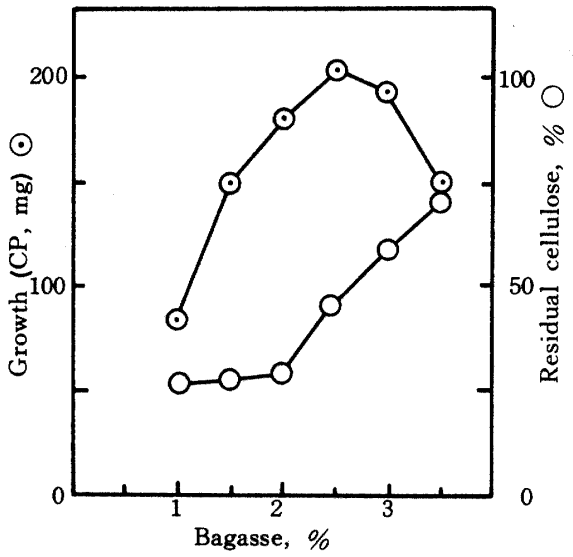

Fig.7 Effect of bagasse concentration in the medium on the growth of *Acremonium* sp. w-398 and bagasse-cellulose degradation

The strain was grown in the medium containing various concentrations of ammonium sulfate and the alkali-treated bagasse with alkali-treated bagasse with alkali extract treated with 0.5% NaOH solution at 120℃ for 20 min. Other conditions are the same as Fig. 2.

Various amounts of bagasse were treated with 0.5% NaOH solution (20:1,liquid:bagasse) at 120℃ for 20 min. The strain was grown on the combined alkali extract. Other conditions are the same as Fig. 2.

7. Bagasse concentration for growth and bagasse-cellulose degration

In the cultivation of the isolate, *Acremonium* sp. w-398, the bagasse concentration in the medium was varied in order to determine the maximum growth and bagasse-cellulose degradation. Various amounts of bagasse were treated with 0.5% NaOH solution (20:1, liquid : bagasse) at 120℃ for 20 min, and the suspensions of the combined alkali-treated bagasse and alkali-extract were used as the carbon source for the growth. Fig. 7 shows the growth and bagasse-cellulose degradation bv the strain cultivated in the medium containing various amounts of bagasse for 72 hr. The growth increased with increase in the concentration of bagasse in the medium, but the extent of growth decreased as the concentration was increased. The maximum growth was obtained at a concentration of 2.5%. The most suitable concentration of bagasse for the cellulose degradation was 1.0 to 2.0%. The degradation decreased with concentrations of bagasse in the region of 2.5% and above.

8. pH for growth and bagasse-cellulose degradation

The effect of initial pH of the medium on the growth of *Acremonium* sp. W-398 and the bagasse-cellulose degradation is shown in Fig. 8. The strain was grown for 72 hr in the medium containing the combined alkali-treated bagasse and alkali-extract treated with 0.5% NaOH solution at 120℃ for 20 min. The most favorable pH range for the growth and cellulose degradation by the strain was from 6.0 to 8.0. At initial pH's of 5.0 and below, there was a marked decrease in the growth. When the strain was grown above pH 6.0, the best cellulose degradation was obtained. Cultures started below pH 4.0 failed to degrade the cellulose.

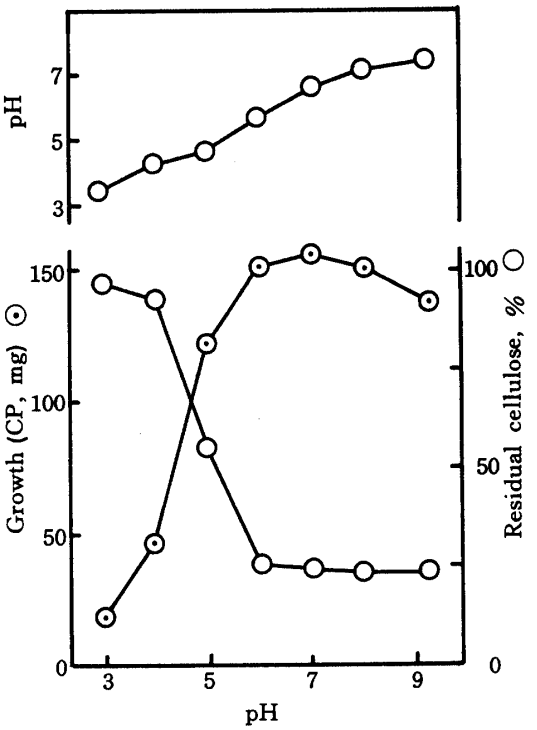

Fig. 8 Effect of pH on the growth of *Acremonium* sp. W-398 and bagasse-cellulose degradation.

The strain was grown at the indicated pH on the combined alkali-treated bagasse and alkali extract. Other conditions are the same as Fig. 2.

IV DISCUSSION

Cellulosic materials in their native forms are quite resistant to microbial and enzymatic degradation. However, pretreated-cellulosic materials are readily utilized as substrates for the growth of microorganisms.[4] Various forms of pretreatment techniques of cellulosic materials have been developed to enhance their degradation by microorganisms and enzymes.[12, 13, 15] Alkali treatment is widely used as a pretreatment method for cellulosic materials.

The fungus *Acremonium* sp. w-398, isolated from soils of sugar-cane fields, could grow on the surface of sugar-cane stalks. It is suggested that this strain can attack the cell-wall components of sugar cane. Native bagasse, ground bagasse, was scarcely utilized by the strain,

whereas the growth occurred when the strain was cultivated on the bagasse treated with NaOH solution. Hence, pretreatment of bagasse with NaOH solution results in its becoming a good substrate for the growth of the strain.

In previous work on the treatment of bagasse with NaOH solution, the treatment removed about 60% of the total dry weight of bagasse, producing solubles.[23] The solubles in the treatment liquor, alkali extract, are chiefly hemicelluloses in addition to some lignin. *Acremonium* sp. w-398 could utilize the hemicelluloses which are mainly xylose. Alkali-treated bagasse, treated solid, was readily utilized by the strain in the presence of alkali-extract in the medium. In the medium of the combined alkali-treated bagasse and alkali-extract, the growth probably occurs on hemicelluloses in the medium. As the hemicelluloses become exhausted in the medium, bagasse cellulose will be degraded. The bagasse cellulose was degraded effectively when the strain was grown on the combined alkali-treated bagasse and alkali extract. At least 75% of the original bagasse cellulose was degraded in the cultivation. When the strain was grown on alkali-treated bagasse without alkali extract, the growth and cellulose degradation were very low. Therefore, both alkali-treated bagasse and alkali extract are necessary to give the best results in the growth and the degradation of bagasse cellulose. This procedure makes efficient use of the carbon source in the original total substrate of bagasse. The strain produces a cellulase system degrading bagasse cellulose during the cultivation, but the enzymes responsible for the degradation have not been characterized.

The maximal growth and bagasse-cellulose degradation by *Acremonium* sp. w-398 were obtained in the medium of the combined alkali-treated bagasse and alkali extract treated with 0.5% NaOH solution at 120°C for 20 min. When the strain was grown on the bagasse treated with NaOH solution of 1.0% or above, there was no marked increase in the growth and cellulose degradation. Little difference in the growth was observed when the strain was grown in the medium of alkali-treated bagasse having various particle sizes. The effect of the alkali treatment of bagasse may be due to the extraction of some lignin and hemicelluloses which are bound with cellulose. Thus, it is apparent that the structural features of bagasse are altered by the treatment so that it becomes susceptible to the microbial degradation. In an earlier paper, we reported that the removal of the ultraviolet-absorbing substances of bagasse by treatment with alkali caused an increase in the degradability.[23] It has been already shown that alkali-treated bagasse is readily hydrolyzed by a cellulase system that produced by *Aspergillus awamori*.[22, 23] Recently, mild alkali-treatments of bagasse have been developed to raise its microbial or enzymatic degradability.[8, 11, 14, 18]

The best growth and bagasse-cellulose degradation by *Acremonium* sp. w-398 occurred within a pH range of 6.0 to 8.0. *Aspergillus awamori* grew most favorably in the range of pH 3.5 to 6.0 as described in our previous report.[21] The production of single-cell protein is being tested by growing microorganisms on agricultral-cellulosic wastes.[16, 17, 19] When *Acremonium* sp. w-398 was grown for 72 hr on the combined alkali-treated bagasse and alkali-extract treated with 0.5% NaOH solution at 120°C for 20 min, the dry matter of the fugal my-

celium and bagasse residue contained 30 to 32% crude protein.

V SUMMARY

A bagasse-cellulose utilizing mold was isolated from soils of sugar-cane fields. The mold was identified as a member of the genus *Acremonium* by its morphological characteristics and named *Acremonium* sp. w-398. The strain was studied to determine the growth behavior on bagasse as a source of carbon and the degradability of bagasse cellulose. Native bagasse was hardly utilized by the strain, whereas the growth occurred when the strain was cultivated on bagasse treated with NaOH solution. Hemicelluloses in the alkali-treatment liquor, alkali-extract, were also utilized by the strain. Bagasse cellulose was degraded effectively when the strain was grown on the combined alkali-treated bagasse and alkali-extract. The maximal growth and bagasse-cellulose degradation were obtained when bagasse was treated with 0.5% NaOH solution at 120℃ for 20 min. At least 75% of the original bagasse cellulose was degraded after 72 hr cultivation. The most suitable concentration of bagasse in the medium for the cellulose degradation was 1.0 to 2.0%. Ammonium sulfate (0.2%) was the most effective nitrogen source for the growth and cellulose degradation. The best growth and cellulose degradation by the strain occurred within a pH range of 6.0 to 8.0.

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REFERENCES

1. Andren, R.K., Mandels, M. and Modeiros, J.E. 1976 Production of sugars from waste cellulose by enzymatic hydrolysis: Primary evaluation of substrates, Process Biochem., October, p. 2～11
2. Ashwell, G. 1957 Determination of pentoses, Methods of Enzymol., 3:87～89
3. Clements, F.E. and Shear, C.L. 1964 "The genera of fungi" p. 200～205, Hafner Publishing Co., New York
4. Cowling, E.B. 1975 Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials, Biotechnol. Bioeng. Symp., No. 5, 163～181
5. Chahal, D.S., Moo-Young, M. and Dhillon, G.S. 1979 Bioconversion of wheat straw and wheat straw components into single-cell protein, Can. J. Microbiol., **25**: 793～797
6. Dekker, R.F.H. and Richards, G.N. 1973 Effect of delignification on the in vitro rumen digestion of polysaccharides of bagasse, J. Sci. Fd. Agric., **24**: 375～379
7. Detroy, R. W. and Hesseltine, C.W. 1978 Availability and utilization of agricultural and agro-industrial wastes, Process Biochem., **13**: 2～8

8. Ghose, T. K. and Bisaria, V. S. 1979 Studies on the mechanism of enzymatic hydrolysis of cellulosic substances, Biotechnol. Bioeng., **21**: 131～146
9. Han, Y. W., Grant, G. A., Anderson, A. W. and Yu, P. L. 1976 Fermented straw for animal feed, Feedstuffs, April **26**, 17～20
10. Han, Y. W. 1975 Microbial fermentation of rice straw: Nutritive composition and in vitro digestibility of the fermentation products, Appl. Microbiol., **29**: 510～514
11. Han, Y. W. and Callihan, C. D. 1974 Cellulose fermentation: Effect of substrate pretreatment on microbial growth, Apll. Microbiol., **27**: 159～165
12. Han, Y. W., Lillehoj, E., Timpa, J. and Ciegler, A. 1980 Solubilization of lignocellulosic materials by gamma radiolysis, Biotechnol. Letters, **2**: 397～402
13. Kristensen, T. P 1978 Continuous single cell protein production from *Cellulomonas* sp. and *Candida utilis* grown in mixture on barley straw, European J. Appl. Microbiol. Biotechnol., **5**: 155～163
14. Martinez, D. V. G., Ogawa, T., Shinmyo, A. and Enatsu, T. 1974 Hyrolytic degradation of bagasse by enzymes produced by *Penicillium variable*, J. Ferment. Technol., **52**: 378～387
15. Millett, M. A., Baker, A. J. and Satter, L. D. 1975 Pretreatments to enhance chemical, enzymatic, and microbiological attack of cellulosic materials, Biotechnol. Bioeng. Symp., No. 5, 193～219
16. Moo-Young, M., Chahal, D. S., Swan, J. E. and Robinson, C. W. 1977 SCP production by *Chaetomium cellulolyticum*, a new thermotolerant cellulolytic fungus, Biotechnol. Bioeng., **19**: 527～538
17. Ramasamy, K., Prakasam, K., Bevers, J. and Verachtert, H. 1979 Production of bacterial proteins from cellulosic materials, J. Appl. Bacteriol., **46**: 117～124
18. Shewale, J. G. and Sadana, J. C. 1979 Enzymatic hydrolysis of cellulosic materials by *Sclerotium rolfssii* culture filtrate for sugar production, Can. J. Microbiol., **25**: 773～783
19. Sidhu, M. S. and Sandhu, D. K. 1980 Single-cell production by *Trichoderma longibrachiatum* on treated sugar-cane bagasse, Biotechnol. Bioeng., **22**: 689～692
20. Toyama, N. and Ogawa, K. 1975 Sugar production from agricultural woody wastes by saccharification with *Trichoderma viride* cellulase, Biotechnol. Bioeng. Symp., No. 5, 225～244
21. Toyama, S., Yonaha, K. and Uehara, H. 1977 Cultivation of *Aspergillus awamori* with sugarcane bagasse as a sole carbon source, Sci. Bull. Coll. Agr. Univ. Ryukyus, **24**: 253～261
22. Toyama, S., Yonaha, K., Ikema, Y. and Uehara, H. 1976 A bagasse-hydrolyzing enzyme of *Aspergillus awamori*, Sci. Bull. Coll. Agr. Univ. Ryukyus, **27**: 79～87
23. Toyama, S., Yonaha, K. and Ishihara, M. 1980 Relationship between conditions for alkali-treatment of sugarcane bagasse and ultraviolet-absorption value of the alkali extract, Sci. Bull. Coll Agr. Univ. Ryukyus, **27**: 79～87
24. Updegraff, D. M. 1969 Semimicro determination of cellulose in biological materials, Anal. Biochem., **32**: 420～424

Acremonium sp. w－398 によるバガスセルロースの分解†

当山清善*・与那覇和雄*・石原昌信*

要　約

砂糖きび畑土壌からバガスセルロースを資化分解できる*Acremonium* sp. w-398が分離された。バガスを炭素源とした培地における本菌株の生育及びバガスセルロースの分解性を調べた結果，NaOH溶液で前処理したバガスが本菌株の良好な基質となり，アルカリ溶液で抽出されるヘミセルロースも資化利用された。本菌株はアルカリ抽出液を含むアルカリ処理バガスで良好な生育を示し，バガスセルロースもよく分解された。本菌株は0.5％NaOH溶液を用いて120℃，20分間処理したバガス培地で最も良好な生育を示し，培養72時間で75％以上のバガスセルロースが分解された。本菌株によるバガスセルロースの分解性からみた培地中のバガス濃度は2.0％が適当で，窒素源として用いる硫酸アンモニウムの濃度は0.25％が適当であった。本菌株はpH 6.0～8.0で良好な生育を示し，バガスセルロースもこの pH 範囲でよく分解された。

† 甘蔗バガスの微生物学的利用に関する研究（第6報）

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