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Studies on Cellulase

Part 1. Isolation of Cellulase Forming Microorganisms and the Properties of Crude Enzymes

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Cellulase에 關한 硏究

(第一報) Cellulase生成菌의 分離와 粗酵素의 諸性質

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要 約

糞尿處理場에서 400餘 菌株를 分離한 結果 그 中 두 菌株가 cellulase生成이 强하였다. 이 菌株는 振盪培養으로 cellulase를 많이 生成하였으며 이들 酵素의 性質은 다음과 같다.

1. 最適 pH는 4.0에서 5.5 범위이다.
2. 安全 pH는 3.5에서 6.5 범위이다.
3. 最適溫度는 40°C에서 45°C이다.
4. 熱安全性은 50°C 이하이며 70°C에서 거의 不活性化 된다.
5. 透析酵素는 Mn^{2+} Co^{2+}로 活性化되며 Hg^{2+}는 강한 阻害劑이다.

Introduction

The earlier literature on cellulase is discussed in the monographs of Siu(1) and Gascoigne and Gascoigne (2). Reese has edited the proceedings of a symposium held under the auspices of the American Chemical Society in 1963(3).

Recently papers on the production of cellulase cover a broad range of microorganisms; a) Bacteria; *Sporocytophage myxocoides*(4, 5), *Ruminococcus albus* (6), b) *a Streptomycetes; Streptomyces antibioticus* (9); c) Molds: *Rhizopus sp.*. (10, 58—60), *Penicillium sp.* (11—14), various of species of *Aspergillus sp.* (15—23), *Trichoderma viride* and *Trichoderma koningi* (24—45).

Four culture methods are in common use for the production of cellulase; a) submerged culture in a liquid medium which is aerated from spargers, b) shaking culture in a liquid medium, c) stationary cultures in liqiud medium and a koji type processed in which the organism is grown on a moist solid medium (e.g. wheat bran) from which enzymes are subsequently extracted.

Japan has become the major center of production of cellulases on an industrial scale and a great variety of enzyme preparation from submerged culture and koji culture of *Rhizopus, Trichoderma, Penicillium, Aspergillus* etc. are now available commercially.

Cellulases are used as cell wall disintegraters in order to increase the digestibility of vegetable foods (46—48) or extractibility of protein, fruit juice, essential oil and agar-agar from sea weeds etc. (49—55). For a long time, cellulase have found applification in the pharmaceutical industry as a digestive aid (56).

During we have studied on cenllulase of *Chaetomium globosum*(89) the author had found strong two strains of cellulase activity which were isolated from night soil as Ku-3371 and Ku-4383.

The present paper described a method of isolated of strains and some of its properties of crude cellulase using CMC (carboxymethyl cellulose) and filter paper as substrates.

Experimental Methods

1—1. Isolation of microorganism

Sample of night soil were spread on modified Czapek-Dox agar plates as the following Table 1, and inoculated at 30°C as long as to develop district colonies to transfer the slant.

Table 1. Isolation Medium

Cellulose powder	10g
C.M.C.	5g
K_2HPO_4	1g
$MgSO_4 \cdot 7H_2O$	0.5g
KCl	0.5g
$FeSO_4$	10mg
Distilled water	100ml.
Initial pH	5.0

Molds isolated were streaked on koji extract agar slant. Strains was incubated abundant at 30°C incubater, and the purest strains was used for cellulase forming tests as the following method.

1—2. Cellulase forming medium and crude enzyme solution

Media were dispensed in amount of 100 ml. of the basal medium given in Table 2 to 500ml shake flask and sterilized by autoclaving at 120°C for 30 minutes. Two strains, Ku-3371 and Ku-4383, were grown on a reciprocal shaker (130 strokes/min.) for 5 days at 30°C. After incubated, medium was centrifuged (9000 r.p.m.) at 5°C for 10 minutes, and its supernatant was filter. The filtrate was used for crude enzyme solution.

Table 2. Cellulase forming medium

Wheat bran	50g
Cellulose powder	10g
KH_2PO_4	10g
$NaNO_3$	3g
$(NH_4)_2SO_4$	3g
$MgSO_4 \cdot 7H_2O$	0.5g
Distilled water	1000ml.
Initial pH	4.5

1—3. Determination of cellulase activity

Evidence for the existence of the multiple forms of cellulolytic enzymes, in particular in microorganism, have been presented by the a number of investigators (61—63) and there were many methods of cellulolytic enzymes activity determination (64).

Analysis for cellulase activity was according to the method of filter paper disintegrating activity, CMC-liquefying and CMC-saccharifying activity(65).

Cellulase activities were determined by the following different procedures.

1) Filter paper disintegrating activity; A modified method of Toyama was used (66). A mixture composed of 7 ml. of enzyme solution and 3 ml of McIlvaine's buffer solution (pH 4.0) was placed into a L shaped glass tube (diameter 17 mm. height 70 mm., lenght 115mm.) (37, 66).

The incubation mixture was then shaked by the Monod shaker apparatus (a rate of the rotation of 48 r.p.m. with maximum amplitude of 4.0 cm.) with two sheets of Toyo Filter Paper Co., 51 A of 10×10mm. at 38°C. The time needed to complete disintegration of the filter paper was represented by minutes.

2) CMC-liquefying acitivity; In Ostwald viscometer, five ml. of 0.1% CMC and 5 ml. of McIlvaine's buffer solution (pH 4.0) was placed. The mixture was preincubated at 40°C for 15 minutes, then one of enzyme solution was added and mixed. The flow time was measured after 10 minutes. The CMC-liquefying activity was calculated from the following equation (67—68).

$$V = \frac{B-A}{A} \times 100$$

note; V: CMC-liquefying activity (decrease in viscosity)

B: The flow time (second) of the mixture of substrate and enzyme solution.

A: The flow time (second) of the mixture of substrate and destroyed enzyme solution.

3) CMC-saccharifying activity; The mixture consisted of one ml. of 0.5% CMC solution, one ml. of McIlvaine's buffer solution (pH 4.0) and 0.5ml. of enzyme solution. After incubation at 40°C for 10 minutes, one ml. aliquot of sample removed from the mixture was analyzed by Somogyi-Nelson methods for reducing sugar (69—72). CMC-saccharifying activity was expressed reducing sugar as

glucose in one ml. of the reaction mixture under the present incubation condition(18, 67—68).

Results and Discussion

(1) Optimum pH

The optimum pH of each Ku-3371 and Ku-4383 was determined for the filter paper disintegrating, CMC-liquefying and CMC-saccharifying activities. The results are shown in Table 3, Fig. 1, Fig. 2 and Fig. 3.

Table 3. Filter paper disintegrating activity

	pH / strains	2.2	3.0	4.0	5.0	6.0	7.0	8.0
Filter paper disintegrating	Ku-3371	180	170	150	165	180	200	250
Activity (minutes)	Ku-4383	160	140	120	145	160	180	230

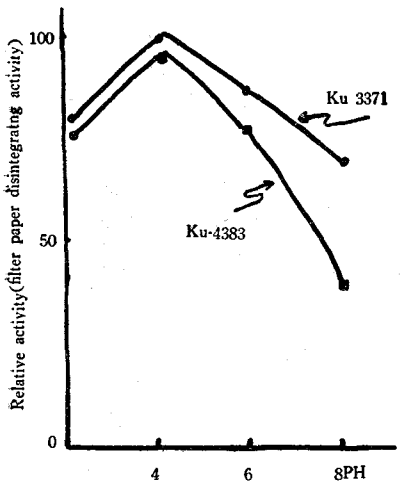


Fig. 1. The pH dependence of crude cellulase activity (38°C)

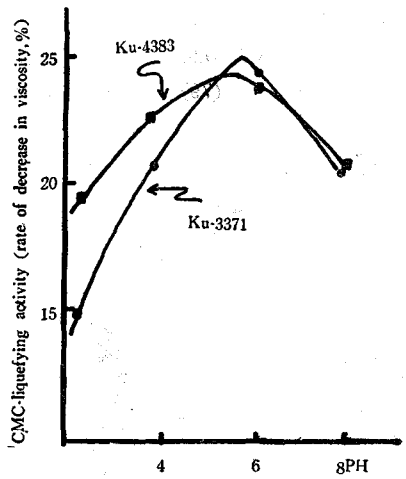


Fig. 2. The pH dependence of crude cellulase activity (40°C)

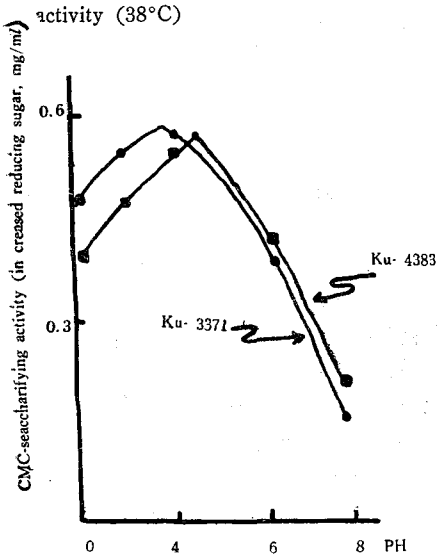


Fig. 3. The pH dependence of crude cellulase activity(40°C)

The optimum pH of filter pater disintegrating activity was the same pH 4.0 for Ku-3371 and Ku-4383. CMC-liquefying activity was the same pH 5.5 for them, but CMC-saccharifying activity was pH 4.0 for Ku-3371 and pH 4.5 for Ku-4383.

The optimum pH of each preparation for the CMC-liquefying activity was approximately by pH 1.0 higher than that for the CMC-saccharifying then there were a little difference among the optimum pH values of the three cellulases. The results from Fig. 3 are shown that CMC-liquefying and saccharifying activities were slightly more resistant those of alkalic pH range. These enzymes were usually stable at acidic pH range.

The optimum pH of fungal cellulase had been found in many experiments to be at 3.0, 50°C with only very slight variation dependings on strains, culture condition and other experimental conditions. The pH optimum of *Irpex lacteus* cellulase was 3.6 for CMC-saccharifying activity (73) and 5.0 for *Aspergillus saitoi* (18—19) and filter paper

disintegration activity had its optimum at pH 4.0 to 6.0 for *Trichoderma sp.* (37).

Fungal cellulases, generally, are stable at 30°C from pH 3.0 to 8.0, active from 3.5 to 7.0 and usually show optimum activity at pH 4.0 to 5.5 in citrate, phosphate or acetate buffer (74). The cellulase from *Curvularia lunata* was the pH optimum of 4.6 to 5.0 (75) and the cellulases of *Irpex lacteus* and *Trichoderma viride* at pH 4.1(76) Maximum activity of *Trichoderma viride* cellulase on foodstuffs was at pH 4.0(77). The pH optimum for the C_1 of *Trichoderma viride* was at pH 4.0 when cotton was the substrate (78) and 4.0 when it acted on hydrocellulose (79), and snail cellulase had the optimum pH of 5.6 (80). Bacterial cellulases show higher pH optima, often around 6.0(81) Nematode cellulase had broad pH optima 5.5 to 8.0(82).

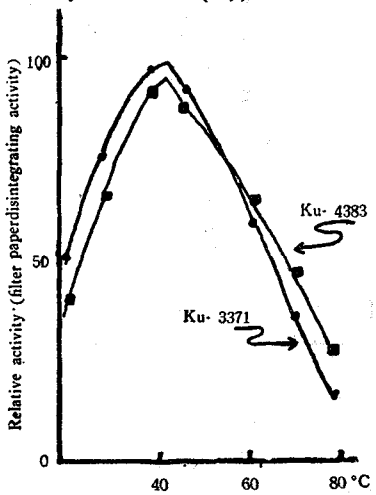


Fig. 4. Temperature dependence of crude cellulase activity (pH 4.0)

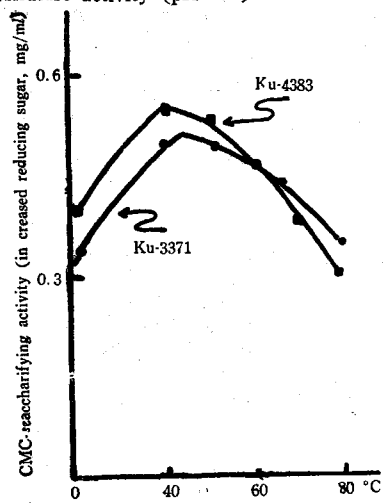


Fig. 6. Temperature dependence of crude cellulase activity (pH 4.0, Ku-3371 and pH 4.5, Ku-4383)

(2) Optimum temperature

Effects of temperature on filter paper disintegrating, CMC-saccharifying and CMC-liquefying activities were measured at various temperature from 20 to 80°C.

As shown Fig. 4, the optimum temperature of the filter paper disintegrating activity are 40°C for Ku-3371 and Ku-4383 at pH 4.0. And the optimum temperature of the CMC-liquefying activity are 40° C for Ku- 3371 and Ku-4383 at pH 4.0 and those of

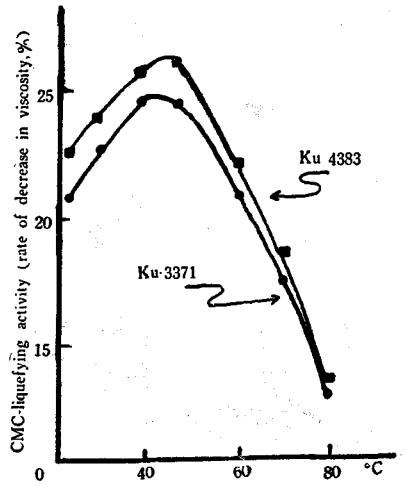


Fig. 5. Temperature dependence of crude cellulase activity (pH 4.0)

the CMC-saccharifying activity are 45°C for Ku-3371, Ku-4383 as shown in Fig. 5 and Fig. 6.

The cellulases of *Irpex lacteus* and *Trichoderma viride* retained 16 to 30 per cent or their original activity after minutes at 99°C (76). A partially purified cellulase form *Poria villantii* lost 44 per cent of its activity in 10 minutes at 70°C (83). The optimum temperature for the C_1 of *Trichoderma viride* acting on cotton was 40° (78) and 43°C when acting on hydrocellulose (79).

Above 43°C, hydrocellulase was rapidly inactivated. Fifty percent of the C_1 activity was lost in 5 minutes at 60°C and in one minute at 70°C. C_x from the same preparation lost 50 per cent of its activity in 15 minutes at 60°C, but was quite stable at 30°C (84). The action of *Myrothcium* cell-

ulase at 50°C was only one half that of its action at 30°C (85). Myers & Morthcote (80) found th-at the C_1 of purified snail cellulase was rapidly inactivated even at 30 and they used their enzyme at 25°C to digest cotton linters. C_x action for this preparation on cellofab. B (CMC) was optimum at 37°C.

(3) pH Stability

Crude enzyme solutions for Ku-3371, Ku-4383 adjusted to various pH values (form pH 2.2 to pH 8.0) with N-HCl, N-NaOH or McIlvaine's buffer solution were incubated at 30°C for 12 hours. And above the enzyme solution readjusted to its optimum pH 4.0, pH 4.5 with McIlvaine's buffer, N-Na OH or N-HCl solution.

The residual activities were estimated by filter paper disintegrating, CMC-saccharifying and CMC-liquefying activity.

As shown in Fig. 7, Fig. 8 and Fig. 9, the stable pH range was within 3.5 to 6.5, especially both of these enzymes activities at acidic range were more stable than neutral. And CMC-saccharifying activity began to cause remarkable inactivation at below pH 3.0 and over pH 7.0.

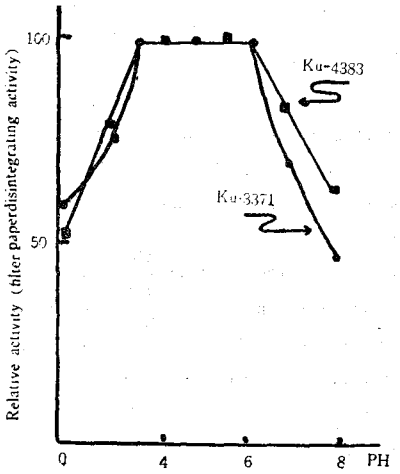


Fig. 7. The pH stability of crude cellulase activity

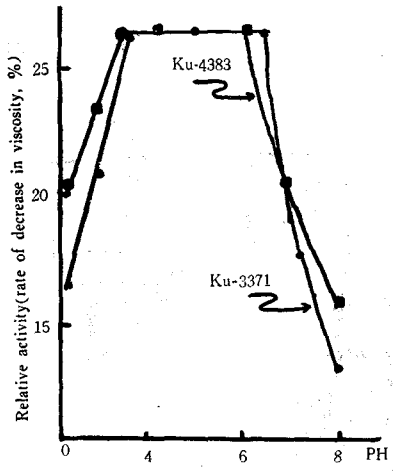


Fig. 8. The pH stability of crude cellulase activity

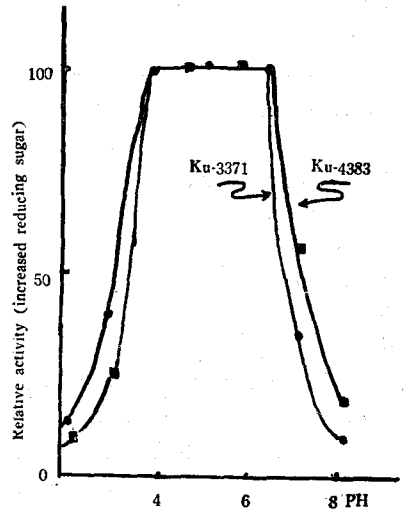


Fig. 9. The pH stability of crude cellulase activity

(4) Thermal Stability

Crude enzyme solution adjusted to pH 4.0 and with N-HCl, N-NaOH or McIlvaine's buffer solution were incubated at 20°C, 30°C, 40°C, 45°C, 50°C, 60°C and 70°C respectively. After 12 hours incubation, the residual activities were determined by filter paper disintegrating, CMC-liquefying and C MC-saccharifying activity.

As shown in Fig. 10, Fig. 11 and 12, the cellulase was fairly stable at below 45°C for 120 minutes on Ku-3371 and Ku-4383. The extent of the heat inactivation was 20—30% at 60°C for CMC-saccharifying activity compared with 10% at 50°C, 85% at 60°C for CMC-saccharifying activity compared with 10% at 50°C, 85% at 60°C for CMC-liquefying activity. But as Fig. 10 shows, at 50°C,

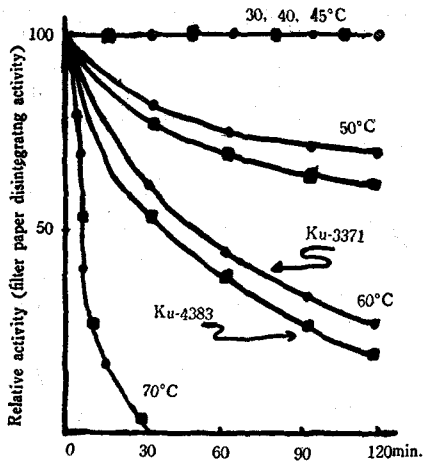


Fig. 10. Thermal stability of crude cellulase activity

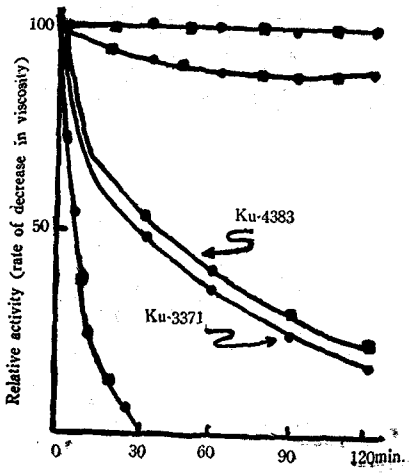


Fig. 11. Thermal stability of crude cellulase activity.

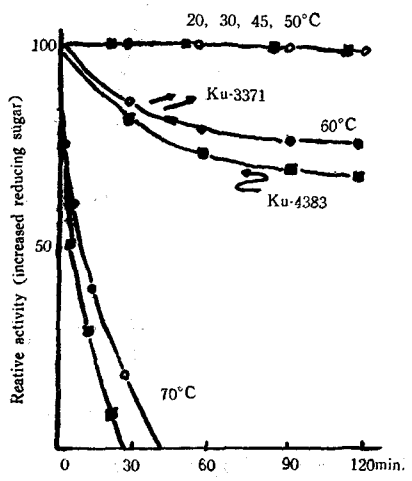
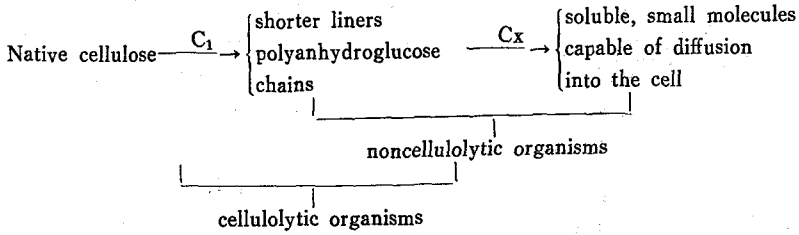


Fig. 12. Thermal stability of crude cellulase activity

filter paper *disintegrating activity* was decreased 25 % *inactivation* for Ku-3371 and 35% for Ku-4383 after 60 minutes. Most of cellulase completely inactivated at 70°C.

These results were indicated that the many cellulases produced from a strain of microorganisms were different in their properties. The data obtained indicated that these microorganisms are hydrolyzed the 1,4-ß-glucosidic linkage of natural cellulose or it derivatives. From these facts as *CMC-liquefying* and CMC-saccharifying activity it is postulated that cellulase, presumably converting native cellulase to sugars, consists of at least two systems. The first step, designated as C_1, occurs preliminary to hydrolysis of straight chain by Cx. The sequence is diagramed as follows.



As currently understood, (78), the degradation of native cellulase involves two types of enzymes designated C_1 and Cx. C_1 acts on crystalline cellulose in such a way that subsequent action by Cx becomes possible. The nature of the action of C_1 is not yet understood. It seems to act by breaking or loosening the forces that hold the cellulose molecules together. This results in hydration of portions of the cellulose chain. The exact relationship of C_1 to "Enzyme A" that is responsible for tensile strength loss and of "Hydrocellulase" that acts on suspensions of crystlline particles is not yet clear.

Cx is complex of enzymes hydrolyzing the β-1, 4-glucosidic bonds in the cellulose molecule (79—86). While they are usually endoenzymes (random acting), they may occasionally be exoenzymes removing glucose of cellobiose successively from the nonreducing end of the cellulose molecule in a manner analogous to that of glucoamylase and β-amylase (83, 87—88).

The cellulolytic microrganism does, of course, produce both C_1 and Cx as it grows on native cellulose. However, C_1 is not found in appreciable amounts in culture filtrates of most cellulolytic organisms, or in most commercial cellulase preparations. The best source of C_1 is *Trichoderma viride*, The cellulase of *Trichoderma viride* is much more effective in hydrolyzing native cellulose than the cellulase of other organisms. These results are in harmony with these observations.

(5) Effect of metallic ions on cellulase activity

Crude enzyme solution was dialyzed during 48 hours at 5°C. The mixture of 0.5% CMC, McIlvaine's buffer and dialyzed enzyme solution was adjust to 10^{-3}M concentration of various metallic ions. The effect of various metallic ions on enzyme activity was determined by CMC-saccharifying activity. These results shown in Table 4.

Table 4. The effect of various metallic ions on enzyme activity

Preparation	Concentration of metallic ions	Relative activity
Crude enzyme activity	none	107
Dialyzed crude enzyme solution	none	100
Dialyzed crude enzyme solution		
$+Ca^{2+}$	10^{-3}M	90
$+Ba^{3+}$	10^{-3}M	85
$+Fe^{2+}$	10^{-2}M	101
$+Fe^{3+}$	10^{-3}M	99
$+Mn^{2+}$	10^{-3}M	126
$+Zn^{2+}$	10^{-3}M	80
$+Co^{2+}$	10^{-3}M	113
$+Mg^{2+}$	10^{-3}M	100
$+Cd^{2+}$	10^{-3}M	80
$+Hg^{2+}$	10^{-4}M	6

As shown in Table 4, the CMC-saccharifying activity was slightly activated by Mn^{2+}, Co^{2+}, at concentration of 10^{-3}M but no effect by Mg^{2+}, Fe^{2+} and cellulase are inhibited by Ca^{2+}, Ba^{2+}, Zn^{2+} and Cd^{2+} at the same concentrations. Hg^{2+} showed remarkable inhibition at concentration of 10^{-4}M.

Generally speaking, cellulases are inhbited by Hg^{2+}, Al^{3+}, Cu^{2+}, Cr^{2+} Pb^{2+} and Zn^{2+} at the concentration of 10^{-3}M (74).

However, there are many exceptions. Especially Hg^{2+} was strong inhibitory (83, 88), *Aspergillus saitoi* carboxymethylcellulase was inhibited by Hg^{2+}, Mn^{2+}, Al^{2+}, Mg^{2+}, Ca^{2+} and Pb^{2+}. Hg^{2+} was most effective followed by Pb^{2+}, Mn^{4+} at concentration of 10^{-2}M. While Mn^{2+} and Ca^{2+} had slightly inhibiting effects. On the other hand, Cu^{2+}(10^{-3}) and Co^{2+}(10^{-4}M) increased activity at the optimum pH to 170 per cent and 160 per cent respectively(21).

Summary

Out of some 400 strains of Microorganisms, cellulase forming organisms was isolated from night soil during the course of screening tests. Two str ains, Ku-3371 and Ku-4383 were found capable of producing cellulase in the shaking culture.

General properties of the crude enzyme were as the following results.

1. The optimum pH values on CMC-saccharifying, CMC-liquefying and filter paper disintegrating activities were 4.0 to 5.5.
2. The stable pH range was within 3.5 to 6.5,
3. The optimum temperature was 40—45°C, the

thermal stability was below 50°C except on paper disintegrating activity and completely inactivated at 70°C.

4. Dialyzed crude enzyme was activated by Mn^{2+} and Co^{2+} repectively but Hg^{2+} was strong inhibitor.

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