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섬유소분해균의 분리및 그의 생리학적 특성

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Isolation of Cellulolytic Microorganisms and their Physiological Characteristics

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ABSTRACT

Cellulolytic microorgnaisms were isolated from the various sources and four of them were identified as *Trichoderma koningi*, *Aspergillus niger*, *Penicillium chrysogenum*, and *Streptomyces* sp. The induction of extracellular cellulase of these species in the liquid culture media containing carboxymethylcellulose (CMC) or Avicel as inducer showed that CMC was a better effective inducer for the production of CMCase (Cx cellulase component) as well as Avicelase (C_1 cellulase component) than Avicel. It is believed that certain hydrolysis products of cellulose (CMC) could serve as an inducer for an enzyme synthesis. In *T. koningi*, *Asp. niger*, and *Streptomyces* sp., the optimum temperature of CMCase on CMC-culture medium was 50° but temperature around 40°C was found to be optimum for the activities of CMCase prepared from *P. chrysogenum*. The optimum temperature for Avicelase activities on Avicel-culture media of *T. koningi* and *P. chrysogenum* was 40°C whereas temperature 50°C was found to be optimum for Avicelase from *A. niger* and *Streptomyces* sp. The optimal activities of these CMCase and Avicelase prepared from. *T. koningi*, *Pen. chrysogenum* and *Streptomyces* sp. were found similarly to be at *p*H's around 5.4 and 6.0, while *p*H 4.8 was optimum for the activities of CMCase and Avicelase from *A. niger*, indicating that *A. niger* in acidic media would yield an enzyme of high activity.

INTRODUCTION

It has been reported that the culture filtrates of cellulolytic microorganisms, such as *T. viride* and *A. niger*, contained multiple cellulase components (Niwa *et al.*, 1965; Okada *et al.*, 1966; Shibata *et al.*, 1969; Nisizawa *et al.*, 1972; Yoshikawa *et al.*, 1974). Although these cellulase components seemed to have a rather varying degrees of specificities toward a series of cellulosic substrates ranging from cotton fibers to cello-oligosaccharides. It was shown that the rates of their activities for Avicel and CMC were remarkably different.

Since the proposition of Reese *et al.* (1950) for the presence of enzyme C_1 and Cx

in cellulase components of *T. viride* that different in their substrate specificities toward cotton fiber, a number of investigators have examined multiplicity of this enzyme in some microorganisms. According to their theories, C_1 can attack native cellulose of higher crystallinity and gives various fragments without a production of reducing sugar, while Cx cannot attack such higher crystalline cellulose, but attack the cellulose fragments which are produced by the action of C_1.

Later this theory was generally accepted and supported by Selby *et al.* (1967) and Wood (1968). These authors have succeeded in fractionating C_1 component from the culture filtrates of *Trichoderma, Penicillium* by combined column chromatography, and they observed a synergism between purified C_1 and Cx components.

However, Nisizawa *et al.* (1972) and Tomita *et al.* (1974) showed in the recent works that C_1 and Cx components, proposed originally by Reese *et al.* (1950), might correspond to Avicelase and CMCase, respectively, and proposed that Cx initiated the degradation of native cellulose, and C_1 could saccharify actively the cellulose fragments produced by Cx. In order to prove this concept, they succeeded in the purification of Avicelase and CMCase, and their theory was supported by the purification and accertainment of these C_1 and Cx cellulase components.

On the other hand, earlier investigations (Shibata *et al.*, 1969; Yamane *et al.*, 1970; Nisizawa *et al.*, 1971) of the cellulase formation in *T. viride* and *Pseudomonas fluorescens* var. *cellulosa* were carried out using cultures on various carbohydrates for a long time The results obtained were very difficult to analyze in detail. The production of C_1 and Cx cellulase components in the microorganisms is influenced by the physiological conditions to which the microorganisms are grown.

It seems therefore very interesting to examine what culture conditions are suitable for the induction of extracellular Cx and C_1 cellulase components of 4 microorganisms isolated and identified by the authors. In order to study this physiological characteristics, CMCase and Avicelase formation of 4 species were measured when the CMC or Avicel was added seperately to culture media as an inducer. In addition, the optimal *p*H's and temperature's on the activities of CMCase and Avicelase were observed with the crude enzyme.

MATERIALS AND METHODS

1. Isolation of cellulolytic microorganisms

The cellulolytic microorganisms were collected from the compost, rotten woods, decayed leaves and soils. These samples were dispersed in saline and spores suspended were streaked on the selective media containing 2% CMC as a sole of carbon source. After 3-day culture at 30°C, colonies grown on the plates were isolated. These microorganisms were tested on the decomposition of filter paper strip (1.5×4 cm) in the basal medium ($NaNO_3$, 3g; KH_2PO_4, 1g; KCl, 0.5g; $MgSO_4$ $7H_2O$, 0.5g; $FeSO_4$ $7H_2O$, 0.01g; in 1,000m*l* distilled water) with 10% wheat bran extract in the test tubes. The activities of inducible emzymes from these microorganisms were measured in liquid culture media using test tubes containing 5m*l* of basal medium with 1% CMC or Avicel.

2. Culture conditions for inducible cellulase

The cellulolytic microorganisms which had been selected were grown on the basal medium (100 m*l*) containing 1% CMC or 1% Avicel as a carbon source in 500m*l* Erlenmeyer flasks. The flasks were incubated at 30°C on a rotary shaker of 200 rpm.

3. Enzyme preparation

After inoculation of microorganisms in liquid culture media, 15m*l* aliquots of the whole culture were removed at time intervals, and centrifuged at 5,000 rpm for 30min. The supernatant was used for the extracellulase assay.

4. Substrates

Sodium-carboxymethylcellulose, CMC (d.S 0.63), was supplied by Daiichi Kogyo Seiyaku Co., Ltd.

Avicel was a commercial product of microcrystalline cellulose powder from Hunakoshi Pharmaceutical Ltd.

Filter paper used in this experiment was No.2 of Toyo Roshi Kaisha, Ltd.

5. Enzyme assay

CMCase or Avicelase activity was measured in the reaction mixture of 0.25 m*l* of 1% CMC or Avicel, 0.65m*l* of 0.02M acetate buffer (*p*H 5.4) and 0.1m*l* of enzyme solution. After incubation at 30°C for 60 min, activities of CMCase or Avicelase were analyzed according to the method of Somogyi-Nelson.

6. Determination of *p*H

The *p*H meter used was of the Perkin Elmer Coleman 28C model.

RESULTS

1. Isolation and identification of cellulolytic microorganisms.

About 350 strains were isolated by the screening method and 4 distinct species of cellulolysis with respect to the decomposition of filter paper strip, hydrolysis of CMC and degradation of Avicel, were used in this experiment. These microoganisms were identified as *Trichoderma koningi*, *Aspergillus niger*, *Penicillium chrysogenum* and *Streptomyces* sp.

2. The change of CMCase activities on CMC-culture media or Avicel-culture media

The CMCase activities of 4 species during the 5-day culture period were observed.

The results are shown in Figs.1 and 2. Fig. 1 illustrates the increase of CMCase activities of 4 microorganisms on CMC-

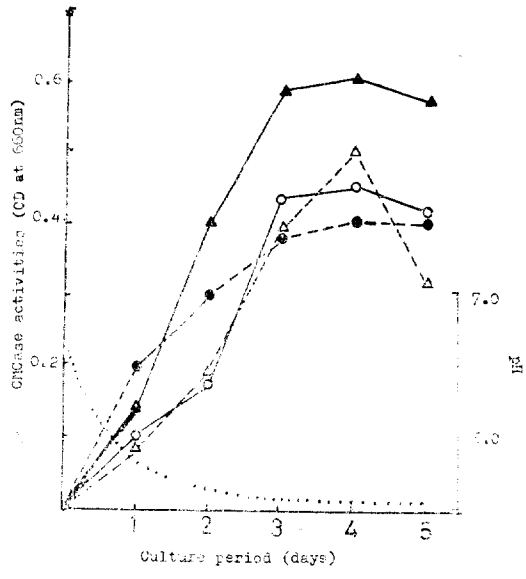


Fig. 1. Time course of the CMCase induction on CMC-culture media of 4 species.

The CMC-culture media were consisted of basal medium and 1% CMC in Erlenmeyer flasks. After the inoculation, the flasks were incubated at 30°C on a rotary shaker of 200 rpm. ▲——▲, *T. koningi;* △·····△, *P. chrysogenum;* ○—○, *Streptomyces* sp.; ●·····●, *A. niger;* ·····, *p*H

culture media up to 4-day culture and the decrease thereafter. *T. koningi* showed the highest activity of CMCase while *Streptomyces* sp., *A. niger* and *P. chrysogenum* showed lower production of CMCase during the culture period.

However, CMCase activities of 4 species when Avicel was used as inducer were significantly lower than those of CMC-culture media as shown in Fig. 2. Especially, in *P. chrysogenum* and *Streptomyces* sp., the activities of CMCase induced in the presence of Avicel were decreased to 50% of those on CMC-culture media. But by the observation of CMCase induction on Avicel, *T. koningi* showed the highest CMCase production and the next highest was *A. niger*.

As shown in Figs. 1 and 2, *T. koningi* gives rise to the most excellent activity among 4 species in CMCase production on either CMC or Avicel as inducer.

Initial *p*H of the culture media (Fig.1—4) was 6.4, but it was acidified to be at 5.6 after 5-day culture.

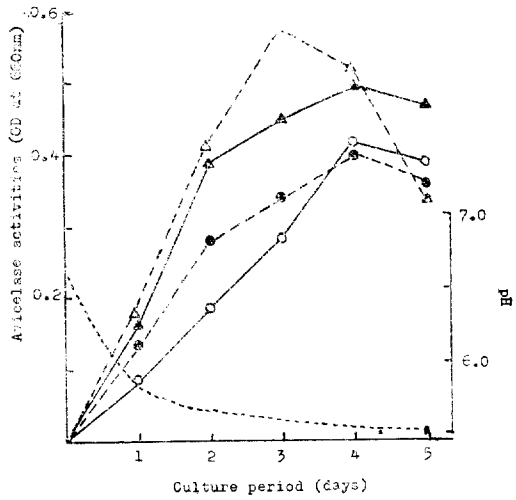


Fig. 3. The change of Avicelase activities on CMC-culture media of 4 species.
All conditions and abbreviations were the same as in Fig. 1

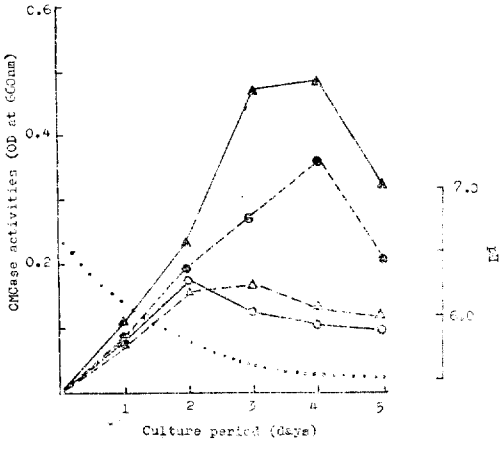


Fig. 2. Time course of CMCase induction on Avicel-culture media of 4 species.
All abbreviations and conditions were the same as in Fig. 1 with the exception of Avicel in place of CMC as an inducer.

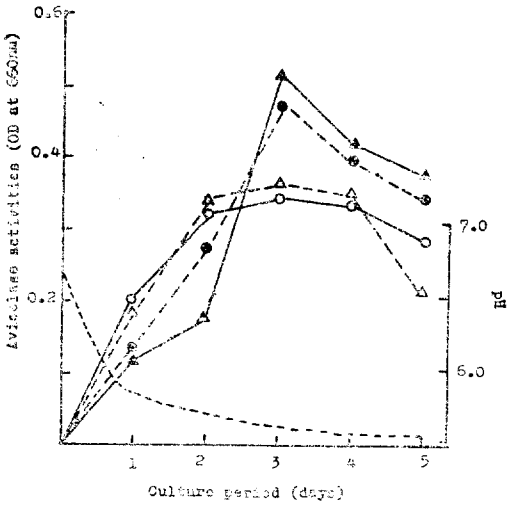


Fig. 4. The change of Avicelase activities on Avicel-culture media of 4 species.
All cenditions and abbreviations were the same as in Fig. 2

3. The changes in Avicelase activities on CMC-culture and Avicel-culture media

The result of the changes in Avicelase activities of 4 species on CMC or Avicel, as an inducer during the culture period were shown in Figs.3 and 4.

The yields of Avicelases in all 4 species grown on CMC-culture media (Fig. 3) were increased till 3 to 4-day period and particularly *P. chrysogenum* showed the highest Avicelase induction among the species. The next highest formation was *T. koningi*. On the other hand, the Avicelase formation of all 4 species grown on Avicel-culture media (Fig.4) was increased till 3-day culture period, and the order of high Avicelase production was *T. koningi*, *A. niger*, *P. chrysogenum*, and *Streptomyces* sp. Comparing Fig.3 with Fig. 4, the result showed that CMC was a better inducer for Avicelase production than Avicel, for example, in *Pen. chrysogenum*, the optical density at 660nm of maximum Avicelase activiy was 0.37 when Avicel was added on culture medium. meanwhile, it was 0.59 when CMC was used as inducer.

From these results as shown in Figs.1—4, CMC was an effective inducer for the production of both CMCase and Avicelase.

4. Optimal temperature's on cellulase activities

The reaction mixtures were incubated at temperatures rising every 10°C from 20°C to 70°C, and the enzyme activities were compared. The results of optimal temperatures for crude CMCase prepared from CMC-culture media of 4 species are shown in Fig. 5.

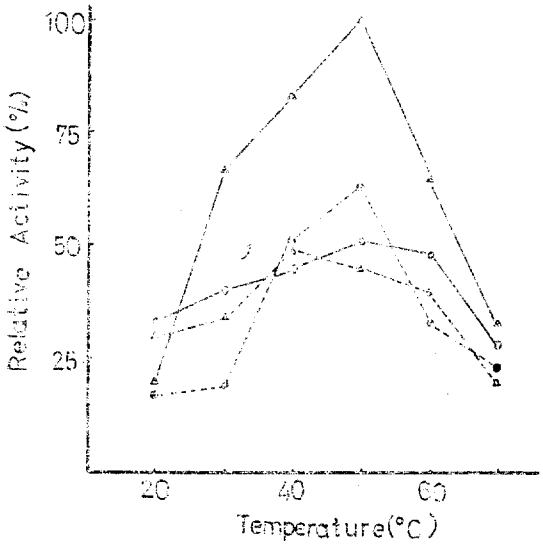


Fig. 5. Influence of temperature on CMCase prepared from CMC-culture media of 4 species.
▲——▲, *T. koningi*; △·····△, *Pen. chrysogenum*; ○——○, *Streptomyces* sp.; ●·····●, *Asp. niger*

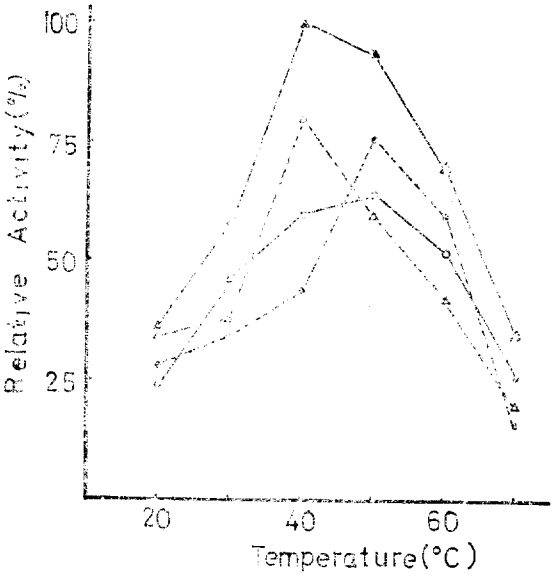


Fig. 6. Influence of temperature on Avicelase prepared from Avicel-culture media of 4 species.
All abbreviations were the same as in Fig. 5

The optimum temperature of CMCases of *T. koningi*, *A. niger* and *Streptomyces* sp. was found to be at 50°C, while that of CMCase of *P. chrysognum* was at 40°C.

Similar results were also obtained from crude Avicelase on Avicel-culture media of 4 species (Fig.6).

According to the results of Figs. 5 and 6, the optimum temperature range of CMCase and Avicelase were similar in *Streptomyces* sp., *P. chrysogenum* and *A. niger* being around 40—50°C. However, in *T. koningi*, the optimum temperature of CMCase was at 50°C, and 40°C was found to be optimum for the activity of Avicelase,

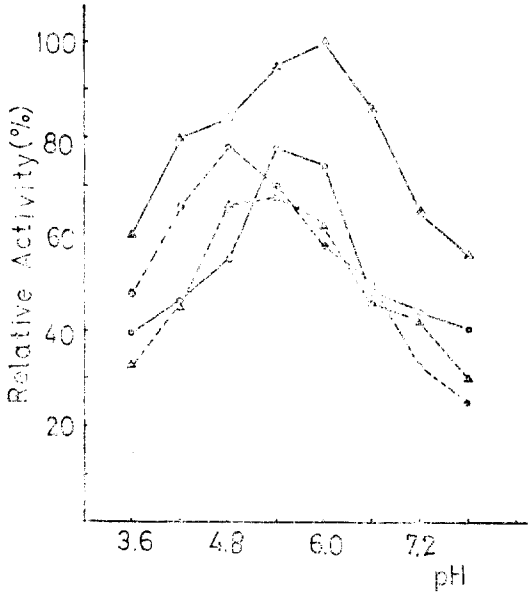
5. Effect of *p*H on cellulase activity

As shown in Figs.7 and 8, the optimal *p*H's on CMCase activity on CMC-culture media and Avicelase activity in culture media of Avicel of 4 species were investigated. The optimal *p*H's of CMCase and Avicelase were examined at 30°C using acetate buffer and phosphate buffer at various *p*H values.

The optimum activity of CMCase on CMC-culture media (Fig. 7) was found at *p*H 5.4—6.0 in *T. koningi*, *Pen. chrysogenum* and *Streptomyces* sp. whereas the highest activity of CMCase of *Asp. niger* was found in acidic condition to be at *p*H 4.8. Moreover the optimal *p*H's of Avicelase of 4 species on Avicel-culture media were similar to those of CMCase activity.

DISCUSSION

The induction of CMCase and Avicelase



Fie. 7. Optimal *p*H's of CMCase prepared from CMC-culture media of 4 species.
All abbreviations were the same as in Fig. 5

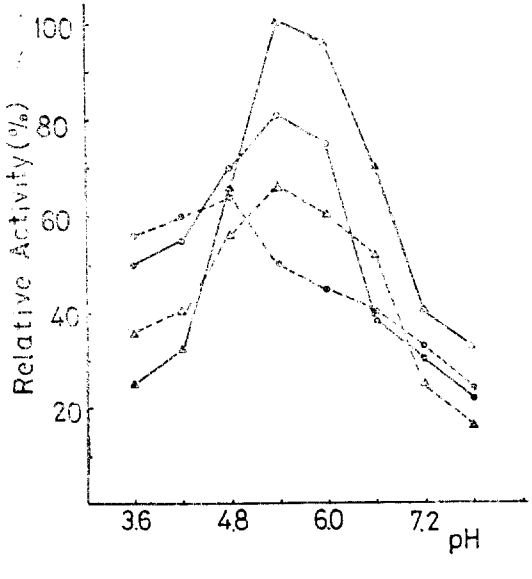


Fig. 8. Optimal *p*H's of Avicelase prepared from Avicel-culture media of 4 species.
All abbreviations were the same as in Fig. 5

production on CMC or Avicel as a carbon source was increased during 3-day incubation, but decreased after 4-day culture. (Figs. 1—4). The result of the decrease of enzyme production during the late culture period seemed that glucose produced by microorganisms based on the degradation of CMC or Avicel, is accumulated and inhibits the new cellulase formation by the mechanism of feedback inhibition and catabolic repression.

Moreover CMC was found as an more effective inducer for the production of C MCase and Avicelase (Cx and C_1 cellulase components) than Avicel. This result may be considered that CMC was more effective for the cellulase formation on the basis of the concept of lactose operon, on the mechanism that certain hydrolysis products of cellulose might serve as an inducer for the enzyme synthesis. Nisizawa *et al.* (1971) had reported the respectable effectiveness for cellulase induction by using sophorose, CMC and Avicel as inducers in *T. viride*, and inducers have been studied to elucidate the overall process of cellulase production in *P. fluorescens* var. *cellulosa*. These experiments using resting cells revealed that sophorose showed a strong inductive potency for cellulase and other cello-oligosaccharides, CMC and Avicel were appreciably effective for induction. This fact that CMC and Avicel were effective for cellulase induction, was coincided with our results.

However, during the experiment of cellulase induction, the change of *p*H in culture media were observed. The initial *p*H was 6.4 and it was lowered to 5.6 with 5-day culture. It is considered that the decrease of *p*H was principally caused by the acid formation from glucose produced by cellulase of microorganisms.

As shown in Figs.5 and 6, the result of the optimal temperature's of CMCase and that of Avicelase prepared from *Streptomyces* sp., *P. chrysogenum* and *A. niger* were similar. Eut the optimum temperature on CMCase activity of *T. koningi* was found at 50°C while 40°C was found to be optimum for the activity of Avicelase. This different result of *T. koningi* was considered that CMCase and Avicelase produced by microbial cells have different properties than any other species. As shown in Figs.5 and 6, cellulases of *Streptomyces* sp. have higher optimum temperature than mesophilic fungi such as *T. koningi* and *P. chrysogenum*. This fact is agreeable with the result of Stutzenberg F.J.(1972).

According to the observation of optimal *p*H's of CMCase and Avicelase as shown in Figs.7 and 8, the optimum *p*H of cellulase activity was at 5.4—6.0 in *Streptomyces* sp., *T. koningi* and *P. crysogenum* while that of cellulase from *A. niger* was at 4.8. This seems to explain the fact that *A. niger* grows well in acidic media.

摘 要

1. 퇴비, 낙엽및 토양등에서 분리한 섬유소분해균 중에서 CMCase (Cx cellulase component)와 Avicelase (C_1 cellulase component)의 활성도가 가장 우세한 4종류의 균주를 선택하여 동정한 결과 *Aspergillus niger*, *Trichoderma koningi*, *Penicillium chrysogenum* 및 *Streptomyces* sp.로 확인되었다.

2. 4가지 균주별로 CMCase와 Avicelase induction에 관한 실험을 실시한 결과 inducer로서 CMC가 Avicel보다 CMCase, Avicelase 생성에 보다 효과적이었다.

특히 *P. chrysogenum*의 cellulase induction은 다른 3균주보다도 CMC가 효과적이었다.

3. CMCase 활성의 최적온도는 *T. koningi*, *A. niger*, *Streptomyces* sp.의 경우 50°C이었고 *P. chrysogenum*은 40°C로 나타났다.

한편 Avicelase 활성의 최적온도는 *T. koningi*와 *P. chrysogenum*은 40°C로 *A. niger*와 *Streptomyces* sp.는 50°C로 나타났다.

4. CMCase의 최적 *p*H는 *T. koningi*, *P. chrysogenum* 및 *Streptomyces* sp.가 5.4—6.0이었고 *A. niger*는 다소 약산성인 4.8로 나타났다. 이것은 *A. niger*가 산성인 조건에서 생육을 잘 할 수 있음으로 최적 *p*H가 낮은 것으로 추측된다.

또한 Avicelase의 최적 *p*H를 조사한 결과는 CMCase의 결과와 대동소이하다.

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