\( \beta \)-Glucosidase Formation in *Cellulomonas* sp.

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**SUMMARY**

To elucidate \( \beta \)-glucosidase formation on various carbon sources by cellulolytic bacteria, *Cellulomonas* sp. CS1-1, the strain was grown on Nutrient Yeast Broth, carboxymethyl cellulose, avicel and cellobiose using a Quickfit FVIL fermentor operated in batch, and the growth characteristics on those substrates and \( \beta \)-glucosidase distribution of extra and intracellular enzyme components were studied. The results were: 1) \( \beta \)-glucosidase was always intracellular, and was formed under all growth conditions tested. ii) but levels of relative activities were higher when the culture was grown on cellobiose and on avicel. iii) the relative activities were always maximum during the growth phase of the organism irrespective of the substrate used.

**INTRODUCTION**

\( \beta \)-Glucosidase (\( \beta \)-D-glucoside glucohydrolase, EC 3.2.1.21), capable of hydrolysing \( \beta -(1\rightarrow 4)\) glucosidic linkage, are known to be widely distributed among microorganisms and plants and to vary the enzymatic properties with the sources. Many studies on these enzymes from microbial origin have been reported as well as those from sheep rumen liquor \(^2\), pig intestine\(^3\) and almond-emulsion\(^4\). Duerksen et al. investigated intensively the properties of yeast \( \beta \)-glucosidase, ie constitutive production by the strains of *Saccharomyces lactis* and their catalytic and immunologic properties \(^{14-15}\); differences in substrate specificity between *Candida tropicalis* and *Saccharomyces cerevisiae*\(^5\). The properties of fungal \( \beta \)-glucosidase from *Stachybotrys atra* were studied by Jermyn \(^{10-12}\) and those from *Myrothecium verrucaria* by Hash et al. \(^7\). Srinivasan et al. purified and characterized \( \beta \)-glucosidase of *Atcaligenes faecalis* (ATCC 21400)\(^{15-19}\). Schaefer reported on the induction of aryl-\( \beta \)-glucosidase and the genetics of the mutant system in *Escherichia coli* K-12 \(^{12-18}\).

The synergistic effects of \( \beta \)-glucosidase with cellulases, \( C_1 \) and \( C_2 \), were often noticed in cellulose degradation \(^{20-22}\). Mutants of the cellulolytic bacteria, *Cellulomonas* sp. CS1-1 produced higher level of \( \beta \)-glucosidase than the parent, and \( \beta \)-glucosidase from one of the mutants which is partially resistant to catabolite rep-

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ression by glucose or cellobiose was markedly induced when grown on glucose\(^{(1)}\). Attempts were made to elucidate i) whether the *Cellulomonas* sp. CSI-1 produce β-glucosidase only on cellulolitic substrates or non-cellulolitic substrates as well, if thus ii) what the enzyme levels on those substrates are, and iii) whether the enzyme released into the culture media.

**MATERIALS AND METHODS**

**Bacterial Strain**

The strain used was *Cellulomonas* sp. CSI-1 isolated from the environment in the previous study\(^{(6)}\) and has been maintained in lyophilized ample.

**Media**

The organism was grown on the following media: i) Nutrient Yeast Broth(NYB) which consisted of 2.5% Oxoid Nutrient Broth and 0.5% yeast extract. ii) 0.5% carboxymethyl cellulose(CM-cellulose, with 0.7-0.8 degree of substitution, BDH Ltd., England) in Dubos' salts solution\(^{(4)}\) supplemented with 0.02% yeast extract, iii) 1.0% avicel (Avicel SF, Asai Kasei, Industrial Co., Japan) in Dubos' salts solution supplemented with 0.02% yeast extract, iv) 0.5% cellobiose (D-Cellbiose, Sigma Chemical Co., U.S.A.) in Dubos' salts solution supplemented with 0.02% yeast extract.

Dubos' medium consists of 0.1g K₂HPO₄, 0.05g MgSO₄·7H₂O, 0.1g NaNO₃, 0.05g NaCl and a trace of FeCl₃ in 100 ml of tap water, the final pH being adjusted to 7.0-7.2, but NaCl was omitted in this work.

**Cultivation**

These growth experiments were carried out in a Quickfit FVIL 1 litre fermentor operated as a batch fermentor at 30°C and without pH control. Air supply was set to give 100% D.O.T. for each medium prior to inoculation, then the rate of air supply was not varied throughout the period of cultivation.

The inoculum was prepared by growing the stock culture on NYB overnight at 30°C on a reciprocal shaker, and washing it in saline to minimize possible carry over of nutrients, then resuspending it in Dubos' salts solution to get the optical density of 0.5 at the wavelength of 610nm. using a Bausch Lomb spectrophotometer. This was added to growth media to a final concentration of 1% by volume. Initial titre of 1 to 3×10⁹ cells/ml of culture was obtained through this procedure.
Enzyme Preparation

Culture samples were taken at the appropriate time intervals and centrifuged at 10,000g for 20 min. at 2°C. The supernatant was then used as the extracellular enzyme extract without further treatment. The pelleted cells were washed in saline and resuspended in McIlvaine buffer, pH 7.0. The cells were then smashed by sonicating for a total of four minutes. The extracts were centrifuged at 15,000g for 20 min. at 2°C to remove cellular debris and the supernatant was used as the intracellular enzyme preparation.

Measurement of Growth

The determination of the number of viable cells in a culture was achieved by a serial dilution in diluent (10% NYB in saline) then plating onto Nutrient Agar. The plates were incubated for 48 to 72 hours at 30°C before counting.

Assay of β-Glucosidase Activity

β-Glucosidase activity was estimated by measuring the release of p-nitrophenol (PNP, BDH Ltd., England) from p-nitrophenyl-β-D-glucoside (PNPG, Sigma Chemical Co., U.S.A.) (9). Enzyme extract (1.0 ml) was added to the preincubated solution (at 40°C for 10 min.) which contained 1.5 ml of McIlvaine buffer, pH 7.0, and 0.5 ml of 0.005 M PNPG. After incubation for 20 minutes at 40°C, the enzyme activity was stopped by adding 2 ml of 1M sodium carbonate. The yellow colour that developed was read at 400nm in a Varian Techtron UV-VIS Recording Spectrophotometer, Model 635.

One enzyme unit is defined as the amount of enzyme which liberates 1 mole of PNP per minute. For specific activity this is calculated per mg of protein.

Protein Determination

Protein was determined by the colorimetric procedure of Lowry et al. (19) with the Folin-Ciocalteau reagent, with crystalline bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Growth Characteristics and β-Glucosudase Formation on Nutrient Yeast Broth.

The results of this experiment are given in Figure 1. Growth reached stationary
phase in approximately 30 hours with a maximum viability of 1 to $1.5 \times 10^9$ cells/ml. Mean generation time in exponential growth phase was 2.57 hours and specific growth rate 0.27 hour$^{-1}$. During the incubation period of 12 days the pH increased from pH 7.0 to pH 8.2 and it was only after 9 days that the total number of viable cells started to fall. But the intracellular protein did not drop after the culture entered in death phase, this implies the cells were not lysed immediately although they lost viability.

![Graph](image)

Fig. 1. Growth and β-Glucosidase Formation on NYB. The strain CSI-I was grown on Nutrient Yeast Broth using Quickfit FVIL fermentor operated in batch. Growth (—○—); intracellular protein (—Δ—); pH (―□―); β-glucosidase, extracellular (—□―) and intracellular (—○—).
No $\beta$-glucosidase was detected in the extracellular preparation, however, this was detected in the intracellular extracts. The level of the enzyme activities was relatively high at the early stage of stationary phase then decreased gradually.

**Growth Characteristics and $\beta$-Glucosidase Formation on CM-Cellulose.**

As shown in Figure 2, the bacterial growth reached stationary phase in approximately 48 hours with a maximum viability of 2.0 to 2.5 x $10^9$ cells/ml of culture. Mean generation time in exponential growth phase was 3.47 hours and specific growth rate 0.2 hour$^{-1}$. Because the strain CS 1-1 has a complex requirement for growth factor, addition of yeast extract to the media was necessary and this should be regarded in interpretation of above data i.e. mean generation time and specific growth rate. Change in pH of the culture was minor and this is possibly due to the buffer action of CM-cellulose. The viability bid not drop during the experimental period of 12 days, owing to pH stability of the media.

![Graphs showing growth and $\beta$-Glucosidase Formation on CM-cellulose.](image)

Fig. 2. Growth and $\beta$-Glucosidase Formation on CM-cellulose. Media was 0.5% CM-cellulose in Dubos' salts solution supplemented with 0.02% yeast extract. The others refer to the legends to Figure 1.
Low levels of intracellular $\beta$-glucosidase was detected but no extracellular enzyme was detected.

Growth Characteristics and $\beta$-Glucosidase Formation on Avicel.

The results were as shown in Figure 3. Stationary phase was obtained after approximately 72 hour with a maximum viability of 8 to $9.5 \times 10^6$ cells/ml. Growth was slow on avicel with mean generation time in exponential growth phase of 7.12 hours and specific growth rate of 0.1 hour$^{-1}$, reminding that these data were also affected by the supplement. It was noticeable that maximum viability of this strain on avicel was approximately 4 times higher than that on CM-cellulose, although the growth rate on avicel was slower than that on CM-cellulose. Cell viability and total enzyme activity started to decrease after 6.5 days when the pH of the culture media dropped to below pH 5.0.

![Graph](image)

*Fig. 3. Growth and $\beta$-Glucosidase Formation on Avicel. Media was 1.0% avicel in Dubos’ salts solution supplemented with 0.02% yeast extract. The others refer to the legends to Figure 1.*
β-Glucosidase was again detected in the intracellular extract but not in the extracelluar preparation. The levels were higher at the late exponential phase and early stationary phase. Marked decrease in β-glucosidase activity after 6.5 days was considered to be due to its unstability in lower pH.

**Growth Characteristics and β-Glucosidase Formation on Cellobiose.**

The results of this experiment are given in Figure 4. Growth reached stationary phase after approximately 24 hours with a maximum viability of $2.5 \times 10^9$ cells/ml of culture. Mean generation time in exponential growth phase was 2.54 hours and specific growth rate 0.27 hour$^{-1}$. Although growth was faster than on any cellulosic substrates used, the maximum viable cell yield of this culture did not exceed that for the CM-cellulose culture. Cell viability and β-glucosidase activity were markedly decrea-
sed after approximately 60 hours when the pH of the culture dropped below pH 5.0. The decrease in pH on avicel and cellobiose culture was found to be mainly due to nonvolatile acids, such as α-ketoglutarate, lactate, pyruvate, etc.\(^{(1)}\). Above results also shows the optimum pH for growth of the strain CS1-1 to be around pH 7.0.

β-Glucosidase was detected in the intracellular extract but no extracellular enzyme was detected.

Han et al. reported that β-glucosidase of *Alcaligenes faecalis* ATCC 21400 had a wide pH range with optimal activity at pH 6.0 to 7.0 and was stable at pH 6.5 to 7.8 when kept at 30°C for 2 hours in solution\(^{(6)}\). But the enzyme from other sources appeared to have different pH optima from above bacterial origin i.e., pH 5.35 for *Myrothecium verrucaria*\(^{(7)}\), pH 5.5 to 5.6 for almond-emulsin\(^{(8)}\), pH 5.4 to 5.8 for sheep rumen liquor\(^{(9)}\).

During the growth experiments illustrated in Figure 1 to 4 it was appeared that the maximum growth on different media varied considerably. The results were recalculated to standardize for the protein content. Prior to assay of the intracellular enzymes the cells were smashed by sonication and cell debris removed by centrifuga-

![Fig. 5. Specific Intracellular β-Glucosidase of NYB(—△—), CM-Cellulose(—□—), Avicel(—Ο—), and Cellobiose(—□—) Culture.](image-url)
tion. The amount of enzyme released by this treatment was assayed as was the total protein released by this procedure. From these data specific activity of the intracellular enzyme was calculated and the results in Figure 5 can be summarized as follows:

i) $\beta$-glucosidase was always intracellular, and was formed under all growth conditions.

ii) but levels of relative activities were higher when the culture was grown on cellobiose and on avicel.

iii) the relative activities were always maximum during the growth phase of the organism irrespective of the substrate used.

Above results were coincide with chromatograms of reducing sugars obtained by Hegott(69) which indicated that the culture media of CS1-1 contained considerable amounts of cellobiose and others but only trace of glucose. It would be possible that the strain CS1-1 excretes enzyme which degrade cellulosic substrate into cellobiose which can be absorbed through cell membrane and utilized.

Bacterial $\beta$-glucosidase was known to be able to hydrolyse cellobiose and aryl-$\beta$-glucoside(69). These experiments did not include tests for substrate specificities of $\beta$-glucosidase from the strain CS1-1, therefore whether $\beta$-galactoside, $\alpha$-nitro-phenyl-$\beta$-D-galactoside and lactose can be hydrolysed by this enzyme was not known. This remained for further study with other biochemical approaches.

REFERENCES

Cellulomonas sp.의 β-글루코시다아제 생성

작용 영역

심유소 분해균 Cellulomonas sp.CS1-1의 가장 흔히 사용되는 β-글루코시다아제 생성을 살펴보기 위하여 Quic-
krift, FVII 발효장치를 이용하고, 뉴트릴로프트 이스트브로스, 간포복분비질물로소, 아미질, 섬유오스
등을 단소원으로 한 배지에 빗치로 배양하여 그 배양액 특성과 세포내 또는 세포외의 β-글루코시다아제의 분
포를 검토한 결과 : 1) β-글루코시다아제는 공시한 모든 배양 조건하에서 생성되었고 세포내 효소로서 배양액
액에서는 검출되지 않았다. ii) 뉴트릴로프트 이스트브로스와 간포복분비질물로소를 단소원으로 한 배 포
타 섬유오스와 아미질을 단소원으로 하는 경우 효소의 비활성도가 높았다. iii) 공시한 모든 기질에서 공
히 균의 세포기(세포기한)에 극히 적의 비활성도를 나타내었다.

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