Control of Catabolite Repression by Limit Feed of Cellubiose in *Cellulomonas* sp.

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**SUMMARY**

As an investigation on the catabolite repression system in cellulase production by *Cellulomonas* sp. CS1-1, the organism was tested on the avicel overlay plates containing glucose or cellubiose at a range of concentration and was grown in continuous culture vessel supplied by cellubiose medium, aiming the enhanced production of extracellular CM-cellulase at low dilution rates. Product inhibition of cellulase action by cellubiose was also tested. The results obtained are: i) no inhibition of CM-cellulase was observed up to 10 mM (3.4 mg/ml) cellubiose in the reaction mixture, however 30\% inhibition was observed at 20 mM and 55\% at 50 mM. ii) the tests of catabolite repression on the solid media were successful and avicel degradation was markedly repressed by glucose or cellubiose. iii) at low concentrations of cellubiose, dilution rate 0.05 and 1.0 hour\(^{-1}\), no significant increase was observed in the production of either intra or extracellular CM-cellulase.

**INTRODUCTION**

The production of cellulosytic enzymes from a number of fungi and bacteria is known to be repressed by the presence of glucose or cellubiose in the growth media\(^1\)\(^2\)\(^3\)\(^4\). Because glucose or cellubiose repress the induction of cellulases unless present in extremely small amount, they are considered to invoke an inducer-repressor mechanism of regulation in enzyme production\(^7\). Therefore the repression can be overcome by limiting the concentration of these sugars, and with this limitation, marked increases are observed in the production of extracellular cellulases\(^6\)\(^7\)\(^8\).

The production of extracellular cellulases by *Cellulomonas* sp. CS1-1 was shown to be inhibited by cellubiose\(^3\). Detailed study was carried out to investigate i) whether this repression can be conformed on solid media, avicel overlay plates, if then ii) what the degree of repression is, iii) whether this repression can be reduced/overcome

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by limit feed of cellobiose at low dilution rates in continuous culture system. This study also included an investigation on product inhibition of cellulase activity by cellobiose.

MATERIALS AND METHOD

Bacterial Strain

Cellulomonas sp. CS1-1\(^{(3)}\).

Media and Cultivation

The controlled feeding of cellobiose to the strain CS1-1 were carried out in continuous culture under controlled pH and D.O.T., and with step change of dilution rate.

The media used in this work was 0.05% cellobiose in Dubos' salts solution supplemented with 0.02% yeast extract. This was prepared in bulk and sterilized by filtration through a membrane filter(Figure 1)\(^{(4)}\).

![Fig. 1. Sterilization Unit. mounted on stainless steel service trolley with heavy duty castors.](image)

The fermentor was a modification of Quickfit FVIL 1 litre culture vessel(Figure 2) and was fitted with an EI Vibromix Stirrer controlled through a variac transformer. The overflow was placed to give an actual working volume of approximately 820 ml. Dissolved oxygen tension was measured using a Johnson-type electrode\(^{(10)}\) and controlled by varying the air flow rate and/or the stirrer speed. Temperature was
controlled by means of the Ether Mine Model 19-90/1 using a Resistance Thermometer type P5 as a sensor and operating a 250W infra red lamp. pH was continuously monitored using a pH probe connected to a Dynaco 21A pH meter and when necessary, was adjusted by sodium hydroxide solution(2M) through a miniature peristaltic pump.

Fig. 2. The Modified Quickfit FVIL 1 litre Fermentor and its Attachments.

Enzyme Preparation, Measurement of Growth, and Protein Determination.

These were achieved by the same methods as described in the previous report\(^{40}\) except for the extracellular enzyme extracts which were dialysed in collagen tubing against a McIlvaine buffer\(^{12}\), pH 7.0 for 48 hours at 4°C.

Determination of Enzyme Activity

i) Assay of CM-cellulase Based on Formation of Reducing Sugar (CM-SA).
The method employed was similar to that developed by Reese et al. where they assayed CM-cellulase action on CM-cellulose by determination of reducing equivalents of the reducing sugars. Reducing sugars were estimated by the Somogyi method\(^{(15)}\) using the chromogen developed by Nelson\(^{(14)}\).

The enzyme preparation (1.0 ml) was added to 1.5 ml of 1% CM-cellulose in McIlvaine buffer pH 7.0. After 20 minutes incubation at 40°C, the reaction was stopped by adding 2.0 ml of the copper reagent. This was boiled for 20 minutes, cooled and mixed with 2 ml of arsenomolybdate colour reagent. After dilution in distilled water to a final volume of 12 ml, the tubes were allowed to stand at room temperature for 20 minutes, centrifuged at 2,500g for 10 minutes then read in the spectrometer at 520 nm.

A unit of enzyme activity is defined as the amount of enzyme that will produce an amount of reducing sugar equivalent to 1 n mole of glucose per minute under the above conditions.

ii) Assay of CM-cellulase Based on Viscosity Reduction of CM-cellulose (CM-VA).

The method used was the Hulme modification\(^{(16)}\) of the method originally developed by Levinson et al. and Tracey\(^{(17)}\).

Two ml of enzyme extract was added to a mixture of 5 ml of 0.88% CM-cellulose and 3 ml of McIlvaine buffer, pH 7.0. Nine ml of this solution was then transferred into an Oswald viscometer, maintained in the 30°C water bath and the flow time were measured at frequent intervals. The rate of increase in fluidity \[ \frac{d}{dt} \left( \frac{1}{\eta_{sp}} \right) \] was calculated by a simplified equation derived by Hulme\(^{(16)}\) where:

\[
\frac{d}{dt} \left( \frac{1}{\eta_{sp}} \right) = \frac{\eta_{sp1} - \eta_{sp2}}{\eta_{sp1} \eta_{sp2}} \times \frac{1}{t_2 - t_1}
\]

\(\eta_{sp1}\) = specific viscosity at time \(t_1\),

\(\eta_{sp2}\) = specific viscosity at time \(t_2\).

One unit of enzyme activity was defined as that quantity of enzyme that causes a change in \[ \frac{d}{dt} \left( \frac{1}{\eta_{sp}} \right) \] of 0.001 units per minute.

**RESULTS AND DISCUSSION**

**Inhibition of CM-cellulase by Cellobiose.**

As the experiment conducted to determine whether one of the products of CM-cellulase, namely cellobiose, could inhibit the enzyme activity, CM-cellulase activity was measured viscometrically in the presence of cellobiose at the range of concentrations indicated in Figure 3. No inhibition was observed up to 10 mM (3.4 mg/ml), however 30% inhibition was observed at 20 mM and 55% at 50 mM. In fungal system, cellobiose was reported to inactivate cellulase. Cx. already formed in cultures of Basidio-
mycelium QM 806. Trichoderma viride, Sporotrichum pruinose. Myrothecium verrucaria, Pestalotiopsis westerdijkii, or Stachybotrys atra producing the enzyme on cellulose when added at concentrations of 0.5 to 1.0% (11), and Halliwel et al. reported that the purified component C was completely inhibited by its reaction product cellobiose (3).

**Catabolite Redression of Cellulase Production by Glucose and Cellobiose.**

The experiments dealing with growth in liquid media suggested that cellobiose may cause catabolite repression of the production of cellulases (3). A convenient method for observing bacterial digestion of cellulose was to prepare and avicel (1%) containing agar overlay and pour it over the surface of a Dubos' +0.02% yeast extract containing plate. The overlay also contains Dubos' salts +0.02% yeast extract. The cellulolytic strains, when streaked onto this media and incubated at 30°C were able to

![Graph](image.png)

**Fig. 3. Cellobiose Inhibition of CM-cellulase.** CM-cellulase activity was assayed using the viscometric method and results are expressed by relative activity.

cause clearing around the bacterial colonies due to the degradation of the avicel layer. Using this as an indication of cellulolytic activity glucose and cellobiose were incorporated into the both base media and the overlay in varying concentrations. The effect of glucose and cellobiose could then be assessed by streaking the test organisms.
Table 1. Catabolite Repression of Cellulase Activity by Glucose and Cellubiose in Wild Type Strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control no sugar</th>
<th>Glucose(%) 0.1 0.5 1.0 2.0</th>
<th>Cellubiose(%) 0.1 0.5 1.0 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCIB 8077</td>
<td>++</td>
<td>- - - -</td>
<td>+ - - -</td>
</tr>
<tr>
<td>CS1-1</td>
<td>++</td>
<td>- - - -</td>
<td>+ - - -</td>
</tr>
<tr>
<td>CS2-1</td>
<td>++</td>
<td>- - - -</td>
<td>+ - - -</td>
</tr>
<tr>
<td>CS3-1</td>
<td>-</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>NCIB8633</td>
<td>++</td>
<td>- - - -</td>
<td>++ + ± -</td>
</tr>
<tr>
<td>NCIB8634</td>
<td>+±</td>
<td>- - - -</td>
<td>+ - - -</td>
</tr>
<tr>
<td>PS1-1</td>
<td>+</td>
<td>- - - -</td>
<td>± - - -</td>
</tr>
<tr>
<td>PS2-1</td>
<td>++</td>
<td>- - - -</td>
<td>++ - - -</td>
</tr>
</tbody>
</table>

Notes on Table 1. i) Lysis of avicel layer was recorded after a total of 10 days incubation. This was compared with other *Cellulomonas* sp. (NCIB 8077, CS2-1 and CS3-1) and *Pseudomonas* sp. (NCIB 8633 & 8634, PS1-1 and PS2-1).

ii) Abbreviations: - no lysis; ± faint clearing; + moderate clearing; ++ good clearing of the avicel layer.

onto the layer then determining the ability to clear the avicel layer.

The results of this experiment are given in Table 1, where the strain CS1-1 was compared with other bacterial strains, wild type, of *Cellulomonas* and *Pseudomonas*. Avicel degradation was markedly inhibited by glucose in all of the wild type strains even at concentrations as low as 0.1% glucose. Inhibition by cellubiose was not as marked as with glucose although cellubiose still caused a considerable reduction in lysis of the avicel layer in all strains tested.

Enzyme Production during Controlled Supply of Cellubiose.

The ability to degrade an avicel layer by CS1-1 was inhibited by either cellubiose or glucose (Table 1). To investigate this repression in more detail an experiment was carried out in continuous culture where it is possible to study cellulase production at a range of different concentrations of cellubiose. The concentration of cellubiose, 0.05%, was chosen by testing growth preliminarily in a range of concentrations of cellubiose. As shown in Table 2, 0.05% of cellubiose supported growth to get a turbidity of the culture 0.47-0.48 at OD610nm in 20 hours, which normally gave viable count of 1 to $5 \times 10^6$ cells/ml and was appeared to be enough for enzymatic study.

When the culture reached stationary phase in the fermentor, 19 hours after inoculation the vessel was switched to continuous operation. pH was maintained at pH 7.0 ± 0.1, temperature at 30°C ± 0.5°C, and D.O.T. at 54 ± 2%. The initial dilution rate
Table 2. Growth of the Strain CS1-1 on Cellulobiose.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Maximum OD at 610nm</th>
<th>Conc. of cellulose at stationary phase (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (mg/ml)</td>
<td>(time required to reach the stationary phase)</td>
<td></td>
</tr>
<tr>
<td>.00(0.0)</td>
<td>.09-.10(10hr)</td>
<td>&lt;12</td>
</tr>
<tr>
<td>.02(0.2)</td>
<td>.28-.31(16hr)</td>
<td>29-35</td>
</tr>
<tr>
<td>.05(0.5)</td>
<td>.47-.48(20hr)</td>
<td>55-68</td>
</tr>
<tr>
<td>.10(1.0)</td>
<td>.71-.76(33hr)</td>
<td>128-172</td>
</tr>
<tr>
<td>.50(5.0)</td>
<td>.91-.98(33hr)</td>
<td>2800-3200</td>
</tr>
</tbody>
</table>

Notes on Table 2. i) The culture was grown in 250 ml side-arm flask containing 50 ml of medium at 30°C on a reciprocal shaker.
ii) One percent (v/v) cell suspension OD 610nm 0.5 in Dubos' salts solution was used as inoculum.

Fig. 4. Enzyme production during Controlled Supply of Cellulobiose by Continuous Cultivation. Characteristics determined: bacterial growth (viable count - □ - ); residual cellulobiose concentration (——△——); CM-Cellulase (CMC-SA, intracellular—○—, extracellular—□—); and β-glucosidase ( — O — )

studied was 0.05 hour⁻¹ and after the culture had reached a steady state a culture sample was withdrawn and a number of parameters determined. The dilution rate was then increased to 0.1, 0.2, 0.3 and 0.4 hour⁻¹. The results of this experiment are given in Figure 4. With very low dilution rates the cellulobiose carbon source was almost completely used up, cell numbers were at a maximum as was both the β-glucosidase and intracellular CM-cellulase. No extracellular CM-cellulase was detected. With
increasing dilution rate the viable counts dropped and the amounts of \( \beta \)-glucosidase and intracellular CM-cellulase fell approximately in proportion to drop in viable organisms. No extracellular CM-cellulase was detected at any dilution rate. With increasing dilution rate the concentration of residual cellobiose increased. Under conditions where only very low concentrations of cellobiose in the culture dilution rate 0.05 and 0.1 hour\(^{-1}\), no significant increase was observed in the production of either intra or extracellular CM-cellulase. This results showed that the strain CS1-1 have a different regulatory system by cellobiose from other cellulolytic bacteria Pseudomonas fluorescens var. cellulosa studied by Yamane et al\(^{(18)}\). They obtained results that the formation of extracellular cellulase was inhaled to a comparable extent to that in cultures containing cellulose or sophorose by the controlled supply of cellobiose and other sugars, and concluded that paucity of absorbable cellulolytic products in the culture medium results in enhanced extracellular cellulase formation. Hulme also reported similar results ie. when glucose level in the culture of Myrothecium verrucaria reduced lower than 0.4mg/ml cellulase activity in the culture filtrate increased markedly\(^{(9)}\). The relationship between bacterial growth and substrate concentration, the strain CS1-1 on cellobiose, did not followed the ideal curves suggested by Herbert et al\(^{(6)}\).

REFERENCES

Cellulomonas sp.에 있어서 설탕비오스의 미량공급에 의한 생성물 저해의 조절

최 우 영

적 요

Cellulomonas sp. CS1-1의 설탕비오스 생산에 있어서 분해생성물에 의한 저해 기구를 연구하고 아울러 호소 작용시 생성물의 저해 작용 여부를 구별하기 위하여 각종 함량의 설탕비오스를 함유한 아미노 중층 한정배지시험, 설탕비오스의 공급을 근소화함으로서 세포의 설탕비오스의 증산을 목적으로 한 연속배양 시험, 그리고 효소기질에 각기 다른 농도의 설탕비오스가 존재할 때의 활성도에 대한 저해 시험을 시도한 결과 i) 효소반응 혼액중 설탕비오스의 농도가 10mM 이하에서는 설탕비오스의 작용이 저해되지 않았으나 20mM에서 30%, 50mM에서 약 55% 저해 됨이 인정되었다 ii) 아미노 중층 배지 시험에서 설탕비오스의 분해 작용은 글루코스 및 설탕비오스에 의해 크게 저해되었다 iii) 연속 배양에 의하여 배지중의 설탕비오스의 잔존량을 극히 적게 한 경우(최식을 0.05 및 0.1hr⁻¹)에도 세포내의 효소가 증산되지 않았다.