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# Cellulase Production in Fed-Batch Culture by *Trichoderma reesei* Rut C30

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**Abstract** Cellulase production by fed-batch cultivation of *Trichoderma reesei* Rut C30 with various initial concentrations of Solka Floc in 1% wheat bran-containing medium was investigated. The cellulase activity and productivity increased with initial Solka Floc concentration up to 5%. When a total Solka Floc concentration of 90 g/l was used for cellulase production, CMC (carboxymethyl cellulose) and FP (filter paper) activities, productivity, and yield were 359.7 U/ml, 30.61 U/ml, 161 FPU $L^{-1}$ $h^{-1}$, and 340 FPU $g^{-1}$, respectively. It was important to maintain a high cell concentration during cellulase production to obtain high cellulase activity and productivity. Cellulase powder was prepared by ammonium sulfate precipitation: FP activity was 396.7 U/g and CMC activity was 6481 U/g.

**Key words:** Cellulase, fed-batch, *Trichoderma reesei*, wheat bran, Solka Floc

Cellulose is the most abundant renewable carbon source that has a great potential as an energy source if it can be easily hydrolyzed into glucose. Cellulase is one of the most extensively studied enzyme systems since it is able to decompose cellulosic materials into glucose. There has been a great interest in the commercial use of cellulases in the agricultural and chemical industries to produce glucose for feed, food, and the manufacture of other chemicals [12, 21, 28]. The major bottleneck in the application of cellulase on an industrial scale is in the high cost of the enzyme. This is due to the low specific activity of cellulases, necessitating a large quantity of enzyme for cellulose hydrolysis. Strain development, optimization of culture conditions, and mode of cultivation were extensively investigated for high cellulase production [5, 11, 19, 22].

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. Functionally complete cellulase enzyme systems can be produced by various microorganisms. Most of the studies on the cellulases have been carried out using fungal cellulolytic systems. Among the best characterized and most widely studied of these systems are the inducible cellulases of the fungus genus *Trichoderma*. The cellulase obtained from *Trichoderma* sp. contains all the components required for hydrolysis of crystalline cellulose. The mutant strain, *T. reesei* Rut C30, has been the most widely used. Compared to other microorganisms, it possesses an ability to produce significantly higher quantities of cellulases and a strong resistance to catabolite repression [18].

Most of the cultivation variables such as pH [3, 19], medium composition [2, 3, 6, 23], temperature [16, 24], and culture method [8, 10, 14] have been thoroughly investigated with regard to their effect on the growth and production of enzymes. Various substrates have been evaluated for cellulase production and Solka Floc has been used often as a carbon source. Various types of cultivation were applied for cellulase production [1, 3, 8, 10, 14]. In order to increase the biomass available for enzyme production, a number of workers tried to increase the initial concentration of the substrate. In the batch system, an initial concentration of cellulose higher than 5% did not improve cellulase activity and led to lower productivity [10, 25]. Continuous cultivation provides high productivity; however, it yields a significantly lower enzyme titre [1, 10]. As many researchers have reported [8, 9, 27, 28], the greatest potential for improving the production of cellulases that comes from developments in fermentation technique appears to be in the use of the fed-batch mode of fermentation.

In fed-batch production of cellulase using *T. reesei* Rut C30, the effect of the size of the substrate injection, the time between injections, and feeding rate were examined [14, 15]. To achieve higher cellulase activity and productivity, it was suggested to maintain a high cell concentration during cellulase production. Earlier results used a mycelial recycled continuous culture, however, low cellulase activity and productivity were reported [8]. McLean *et al.* [14] claimed a cell concentration of 12 g/l

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as an optimal concentration for cellulase production during fed-batch culture. In this paper, the effect of the initial concentration of Solka Floc in wheat bran-containing medium and a feeding strategy for the production of cellulase during fed-batch cultivation were investigated. The relation between maintaining a high cell concentration during cellulase production and cellulase activity and productivity are also presented.

## MATERIALS AND METHODS

### Microorganism

*Trichoderma reesei* Rut C-30 (ATCC 56765) was used in this study. It was grown on potato dextrose agar (Difco Lab., Detroit, U.S.A.) slants at 30°C for 3 days and then stored at 4°C until use. The organism was transferred to new agar slants every month.

### Media Preparation

Seed culture medium contained: 10 g/l Solka Floc (Fiber Sales and Development Co., Green Brook, U.S.A.), 10 g/l wheat bran (Donga Meal Co.), 2 g/l $(NH_4)_2SO_4$, 0.5 g/l yeast extract (Difco Lab.), 3 g/l proteose peptone (Difco Lab.), 4 g/l $KH_2PO_4$, 0.3 g/l $CaCl_2 \cdot 2H_2O$, 0.3 g/l $MgSO_4 \cdot 7H_2O$, and 0.2 ml/l Tween-80. Cellulase production medium contained: 10 g/l wheat bran, 2 g/l $(NH_4)_2SO_4$, 0.5 g/l yeast extract, 3 g/l proteose peptone, 4 g/l $KH_2PO_4$, 0.3 g/l $CaCl_2 \cdot 2H_2O$, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 0.2 ml/l Tween-80, and 0.3 ml/l Antifoam 204. Various concentrations of Solka Floc were added after sterilization. To determine CMC and FP activities, CMC (Sigma Chemical Co., St. Louis, U.S.A.) and filter papers, No. 1 (Whatman Lab., Hillsboro, U.S.A.) were used. Reagent chemicals used in this study were obtained from Sigma Chemical Co.

### Fermentation Conditions and Control

The seed culture was prepared for two days and inoculated to the production medium. Cultivations were carried out in a 2.5-l fermenter with an operating volume of 1.6 l. Temperature was maintained at 28°C for the first two days to support rapid growth and 25°C for the rest of the fermentation to provide slow growth and prolong the period of enzyme production [20]. Medium pH was held at 3.5 by the addition of $NH_4OH$. Dissolved oxygen was maintained above 20% of the saturation value for the medium by varying the aeration rates and agitation rates in response to changes in the dissolved oxygen tension. Intermittent additions of Solka Floc slurry were made so that the total concentration of Solka Floc in the fermenter was 2%, whenever the cell growth was observed to be slower as demonstrated by a decrease in the base addition rate [10]. Cultivation was terminated when there was an increase or no change in culture pH.

### Analytical Methods

Carboxymethyl cellulose (CMC) and filter paper (FP) activities were determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC). The amount of reducing sugar liberated was determined by the dinitrosalicylic acid (DNS) method. One unit of enzyme activity was defined as the amount releasing 1 μmol of reducing sugar per minute [7].

Mycelial dry weights were determined by the procedures as follows [19]: 3 ml culture broth and 3 ml 1 N perchloric acid solution were mixed together and boiled for 20 min. After cooling down to room temperature, the sample was centrifuged and the optical density of the supernatant was measured at 260 nm ($OD_{260}$). The optical density of the blank was determined by the same procedures using the culture filtrate ($OD^0_{260}$). One gram of mycelial dry weight per liter corresponded to $0.65 \times (OD_{260}\text{-}OD^0_{260})$.

### Preparation of Cellulase Powder

Fungal mycelium and solid particles were removed from the culture broth by filtration using four layers of gauze. 60% (w/v) $(NH_4)_2SO_4$ was added to the cell extract and placed in a refrigerator (4°C) overnight. The precipitate was filtered by Whatman No. 1 filter paper and dried in a vacuum drying oven for 2 days at 45°C.

## RESULTS AND DISCUSSION

### Effect of the Initial Concentration of Solka Floc

To study the effect of the initial cellulose concentration on cellulase production in a fed-batch culture, *Trichoderma reesei* Rut C30 was grown on various concentrations of Solka Floc- and 1% wheat bran-containing medium. Wheat bran was added to the medium since it was found to be a good substrate for cellulase production. Its nutrients, starch, proteins, and lignocellulosic materials are readily available for microbial growth and cellulase synthesis [4]. In our batch culture study, it was found that the cellulase activity and productivity were highest at 5% Solka Floc and 1% wheat bran and a further increase in Solka Floc concentration resulted in lower cellulase activity and productivity [26]. In this study, *T. reesei* was grown at 5 different Solka Floc concentrations: 1.5, 2, 3, 4, and 5%. When the base addition rate decreased, Solka Floc was added so that the total Solka Floc concentration in the fermenter was approximately 2%. The results are shown in Figs. 1 to 5 and summarized in Table 1. Additions of each new portion of Solka Floc slurry are marked by an arrow in the figures. The highest cellulase production was achieved when the initial Solka Floc concentration was 5% and Solka Floc slurry was added to the culture twice during fermentation (a total of 90 g/l Solka Floc was used

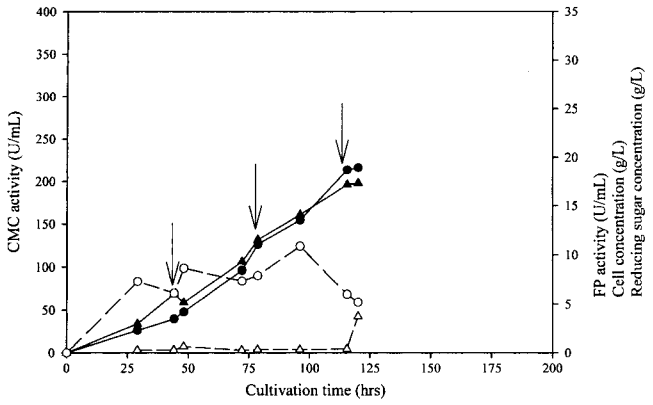


**Fig. 1.** Production of cellulase by intermittent feeding fed-batch culture (1.5% Solka Floc + 1% wheat bran).
●: CMC activity; ▲: FP activity; ○: Cell concentration; △: Reducing sugar concentration.

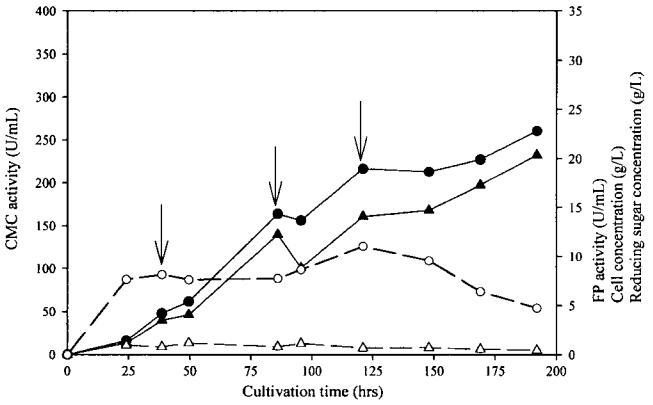


**Fig. 2.** Production of cellulase by intermittent feeding fed-batch culture (2% Solka Floc + 1% wheat bran).
●: CMC activity; ▲: FP activity; ○: Cell concentration; △: Reducing sugar concentration.

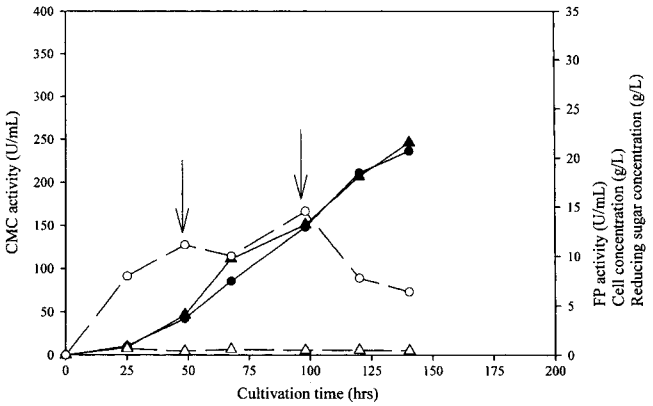


**Fig. 3.** Production of cellulase by intermittent feeding fed-batch culture (3% Solka Floc + 1% wheat bran).
●: CMC activity; ▲: FP activity; ○: Cell concentration; △: Reducing sugar concentration.

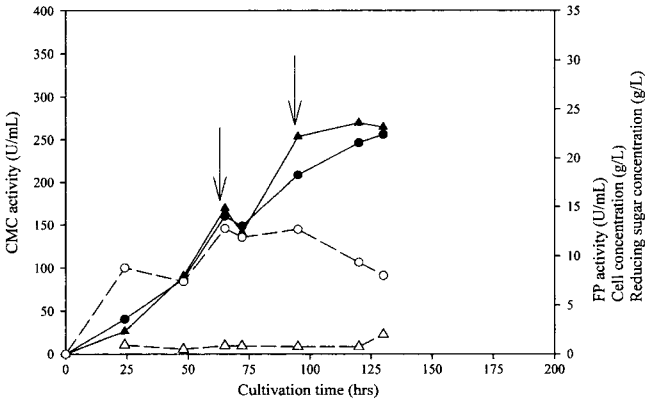


**Fig. 4.** Production of cellulase by intermittent feeding fed-batch culture (4% Solka Floc + 1% wheat bran).
●: CMC activity; ▲: FP activity; ○: Cell concentration; △: Reducing sugar concentration.

during the fed-batch culture): CMC and FP activities, productivity, and yield were 359.7 U/ml, 30.61 U/ml, 161 FPU $L^{-1}$ $h^{-1}$, and 340 FPU $g^{-1}$, respectively. These are much improved results compared to those obtained from batch fermentation [26]: FP activity of 21.25 U/ml, CMC activity of 232.4 U/ml, and productivity of 143.6 FP $L^{-1}$ $h^{-1}$. Also, the results obtained in this study are higher than those obtained from other fed-batch cultivations [10]: FP activity of 30 U/ml, productivity of 106 FPU $L^{-1}$ $h^{-1}$, and yield of 203 FPU $g^{-1}$.

As shown in Table 1, a total Solka Floc concentration of 70 g/l to 90 g/l was used for cellulase production, although the initial concentration of Solka Floc varied from 1.5 to 5%. The increase in cellulase production was due to substrate inhibition [26].

Cellulase production was also significantly influenced by cell concentration. As shown in Figs. 1 to 5, cell growth could be divided into three phases: exponential phase, stationary phase, and death phase. When 5% Solka Floc (initial substrate concentration) was used, the highest maximum cell concentration of 15.4 g/l and highest stationary phase cellulase concentration of 12.8 g/l were obtained. As shown in Fig. 5, a higher cell concentration (15.4 g/l) was maintained for a longer time (approximately 132 h) than in other experiments and this probably contributed to the high cellulase activity attained during cellulase production. This implies that it is important to maintain a high cell concentration to achieve high cellulase production. When the results of cellulase production performed with initial Solka Floc concentrations of 2% and 4% are compared, the total amount of Solka Floc (80 g/l) added for cellulase production were the same. However, the stationary phase cell concentration obtained from 2% Solka Floc (10.11 g/l) was higher than that obtained from 4% Solka Floc (7.94 g/l) and resulted in the higher FP activity (23.63 U/ml for 2% Solka Floc and 20.38 U/ml for 4% Solka Floc). Consequently, it is a key factor to maintain a high cell concentration for high

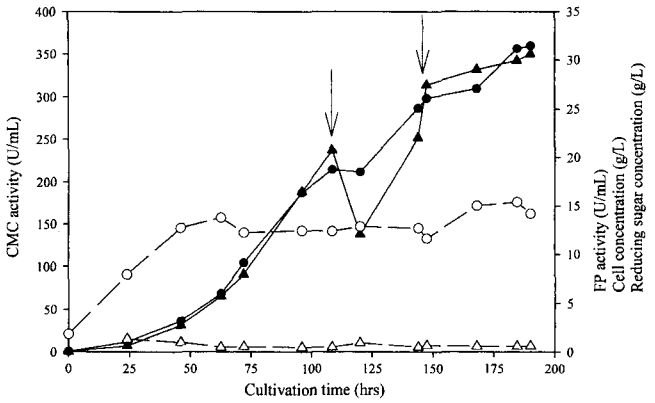


**Fig. 5.** Production of cellulase by intermittent feeding fed-batch culture (5% Solka Floc + 1% wheat bran).
●: CMC activity; ▲: FP activity; ○: Cell concentration; △: Reducing sugar concentration.

cellulase production whether it is carried out in a batch mode or a fed-batch mode.

### Variations in Cell Mass and Cellulase Activity During Fed-batch Culture

As shown in Figs. 1 to 5, there was not any significant cell growth after each feeding of Solka Floc except in the fed-batch culture started with 1.5% Solka Floc. When cell growth entered the death phase, cell concentration kept decreasing even after the addition of Solka Floc slurry. However, fed-batch culture of *Trichoderma reesei* Rut C30 performed by McLean and Abear showed an increase in cell concentration after each feeding [14]. This is probably due to the difference in feeding time during fed-batch culture. In this study, the feeding time was determined by observing cell growth. The intermittent feeding was made, whenever cell growth was observed to be decreasing, as demonstrated by a decreasing base addition rate. However, McLean and Abear fixed the feeding time. They started their first feeding after 4 days and made additional feedings of cellulose at every 1 or 2 days. It is probable that the carbon source was totally exhausted and the cells were in the death phase around their feeding time. The relationship between feeding time and cellulase production will be discussed more in a later section. The concentration of reducing sugar remained very low during fed-batch cultivation and this implies that the amount of cells and the uptake rate of reducing sugar were always sufficient to fully utilize the sugars produced from enzymatic hydrolysis of the cellulose.

During the cultivation of *T. reesei*, a very viscous culture broth was observed, usually after 1 day of cultivation. This was due to the large amount of hyphae produced during cell growth and it caused difficulties in agitation during fermentation. As mentioned earlier, high cellulase activity is obtained when high cell concentration is maintained for a long time. However, a highly viscous culture broth exerts a negative effect on mass transfer during fermentation and requires high power input and this results in limitations for further increase in cellulase production. This implies the existence of an optimal cell concentration for the cellulase production. However, the mass transfer can probably be improved by applying a better fermenter design and by adding cationic polymers to change the fungal morphology from dispersed filaments to pellets in order to decrease the viscosity of culture broth [17]. Serious foaming during fermentation was reported during cellulase production by fed-batch culture [14, 22]; however, foaming was not found to be severe in our experiments.

### Feeding Strategy During Fed-batch Culture

The C/N ratio of the cellulase production medium is usually high since cellulase is synthesized under nitrogen limited conditions [22]. Acid production is directly related to the consumption of Solka Floc and cell growth. Ammonium hydroxide was used for pH control and also ensured a sufficient supply of nitrogen. Consequently, the rate of base addition reflects the Solka Floc concentration in the medium. Rapid addition of ammonium hydroxide indicates that there are large amounts of Solka Floc and very slow addition of ammonium hydroxide implies that there is only a little Solka Floc left in the medium. As a result, Solka Floc concentration in the culture could be indirectly indicated by the variation in base addition rate. Solka Floc was added repeatedly whenever a decrease in the rate of base addition was observed as a result of Solka Floc depletion in the medium.

**Table 1.** Effects of initial Solka Floc concentration on cellulase production in fed-batch culture.

| Solka Floc concentration | | FP activity | CMC activity | Productivity | Yield | Cell concentration (g/l) | |
|---|---|---|---|---|---|---|---|
| Initial (%) | Total (g/l) | (U/ml) | (U/ml) | (FPU $L^{-1}$ $h^{-1}$) | (FPU/g) | Maximum | Mean* |
| 1.5 | 75 | 17.34 | 226.4 | 145 | 231 | 10.9 | 7.4 |
| 2 | 80 | 20.38 | 260.7 | 106 | 255 | 11.0 | 7.9 |
| 3 | 70 | 21.55 | 236.6 | 153 | 303 | 14.6 | 9.7 |
| 4 | 80 | 23.63 | 256.1 | 182 | 295 | 12.8 | 10.1 |
| 5 | 90 | 30.61 | 359.7 | 161 | 340 | 15.4 | 12.8 |

1% wheat bran was added to all media.
Mean* (value) was calculated based on the cell concentration during the stationary phase.

**Table 2.** Results of cellulase preparation by ammonium sulfate precipitation.

| Culture volume (ml) | Activity (U/ml) | | Weight of cellulase powder (g) | Activity (U/g) | | Recovery (%) | |
|---|---|---|---|---|---|---|---|
| | FP | CMC | | FP | CMC | FP | CMC |
| 1600 | 30.61 | 359.7 | 59.7 | 396.7 | 6481 | 48.4 | 67.2 |

Feeding time during fed-batch culture was dependent on both the initial concentration of Solka Floc and cell growth. When there is a large amount of Solka Floc in the medium initially, the first feeding time was delayed. At higher substrate concentrations, longer fermentation times were required. The first feeding time for fed-batch culture of *T. reesei* on 2%, 3%, 4%, and 5% Solka Floc (initial concentration) were 38.5, 48.5, 65, and 108.5 h, respectively. However, cellulose was consumed rapidly when cell concentration or cellulase activity was high. In this case, the time interval for the first feeding could be shortened. As a result, the time interval between feedings was dependent on cell concentration and cellulase activity. In this study, the feeding interval varied from 30 h to 48.5 h.

### Extraction of Cellulase

Ammonium sulfate precipitation was used to prepare dry cellulase powder from the culture broth (Table 2). The precipitation of enzyme proteins with ammonium sulfate, followed by centrifugation and solubilization of protein in sodium acetate buffer, resulted in a 48.4% recovery in FP activity and 67.2% recovery in CMC activity in comparison with those activities obtained from the culture broth. FP activity represents the enzyme activity of the entire cellulase complex and CMC activity represents the endo-cellulase activity [13]. As a result, the higher recovery of CMC activity than FP activity probably implies that less endo-cellulase was adsorbed on cotton gauze and filter paper than other enzymes in the cellulase system during cellulase powder preparation.

## Conclusions

Cellulase production by fed-batch culture of *Trichoderma reesei* Rut C30 on various initial concentrations of Solka Floc was examined. The initial concentration of Solka Floc had a significant effect on cell growth and cellulase production. The highest cellulase titre was obtained from the fed-batch cultivation of *Trichoderma reesei* Rut C30 on 5% Solka Floc and 1% wheat bran medium (initial concentration) with intermittent feeding. CMC and FP activities and productivity were 359.7 U/ml, 30.61 U/ml, and 161 FPU $L^{-1}$ $h^{-1}$ respectively. It is important to maintain a high cell concentration for high cellulase production. Maximum cellulase production occurred during the stationary phase of cell growth and cellulase production was high when the stationary phase was prolonged. A high titre of cellulase could be prepared from culture broth by ammonium sulfate precipitation.

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