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Production of Cellulase by *Trichoderma reesei* Rut C30 in a Batch Fermenter

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Abstract Cellulase production by batch culture using the *Trichoderma reesei* Rut C30 strain with various concentrations of Solka Floc with 1% wheat bran was studied in a 2.5 l fermenter. The cellulase activity increased with Solka Floc concentration up to 5%. When 5% Solka Floc and 1% wheat bran were contained in the medium, carboxymethyl cellulose (CMC) and filter paper (FP) activities were 232.4 U/ml and 21.25 U/ml, respectively. The productivity was 143.6 FPU $l^{-1}h^{-1}$ and the yield was 425 FPU/g. The colonial morphology of *T. reesei* Rut C30 grown on Avicel agar plates and the changes in mycelial morphology of *T. reesei* Rut C30 with culture time are also presented.

Key words: Cellulase, *Trichoderma reesei*, wheat bran, Solka Floc, morphology

Cellulose is the most abundant renewable carbon source in the world. There has been great interest in the hydrolysis of cellulose for the production of glucose, which can be used for fuel, food, and chemical production. Other applications of cellulase can be found in many fields such as the food, feed, textile, detergent, and pulp industries. The major bottleneck to application of cellulase on an industrial scale is the high cost of enzymes. This is due to the low specific activity of cellulases, necessitating a large quantity of enzyme for extensive hydrolysis. Strain development, optimization of culture conditions, and mode of cultivation were extensively investigated for high cellulase production [2, 5, 9, 11].

Most of the studies on cellulase have been carried out using fungal cellulolytic systems. The cellulase obtained from *Trichoderma* sp. contains all the components required for hydrolysis of crystalline cellulose and is resistant to chemical inhibitors and stable in stirred tank reactors at pH 4.8. *Trichoderma reesei* Rut C30 and other mutants have been used by many investigators to increase cellulase yield productivity [8, 12]. The preferred substrates used by most researchers are pure celluloses such as cotton, Solka Floc, Avicel, or sulfite pulp. Also, it was reported that the addition of wheat bran to purified cellulose enhanced the cellulase activity [15]. Usually, high cellulase production is obtained in fed-batch and continuous cultivations [4, 6, 7, 13, 14]. However, cultivation with solid substrates is rather difficult due to technical problems in continuous feeding. Higher productivity was obtained in continuous cultivation but the cellulase titre was significantly low [1, 4].

The objective of this study is to obtain optimal concentration of Solka Floc for the production of cellulase in wheat bran-containing medium during batch fermentation. This article also presents the variations in mycelial morphology during cell growth and cellulase production.

MATERIALS AND METHODS

Microorganism

T. 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 0.3~0.4 ml/l Antifoam 204. Different

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concentrations of Solka Floc were added to the above medium before autoclaving. Avicel agar plate contained: 10 g/l Avicel (Fluka Co., Buchs, Switzerland), 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 $MgSO_4 \cdot 7H_2O$, 1 ml/l Triton X-100, and 20 g/l agar. To determine CMC and FP activities, CMC (Sigma Chemical Co., 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 1.5 l. Temperature was maintained at 28°C for the first two days to give rapid growth and at 25°C for the rest of the fermentation to give slow growth and prolong the period of enzyme production [10]. 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.

Morphology Study

An image analysis system was used to observe the mycelial morphology during cultivation. A digitized image was captured by a RHYTHM TS-200 CCD color video camera that was linked to a microscope (CORRECT, Seiwa Optical, Co., Tokyo) and a personal computer. Microscope samples were prepared by diluting the culture broth by 250 times with distilled water.

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 (IU) of enzyme activity was defined as the amount releasing 1 μmol of reducing sugar per minute [3].

Dry cell weight (DCW) of fungal mycelium was measured as follows [9]: 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 procedure using the culture filtrate (OD^0_{260}). One gram of DCW per liter corresponded to $0.65 \times (OD_{260}-OD^0_{260})$.

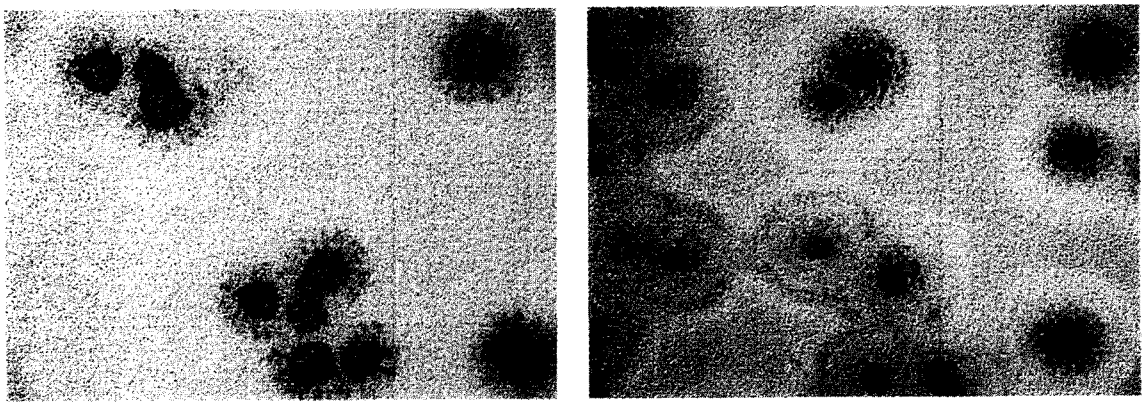
Results and Discussion

Characteristics of Colonial Morphology of *T. reesei* Rut C30

The colonial morphology of *T. reesei* Rut C30 was different from that of *T. reesei* QM9414 as shown in Fig. 1. After 7 days cultivation of *T. reesei* Rut C30 on Avicel agar plate at 30°C, green spores were formed on the center of the colony and had circular colony spores around the center colony spore. However, *T. reesei* QM9414 had green spores on the center of the colony and the edge of the spreading colony was white hyphae. It did not have circular spores around the center spore.

Production of Cellulase on Various Concentrations of Solka Floc and 1% Wheat Bran

Wheat bran is a good substrate for microorganism growth because its nutrients, starch, proteins, and lignocellulosic



(a) (b)

Fig. 1. Colonial morphology of *Trichoderma reesei* Rut C30 and QM9414 on Avicel agar plates (cells were grown on 1% Avicel agar medium for 7 days at 30°C).

(a) *T. reesei* Rut C30, (b) *T. reesei* QM9414,

materials are readily available for microbial growth and cellulase synthesis. In our previous study, it was found that the addition of wheat bran to a pure form of cellulose, such as Solka Floc, enhanced cellulase production in shake flask cultures, although wheat bran itself did not show high cellulase productivity [15].

In this study, various concentrations of Solka Floc and 1% wheat bran were used as carbon sources for cellulase production. Cellulase was produced by using a 2.5 l fermenter with a 1.5 l working volume. Five runs of batch fermentation were carried out using 2, 3, 4, 5, and 6% Solka Floc with 1% wheat bran in the medium. The results are shown in Figs. 2 to 6. Maximum cell concentration (13.82 g/l, 72 h) as well as highest CMC activity (232.4 U/ml) and FP activity (21.25 U/ml) were obtained at 5% Solka Floc with 1% wheat bran (Table 1). Much lower specific FP activity (0.79 IU/mg) was

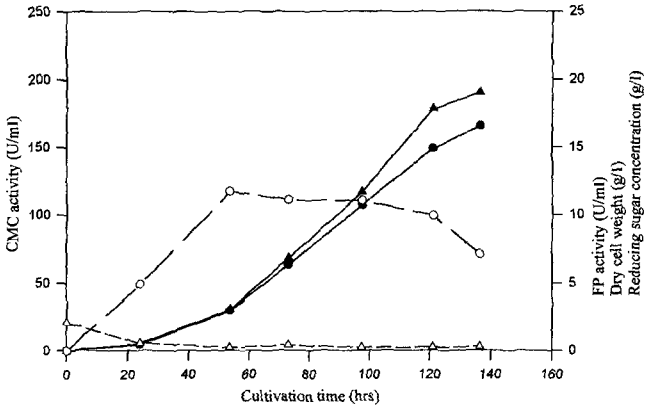


Fig. 4. Cellulase production by *T. reesei* Rut C30 during batch fermentation (4% Solka Floc and 1% wheat bran).
●, CMC activity; ▲, FP activity; ○, dry cell weight; △, reducing sugar.

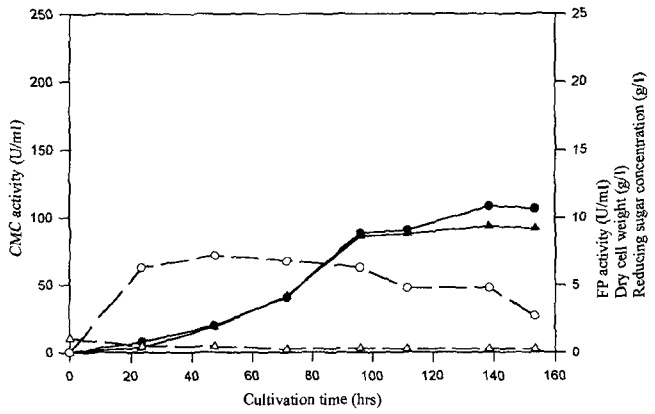


Fig. 2. Cellulase production by *T. reesei* Rut C30 during batch fermentation (2% Solka Floc and 1% wheat bran).
●, CMC activity; ▲, FP activity; ○, dry cell weight; △, reducing sugar.

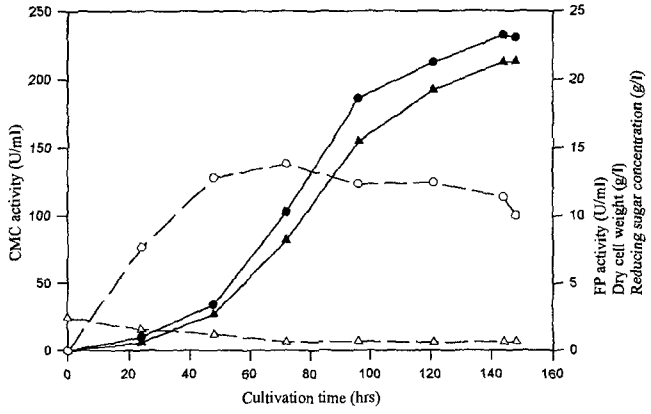


Fig. 5. Cellulase production by *T. reesei* Rut C30 during batch fermentation (5% Solka Floc and 1% wheat bran).
●, CMC activity; ▲, FP activity; ○, dry cell weight; △, reducing sugar.

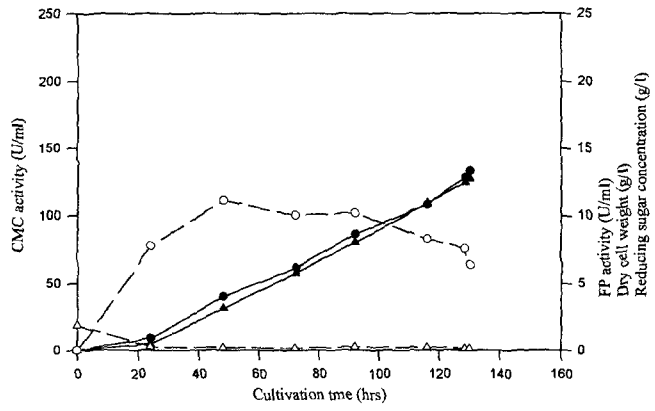


Fig. 3. Cellulase production by *T. reesei* Rut C30 during batch fermentation (3% Solka Floc and 1% wheat bran).
●, CMC activity; ▲, FP activity; ○, dry cell weight; △, reducing sugar.

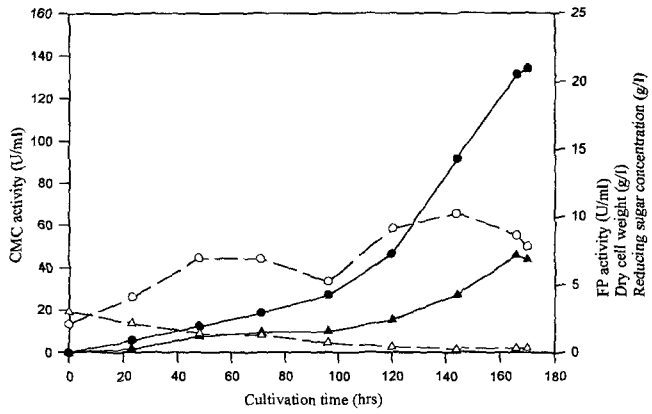


Fig. 6. Cellulase production by *T. reesei* Rut C30 during batch fermentation (6% Solka Floc and 1% wheat bran).
●, CMC activity; ▲, FP activity; ○, dry cell weight; △, reducing sugar.

Table 1. The influence of substrate concentration on cellulase activities in batch fermentation.

Solka Floc concentration* (%)	CMC activity (U/ml)	FP activity (U/ml)	Specific CMC activity (U/mg)	Specific FP activity (U/mg)
2%	108.6	9.37	22.7	1.96
3%	133.5	12.72	17.5	1.67
4%	190.1	16.56	19.1	1.66
5%	232.4	21.25	20.5	1.87
6%	133.7	6.83	15.5	0.79

*All media contained 1% wheat bran.

Table 2. The influence of substrate concentration on cellulase production in batch fermentation.

Solka Floc concentration* (%)	Time (h)	Productivity (FPU $l^{-1}h^{-1}$)	Yield (FPU/g)
2%	153	61.2	468.5
3%	130	97.9	424.0
4%	136	121.8	414.0
5%	148	143.6	425.0
6%	170	40.2	113.8

*All media contained 1% wheat bran.

observed at 6% Solka Floc with 1% wheat bran. The computed cellulase productivity and yield are shown in Table 2. Highest productivity (143.6 FPU $l^{-1}h^{-1}$) was obtained at 5% Solka Floc with 1% wheat bran. Cellulase activity and productivity increased with Solka Floc concentrations of up to 5% Solka Floc. However, yield was highest when 2% Solka Floc and 1% wheat bran were present in the medium. The cellulase titre and productivity were higher than the results reported by Ryu and Mandels [12]. Ryu and Mandels obtained FP activity of 15 U/ml and productivity of 45 FPU $l^{-1}h^{-1}$ by *T. reesei* Rut NG14 using 60 g/l 2 roll-milled cotton as a substrate. When 6% Solka Floc with 1% wheat bran was contained in the medium, the cells grew slower and cell concentration was lower. Also, cellulase activity, productivity, and yield were significantly lower than those obtained at 5% Solka Floc and 1% wheat bran. When 6% Solka Floc was used with 1% wheat bran, the low initial cell growth rate is probably due to the strong inhibition exerted by the high concentration of Solka Floc in the medium (Fig. 6). Also, the mass transfer limitations during batch fermentation contribute to the lower cellulase activities at high cellulose concentration (Table 1). The increase in cell growth rate and cellulase production could be observed after 5 days of cultivation. However, after such a long cultivation time, cells entered the death phase earlier than in other experiments and the increase in cellulase activity ceased. It indicates that high initial cell growth rate and cell concentration are critical for successful cellulase production. Consequently, 5% Solka Floc and 1% wheat bran were optimal for the production of cellulase in batch fermentation. At this level of Solka Floc concentration, cells grew well in the early stage of cultivation and the substrate inhibition did not occur and maximum cell concentration could be achieved. It is important to obtain high cell concentration for high cellulase production since a high level of biomass can enhance productivity per culture volume. The decrease in cell growth rate and cellulase production at high Solka Floc concentration suggests that the application of fed-batch fermentation for further increases in cellulase titre.

According to the analysis of the growth profile of *T. reesei* Rut C30, it could be divided into four periods: lag phase (from 0 to 10 h), exponential phase (from 10 to 48 h), stationary phase (from 48 to 120 h), and death phase (after 120 h). The time required for the cell to reach maximum cell concentration was approximately 48 h (2% and 3% Solka Floc). However, it was delayed with increasing Solka Floc concentration (4, 5, and 6%) as shown in Figs. 2 to 6. By comparing the cellulase production profile with the cell growth profile, it was found that the maximum cellulase activity was obtained during the stationary phase. The concentration of reducing sugar existing in the culture broth during this period was less than 1 g/l. This implies that most of the reducing sugars produced from enzymatic hydrolysis of the cellulose is utilized by cells.

Changes in the Morphology of *T. reesei* Rut C30 with Cultivation Time

In order to investigate the changes in the mycelial morphology of *T. reesei* Rut C30 during growth and cellulase production, the culture broth obtained from the cultivation of *T. reesei* Rut C30 on medium containing 5% Solka Floc and 1% wheat bran was observed by image analyzer. The images of mycelial morphology are shown in Fig. 7. At 24 h, cell growth was approximately at the end of the lag phase and the fungal hyphae were not very long. At that point, filamentous fungi exponentially grew by apical extension of the hyphae and branching and at this stage the culture broth became viscous. At 48 h, the hyphae became very thick and long since there were sufficient nutrients in the medium. From 48 to 100 h, cell growth was at the stationary phase. The culture broth was very viscous during this period. There was not much change in the morphology of *T. reesei* Rut C30 between 72 h and 96 h. However, a large amount of cellulase was produced during this period. Thereafter, viscosity of the culture broth decreased gradually until 120 h, which is about the end of the stationary phase. At 120 h, the hyphae were thinner and shorter and then the culture broth became less viscous as the culture entered the death phase at which autolysis occurred. At

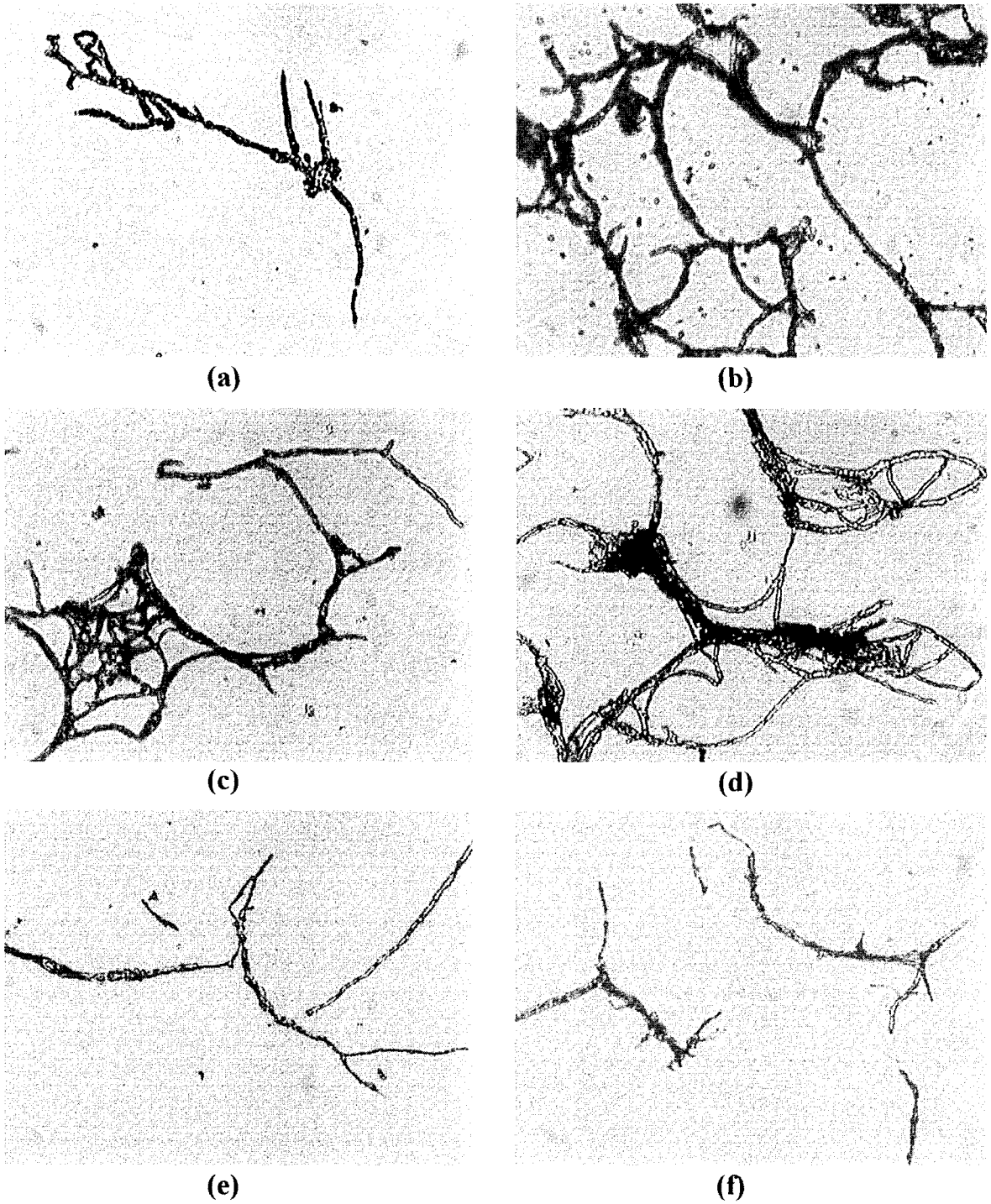


Fig. 7. Changes in mycelial morphology of *T. reesei* Rut C30 with cultivation time (original magnification, 200×).
(a) 24 h, (b) 48 h, (c) 72 h, (d) 96 h, (e) 122 h, (f) 148 h.

148 h, hyphal fragmentation occurred and short hyphal fragments were observed as a result of the exhaustion of nutrients.

As a result, long and thick hyphae (between 48 h and 100 h) were the mycelial form when the cellulase production rate was at its maximum. However, this morphological form significantly influences the rhelogy of the fermentation broth, which leads to a highly viscous culture broth. During this period, it was observed that the upper part of the culture broth was not agitated sufficiently and this probably caused some difficulties in mass transfer during fermentation.

Conclusions

Cellulase production by *T. reesei* Rut C30 was carried out using a batch fermenter. Cellulase production increased with increasing concentrations of Solka Floc up to 5% in 1% wheat bran-containing medium and a further increase in Solka Floc concentration resulted in a significant decrease in cellulase production. When 6% Solka Floc was used with 1% wheat bran, the cell concentration was significantly lower than that with 5% Solka Floc and 1% wheat bran. The highest cellulase titre and cellulase productivity were obtained when 5% Solka Floc and 1% wheat bran were contained in the medium: CMC and FP activities were 232.4 U/ml and 21.25 U/ml, respectively, and productivity was 143.6 FPU $l^{-1}h^{-1}$. The maximum rate of cellulase production occurred during the stationary phase. During this period, long and thick hyphae were observed and cellulase activity was highest.

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