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Effect of Thickening Agents on the Penicillin Fermentation

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ABSTRACT

The effects of thickening agents (polyethylene glycol [PEG] 20000 and carboxymethylcellulose [CMC]) on the penicillin fermentation by *Penicillium chrysogenum* were investigated. By adding the thickening agents to the fermentation medium, the growth form of the mold can be manipulated. Depending on the amount of thicking agent added, the change in morphology is from compact smooth pellets to various intermediate forms, and finally to filamentous mycelia. It was found that better penicillin production was obtained when the mold was in small, fluffy, loose pellets. The penicillin fermentation is not only affected by the thickening agents, but also the status of inoculum and agitation. Under the condition that the mold will otherwise grow in large pellets (e.g., under a low level of spore inoculum), the enhancement in the penicillin production through addition of the thickening agents may be more significant. In tank fermentation, the thickening agent was introduced in the stage of preculture, rather than main culture. The increase in the broth viscosity caused by addition of the thickening agent resulted in a decrease in dissolved oxygen level, which could be compensated in the case of PEG 20000.

Index Entries: Penicillin fermentation; morphology; thickening agent; polyethylene glycol (PEG); carboxymethylcellulose (CMC).

INTRODUCTION

The growth form of mold, the morphology, has been regarded as an important variable in penicillin fermentations. The morphology can be

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classified into pellet and filamentous forms. In the literature, many reports suggested that pellet cultures of *Penicillium chrysogenum* are more favorable for penicillin production than filamentous mycelia *(1–3)*. This is mainly because the broth viscosity is low and the aeration efficiency is high when pellets are formed. Moreover, it has been illustrated that the supply of oxygen to the interior of pellets is by molecular diffusion *(4)*. Because oxygen can be more readily transferred to the interior of smaller pellets, large pellets usually show low penicillin productivity. In other words, it implies that the growth form of small pellets favors penicillin fermentations. Unfortunately, this point is not consistent in the literature. Pirt and Callow *(5)* reported that the rate of penicillin production of the pellet form is much lower than that of the filamentous form. In a repeated fed-batch cultivation, Nielsen et al. *(6)* claimed that there was no relation between macroscopic morphology and penicillin production. Obviously, more information on this issue is necessary for a clear picture to be drawn.

The growth form of *P. chrysogenum* is influenced by a number of factors, e.g., agitation, inoculum, and polymer additives *(7)*. General tendencies for these factors may be drawn as follows. First, as the agitation intensity becomes stronger, the mold grows in smaller pellets and finally shifts to filamentous form *(8)*. Second, increasing the concentration of spore inoculum decreases the pellet size and, eventually, filamentous growth occurs *(9–11)*. Third, addition of polymer additives can result in a decrease in pellet size or even a filamentous growth *(12,13)*. It should be noted that the penicillin fermentation is affected mutually rather than individually by these factors. However, in the literature there is still not much information concerned with these mutual effects, especially with polymer additives.

In the present work, the effect of thickening agents (polymer additives) on the penicillin fermentation by *P. chrysogenum* was investigated. By adding a thickening agent to the fermentation medium, the growth pattern of the mold can be changed to facilitate the examination of the influence of the morphology on penicillin production. This study attempted to explain and elucidate the effect of thickening agent under different conditions of inoculum and agitation. The thickening agents used were PEG 20000 and CMC. Owing to comparison of these two polymer additives, the role of thickening agents in this fermentation could be more clearly understood.

MATERIALS AND METHODS

The strain used in this work was *P. chrysogenum* ATCC 48271, which was obtained as a lyophilized culture from the Culture Collection and Research Center, Hsinchu, Taiwan.

The mold was grown on agar slants with the following composition (g/L): lactose, 15.0; corn steep liquor (Sigma, St. Louis, MO), 3.1; peptone

(Difco, Detroit, MI), 5.0; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 0.25; KH_2PO_4, 0.6; $FeCl_3$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.002; agar (Difco), 30. The surface culture was mature after about 12 d at 25°C. The spores were rubbed off using sterile water and stored at –30°C. The spore concentration was estimated by absorbance at 250 nm using a spectrophotometer (UV-1201, Shimadzu, Japan), which was correlated with spread plate count.

Shake-flask cultivations were performed in 500-mL Erlenmeyer flasks with 100 mL growth medium and various amount of PEG 20000 or CMC. The growth medium was composed of (g/L): glucose, 20.0; yeast extract (Difco), 10.0; corn steep liquor, 5.0; beef extract (Difco), 0.075; peptone, 0.125; $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 3.0; $ZnSO_4 \cdot 7H_2O$, 0.01; and $MgSO_4 \cdot 7H_2O$, 2.3. After inoculating with a predetermined amount of spores, the resulting mixture was incubated at 25°C and 125 rpm on a rotary shaker. When glucose was depleted, 0.4% (w/v) lactose and 0.1% (w/v) phenylacetate were added to the medium daily to induce the production of penicillin G.

Tank fermentations were carried out in a 2-L fermentor (M-100, Tokyo Rikakikai, Japan) by seeding 900 mL of the growth medium with a 60-h preculture. Precultures were performed under the same conditions as in the shake-flask cultivations. The conditions of tank fermentation were: agitation speed 350 rpm, aeration rate 0.5 vvm, temperature 25°C and pH 6.5. The control of pH was achieved by adding dropwise solution of 3 *N* NaOH or 2 *N* H_2SO_4 [containing 0.5 *M* $(NH_4)_2SO_4$]. Foaming was controlled by 20% KM-70 (Shin-Etsu, Japan). The induction of penicillin G synthesis was the same as in shake-flask cultivations.

Culture samples of 20-mL volume were collected at specific time intervals, in which 1 mL was used for observation of macroscopic morphology. Dry-cell weight was measured after 19 mL of fermentation broth was filtered, washed with demineralized water, and dried at 80°C to a constant weight. Glucose was assayed as reducing sugar by the method of Miller *(14)*. Penicillin titers were analyzed by high-performance liquid chromatography (HPLC; Shimadzu LC-6A) using a RP-8 column (Merck, Darmstadt, Germany, 4 mm i.d. × 25 cm length; 5 μm particle size) at 50°C. The injection size was 20 μL. The mobile phase consisted of 85% (v/v) 0.075 *M* sodium phosphate buffer, pH 5.0, and 15% (v/v) acetonitrile (Merck, HPLC grade). The flow rate of the mobile phase was 1 mL/min. The eluted peaks were detected at 220 nm in a UV spectrophotometer (Shimadzu SPD-6A) and the peak areas determined with an integrator (Shimadzu C-R6A).

RESULTS AND DISCUSSION

Figure 1 shows the effect of PEG 20000 on penicillin production in shake flask cultivations. The concentration of spore inoculum was 1.3×10^6 spores/L. To each flask, 0, 3, 5, 10, and 20% (w/v) PEG 20000 was added

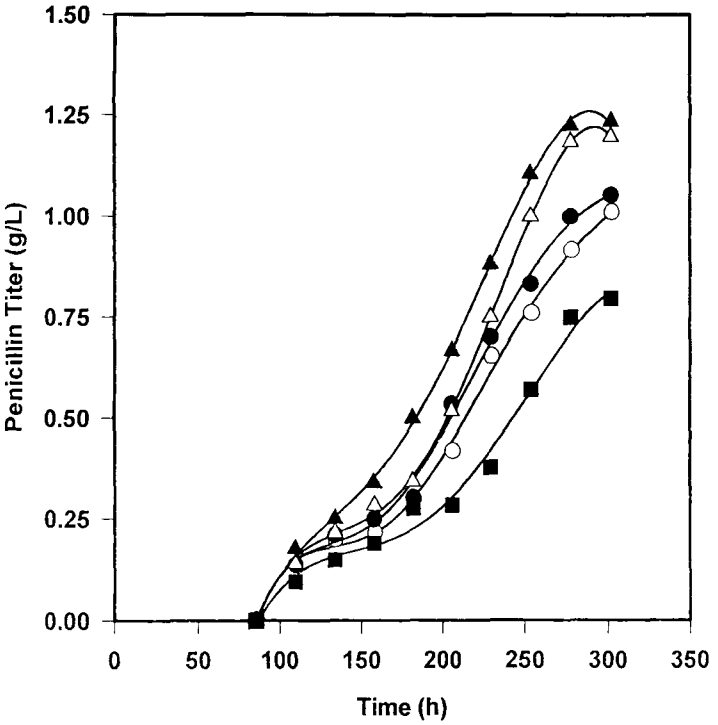

Fig. 1. Effect of PEG 20000 on penicillin production in shake-flask cultivations with a low level of spore inoculum. Amount of PEG 20000 added (w/v): (○) nil, (●) 3%, (△) 5%, (▲) 10%, (■) 20%.

separately. The mold was found to grow in pellets except in the flask of 20% PEG 20000, where it was in filamentous form. In general, addition of PEG 20000 to the fermentation medium resulted in more and smaller pellets, when compared with the control. The penicillin production was in the sequence 10% > 5% > 3% > nil > 20%. These experimental findings can be summarized as follows: thickening agents decrease the pellet size or even cause filamentous growth, small pellets favor penicillin synthesis, and filamentous mycelia provide poor penicillin production. Similar phenomena were also observed with CMC as the thickening agent, where the spore inoculum concentration was 9.4×10^5 spores/L and the amount of CMC added was 0, 0.5, 1, 1.5, and 2% (w/v), respectively. The mold also grew in pellets except in the case of 2% CMC. However, as shown in Fig. 2, the sequence of penicillin titer was somewhat different: 0.5% > 1% > 1.5% > nil > 2%; the 0.5% CMC and not the 1.5% one resulted in the highest penicillin titer. The explanation for the sequences of penicillin titer is given in the next paragraph. Nevertheless, it is interesting that the existence of an optimum amount of thickening agent for penicillin production is common between PEG 20000 and CMC.

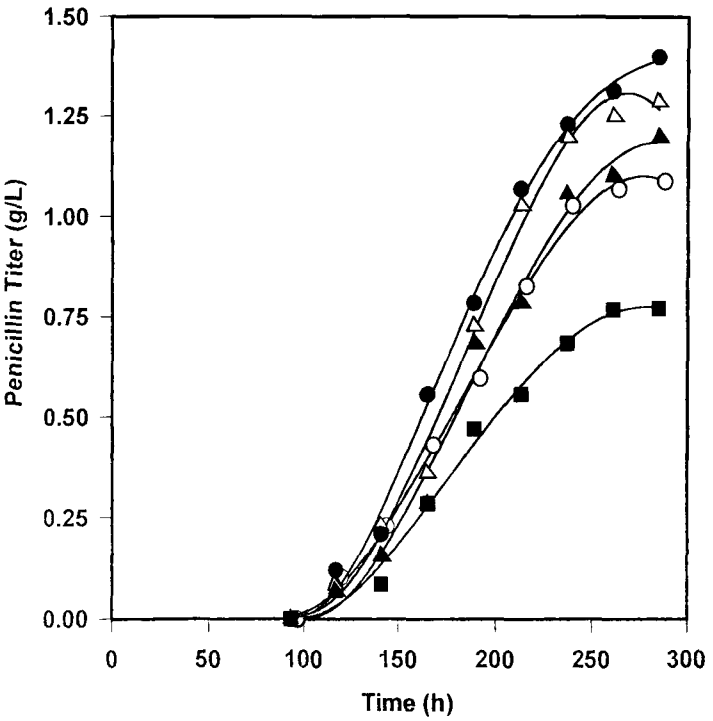

Fig. 2. Effect of CMC on penicillin production in shake-flask cultivations with a low level of spore inoculum. Amount of CMC added (w/v): (○) nil, (●) 0.5%, (△) 1%, (▲) 1.5%, (■) 2%.

The existence of an optimum in penicillin production indicates that there must be two opposing effects, favorable and adverse, acting at the same time when a thickening agent is added to the medium. The favorable effect comes from two aspects: one, reducing agglomeration of spores and hyphae, which results in more and smaller pellets, thus enhancing the mass transfer rate into the interior of the pellets; the other, depressing local velocity gradient, which prevents the cells from damage *(12)*. Because shear stress is considered to be gentle in the shake flask cultivations *(11)*, the former should be predominant. On the other hand, the adverse effect is an increase in broth viscosity, which leads to a lower oxygen solubility, a slower rate of external mass transfer, or even a filamentous growth. Whether the apparent influence of addition of a thickening agent is favorable or adverse depends on the relative strength of these two opposing effects. As shown in Figs. 1 and 2, the highest net positive influence of thickening agent on penicillin production occurred at 10 and 0.5% for PEG 20000 and CMC, respectively.

Addition of the thickening agents caused an alteration in the pellet structure. In the control run, the mold grew in a nonuniform morphology,

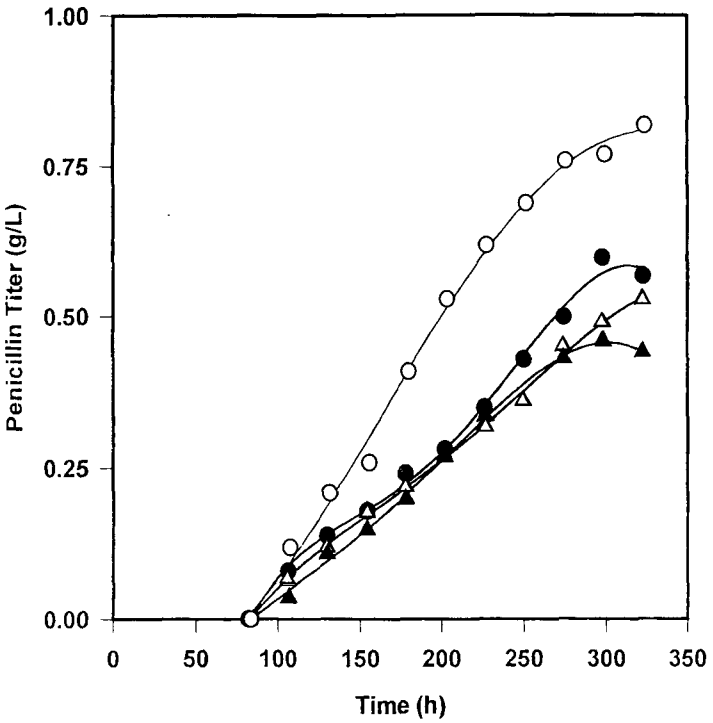

Fig. 3. Effect of PEG 20000 on penicillin production in shake-flask cultivations with a high level of spore inoculum. Amount of PEG 20000 added (w/v): (○) nil, (●) 3%, (△) 5%, (▲) 10%.

with a large portion existing as compact smooth pellets. As the fermentation proceeded, the pellets became bigger, and eventually, broke into clumps. In the presence of PEG 20000 or CMC, however, the mold first grew in quite uniform, relatively small, fluffy, loose pellets, i.e., pellets having a compact center and a much looser outer zone (*7*). As these pellets grew, the center of the pellets became looser and looser, and finally, the mold turned filamentous. It was found that the variation of pellet structure was more obvious as more thickening agent was added. Because small fluffy loose pellets occurred in the cases of higher penicillin productions (for example, 10% PEG 20000 in Fig. 1 and 0.5% CMC in Fig. 2), this morphology is therefore favorable for penicillin production.

As mentioned earlier, the pellet size decreases with increasing concentration of spore inoculum. Therefore, under the same gentle agitation, the favorable effect owing to addition of a thickening agent should be less pronounced, if a higher spore inoculum level is used. On the other hand, the adverse effect (increase in broth viscosity) would remain the same in spite of the inoculum level. In other words, addition of a thickening agent may even lower the penicillin titer in cases of high-inoculum levels. This

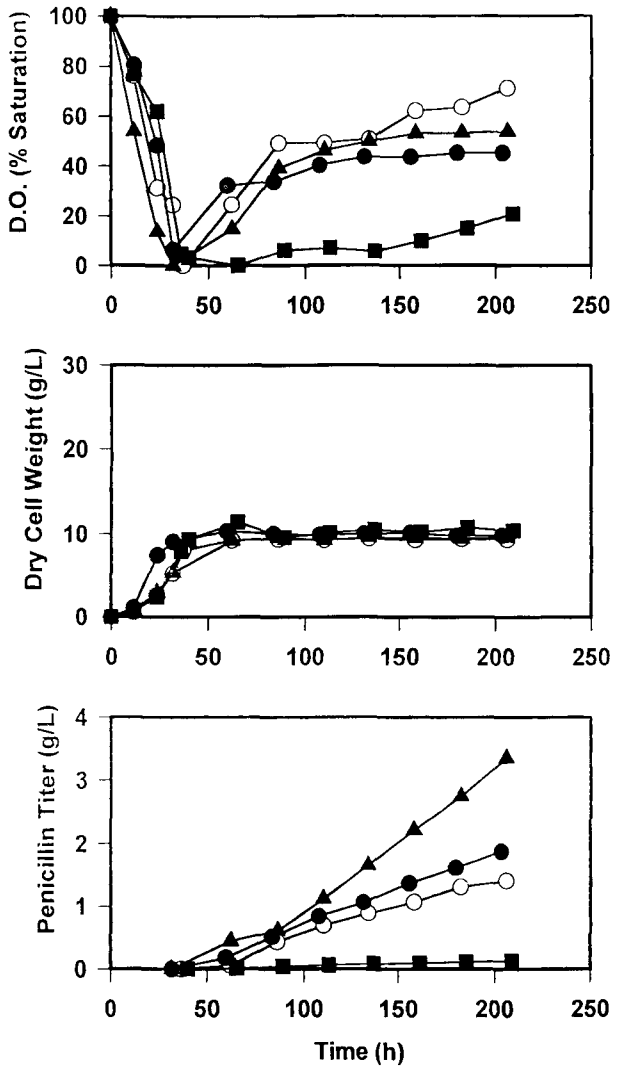

Fig. 4. Comparison of different strategies of applying PEG 20000 in tank fermentations. ○, control; ●, 10% PEG 20000 in preculture; ▲, 5% PEG 20000 in preculture; ■, 5% PEG 20000 in both preculture and main culture.

rationale is demonstrated in Fig. 3 for PEG 20000, where, compared with Fig. 1, a higher-inoculum level of 1.9×10^6 spores/L was used. As one can see, the influence of the thickening agent was negative for penicillin production, although it may be favorable under lower levels of spore inoculum. It thus suggests that enhanced penicillin production owing to addition of a thickening agent can be obtained only with low spore inoculum levels, or more strictly, under the conditions that the mold would otherwise grow in large pellets.

In the operation of a tank fermentation, the process is usually divided into two stages: preculture (inoculum) and main culture. Because the status of inoculum is known to be of prime importance for successful penicillin fermentations, the timing of employing the thickening agent was therefore considered not to exclude this stage. Fig. 4 shows the comparison of different strategies of applying PEG 20000 to the fermentation. The concentration of spore inoculum in the preculture was 8.8×10^6 spores/L. For the first case, with 5% PEG 20000 in the preculture, the penicillin production was increased by 130%. However, for the second case, the penicillin titer was only 10% of the control when 5% PEG 20000 was in both preculture and main culture. As discussed earlier, the enhanced penicillin production in the first case is owing to smaller pellets encountered in the preculture, whereas the reduced production in the second case is because of an increased broth viscosity in the main culture. From the microscopic observation, we concluded that this increased viscosity led to filamentous growth. Moreover, it can be seen in Fig. 4 that the dissolved oxygen level in the second case was less than 20% air saturation, which, according to Vardar and Lilly *(15)*, would impair penicillin synthesis.

The agitation intensity is much stronger in tank fermentations than in shake flask cultivations. As mentioned earlier, higher extent of agitation makes the pellet size smaller or even causes filamentous growth. Therefore, the optimum amount of a thickening agent for enhancing penicillin production should be smaller in tank fermentations than in shake-flask cultivations, other conditions being equal. In other words, it suggests that the quantitative results obtained in shake flasks should be re-evaluated for tank fermentors. In Fig. 4 it can be seen that in tank fermentations, penicillin productivity with 10% PEG 20000 in the preculture was smaller than that with 5% PEG 20000. This is attributed to the fact that part of the pellets turned filamentous in the former case. It therefore indicates again that filamentous mycelia provide poor penicillin production. The inoculum effects on morphology in shake flasks and agitated bioreactors, reported in the work of Tucker and Thomas *(11)*, can be explained based on an essentially similar rationale.

An additional favorable effect of PEG 20000 in the penicillin fermentation is its ability of suppressing foaming. The control run required about 90 mL of 20% KM-70 to control foaming, whereas with 5% and 10% PEG 20000 in the preculture, KM-70 volumes used were 10 mL and nil, respectively. In a previous report *(16)*, KM-70 was shown to exert a hindrance in the oxygen-transport process. Less amount of KM-70 used thus compensates the decrease in dissolved oxygen level due to PEG 20000 in the medium. As shown in Fig. 4, with 5% and 10% PEG 20000 in the preculture the dissolved oxygen level did not decrease significantly. Unlike PEG 20000, CMC functions as a foam stabilizer *(17)* and does not cause such a compensatory effect.

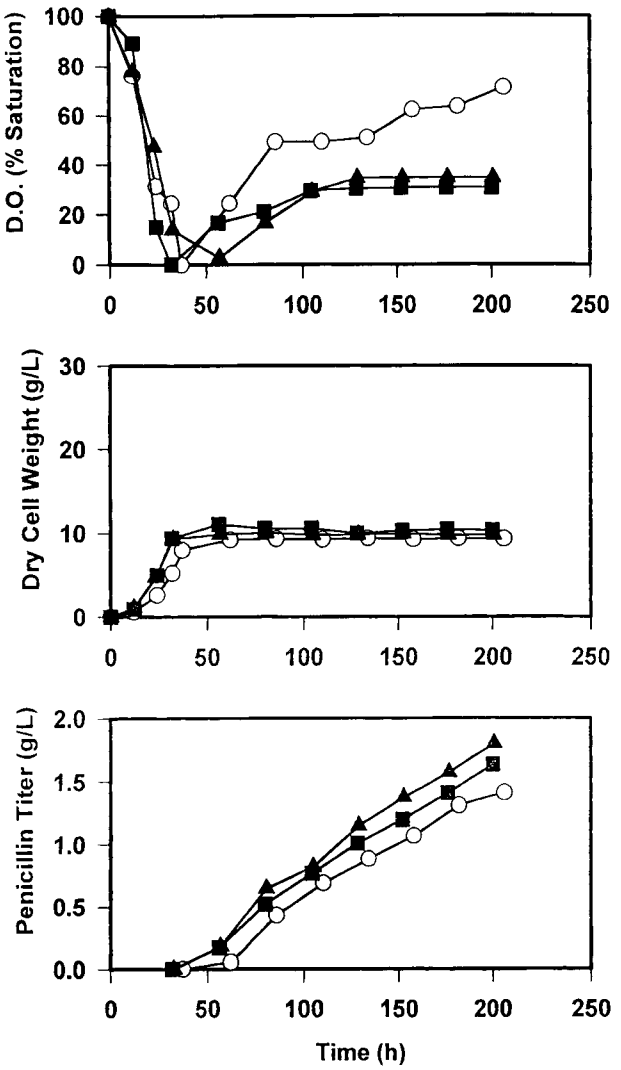

Fig. 5. Effect of CMC in preculture on the penicillin fermentation. ○, control; ▲, 1% CMC; ■, 3% CMC.

In Fig. 5, it is shown that 1% and 3% CMC in the preculture caused significant decreases in dissolved oxygen level; however, penicillin production was still higher than the control. This can be attributed to the even stronger effect of favorable morphology for the mold.

CONCLUSIONS

We have illustrated that the growth form of *P. chrysogenum* can be manipulated by additions of PEG 20000 and CMC to the culture broth.

Thus the influence of morphology on the penicillin production can be examined. Better penicillin production was obtained when the mold was in small, fluffy, loose pellets. Because addition of a thickening agent to the medium results in two opposing effects, the amount added should be optimized. This optimum amount is affected by the level of spore inoculum and agitation intensity. In general, improved penicillin production can be obtained when the mold will otherwise grow in large pellets. In tank fermentations, the employment of a thickening agent should be in the preculture but not in the main culture. As a thickening agent for improving the penicillin fermentation, PEG 20000 is more adequate than CMC. This is because PEG 20000 can suppress foaming, and CMC cannot.

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