

Full Length Research Paper

Optimal control of a batch bioreactor for the production of a novel antifungal substance CF66I

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More than 80% of the plant diseases are caused by fungi. Usually, fungi not only destroy the plants, but also produce mycotoxins that are harmful to human health. At present, chemical fungicides are mainly used for the prevention of fungi-related plant diseases, however, research and development of biological prevention and controlling are of great importance. In this work, the effects of pH and temperature on cell growth and CF66I formation in batch culture of *Burkholderia cepacia* CF-66 were studied. The pH value has a marked effect on cell growth and production of CF66I. The lag phase was much longer when pH set lower (e.g.5.0) or higher (e.g.8.0). For earlier phase, optimal pH value was 6.0, because the lag phase can be shortened and the whole fermentation phase can also be shorten and then quickly goes into CF66I production phase. In the late phase, the higher pH is in favor of the production of CF66I. Different temperature have different effect on cell yield, specific growth rate, CF66I yield and specific synthesis rate. In the prophase of fermentation, it is better to set higher temperature to make the cell growth maximizing as soon as possible. However in mid-anaphase, lower temperature shortens the fermentation time, reduce heating energy and the cost. According about results, an optimal control strategy was constructed.

Key words: *Burkholderia cepacia*, antifungal activity, optimal control, CF66I.

INTRODUCTION

Every year, plant-pathogenic fungi such as *Fusarium* sp., *Pythium* sp. and *Rhizoctonia solani* cause millions of dollars worthy of crop damage all over the world despite the extensive use of chemical pesticides. Meanwhile, concerns about food safety, environmental quality and pesticide resistance have dictated the need for more useful techniques (Copping and Menn, 2000). One of most promising alternatives in the context of agricultural world is the use of bioproducts from microorganisms, playing an important role in agriculture sustainable models. Recently, many reports have been published concerning to new antibiotics having biocontrol activity

including some kinds of bacteriocins, alkaloids, lipopeptides and polypeptide (Arima et al., 1968; Cupples and Sen, 1978; Wakayama et al., 1984; El-Banna and Winkelmann, 1998). Some antibiotics, such as polyoxin, validamycin, amipirimycin and abomycin, have been extensively used in agricultural biocontrol (Cabib et al., 1991; Yu et al., 2005). However, there are many problems due to the limited antifungal spectrum, long time to become effective against the pathogenic fungi and their instability. Most of all, the inhibitory efficacy against soil-borne plant pathogens is associated with the productivity of antibiotics, however, in general the productivity is low because they are secondary metabolites.

The bacterium *Burkholderia cepacia* is a plant growth promoting rhizobacteria. Early in 1990s, it has already been used as a biocontrol compound for protecting crops from fungal disease and certain isolates of the *B. cepacia* complex are commercially available for control of plant

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pathogenic fungi and nematodes (DenyTM, Stine Microbial Products, USA). Different *B. cepacia* strains have been reported to be an effective biocontrol compound for *Pythium*-induced damping off and *Aphanomyces*-induced root rot of pea (Parke et al., 1991; King and Parke, 1993; Bowers and Parke, 1993), *Botrytis*-induced gray mold of apple (Janisiewicz and Roitman, 1988), *Rhizoctonia solani* induced root rot of *Poinsettia* (Cartwright and Benson, 1994), and other fungal diseases (Fridlender et al., 1993), because it can produce many secondary compounds such as pyrrolnitrin, altericidins, cepacin and other unidentified volatile or nonvolatile compounds which showed strong antifungal activity (Kirinuki et al., 1977; Hwang et al., 2002; Parker et al., 1984).

Recently, a strain identified as *B. cepacia* CF-66 with strong antifungal activity has been isolated from the compost samples, which strongly inhibits the growth of many plant pathogens such as *R. solani*. A novel antifungal compound CF66I was also isolated from the culture of *B. cepacia* CF-66 strain and preliminary spectra data suggested it was most probably a polyether antibiotic with aliphatic chains (Quan et al., 2005; Quan et al., 2006). The effect of medium components on CF66I production was investigated by shaking-flask culture through statistics-based experimental design, previously. A fractional factorial design augmented with center points revealed that sodium citrate and yeast extract were the most significant factors, which influenced CF66I production positively, while the other factors were not important with the levels tested.

The method of steepest ascent was also used to approach the proximity of optimization. The optimized medium allowed the activity of CF66I to be increased from 2.12 U/ml to 6.24 U/ml, about 200% higher than the original medium (Zeng et al., 2007). Effects of oxygen transfer coefficient (K_{La}) and dissolved oxygen concentration (DO) on batch fermentation of CF66I were investigated with 3.7-L jar fermentor in the previously study. It was found that high K_{La} level was disadvantageous to the cell growth and the CF66I production at the beginning of the fermentation. The K_{La} value was optimized at 121.2 h^{-1} and the DO concentration was optimized at 15% air saturation. Consequently, a two-stage oxygen supply control strategy was applied in which K_{La} value was fixed at 121 h^{-1} at the first 28 h, then the DO value was changed to 15% air saturation by varying the stirring speeds and the aeration rate until the end of the fermentation to increase the production of CF66I. By applying this strategy, the maximal antifungal activity of CF66I had an improvement and reached 7.360 U/ml, which higher than those of constant operations.

Medium pH and culture temperature are key operating parameters of CF66I production process. The object of this work was to evaluate the effects of pH and temperature on cell growth and CF66I formation in batch fermentation of *B. cepacia* CF-66. An optimal pH and temperature control strategy, which maximize antifungal activity of CF66I and CF66I productivity for batch CF66I

fermentation was demonstrated.

MATERIALS AND METHODS

Chemicals

Reagents for cultivation such as yeast extract, beef extract, peptone and agar were purchased from Wako, Japan. D-Glucose, $MgCl_2 \cdot 6H_2O$, NH_4Cl , $Fe_2SO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, K_2HPO_4 and KH_2PO_4 , were obtained from Shenlian Chemical, China. All other reagents used were of the highest grade available unless otherwise indicated.

Microorganism and culture maintenance

B. cepacia CF-66 isolated from compost was used throughout this study. The strain was cultivated on PDA (potato-dextrose-agar) slants and incubated at 30°C for 12 h, then stored at 4°C. *Pichia membranaefaciens* was used as indicator microorganism. This yeast was grown on a medium of glucose 30 g/L, yeast extract 4 g/L and peptone 5 g/L, at 28°C for 12 h on a reciprocal shaker at 200 rpm.

Cultivation media

Medium S composed of yeast extract 8 g/L, Na-Citrate 7.5 g/L, NH_4Cl 2.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, K_2HPO_4 1.5 g/L, KH_2PO_4 0.6 g/L was used seed cultivation. Medium F was an optimal medium developed by Medium S in shake flask culture for enhanced production of CF66I by *B. cepacia* CF-66 (Zeng et al., 2007), which composed of yeast extract 19.61 g/L, Na-Citrate 34.48 g/L, NH_4Cl 2.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, K_2HPO_4 1.5 g/L, KH_2PO_4 0.6 g/L.

Culture methods

A loop of cells grown on a PDA slant was inoculated into 1000 ml-flask containing 200 ml medium S. After 12 h cultivation at 30°C and 200 rpm on a rotary shaker, the seed was inoculated into a 3.7-L stirred tank bioreactor (KLF2000 3.7 L, BioEngineering, Switzerland), equipped with a pH probe (Type 465-35-90-K9; Mettler Toledo) and a dissolved oxygen probe. The aeration rate was 250 L/h and the agitation speed was at 550-650 rpm. To control pH at a set level, 2 N NaOH and 2N H_2SO_4 was automatically added to the culture broth. Generally, foaming appeared in the culture medium after 12 h of cultivation, a situation leading to unstable culture conditions that can be avoided by addition of anti-foaming agent KM-70 (ShinEtsu Chemical Co. Ltd., Tokyo). The temperature was controlled according to the need of the experiments. During the fermentation, the pH and DO was monitored on line, and cell growth, residual carbon source and antifungal activity was analyzed off-line by the method described below. For each experiment, 13 samples were taken and analyzed for three times.

Analytical methods

For the measurement of the concentration of the cells, biomass concentration was estimated from optical density using a predetermined correlation between absorbance at 660 nm and dry mass weight (DCW). The amount of sodium citrate in the culture broth was determined using high performance liquid chromatography (HPLC) following by the method (Marsili et al., 1981). The HPLC system for analysis of sodium citrate was composed of a Hitachi L-6200 solvent delivery controller, a Hitachi 4200 H UV-vis detector, a Hitachi-D-2500 Chromato-Integrator and

a Aminex® HPX-87H (BIO-RAD, U.S.A.) column. The injection volume was 20 µl. The sample was eluted with a mobile phase comprising 0.05 M H₂SO₄ at a flow rate of 0.6 ml/min. The chromatogram was monitored at 210 nm.

The antimicrobial activity of CF661 in the culture broth was measured by Hultmark method (Lee et al., 2004) with slight modification. The culture broth was centrifuged for 10 min at 15,000 rpm and then their supernatant was filtrated through a 0.22-µm filter. The indicator microorganism cultivated into log growth phase was diluted with liquid medium, in which absorbance at 554 nm was 0.2. Five-hundred microlitre of above culture broth sterilized with micro-filter was added into 2 ml of indicator diluents, cultivated for 2 h at 30°C, and then cooled in ice bath for 20 min before determine the change of absorbance at 554 nm. For the control, 500 µl of medium F was added. Antimicrobial activity was calculated as follow $U = (\Delta A_0 - \Delta A_s) / \Delta A_0 \times N$, in this formula ΔA_0 and ΔA_s are absorbencies of control and sample culture broth and N is dilution multiple.

RESULTS

Effects of pH on cell growth and CF661 production

Time-course data on cell, CF661 production and sodium citrate concentration of one batch culture with initial pH 6.0 and four pH controlled batch cultures with pH set point ranging 5.0 to 8.0 are shown in Figures 1 and 2. While culture pH was uncontrolled it increased gradually from 6.0 to 8.5 at 16 h of fermentation and kept higher than 8.0 until the end of fermentation. As the results, the cells reached stationary phase at 12 h of fermentation with a maximum concentration of 2.04 g/L and quickly turned into death phase. However, the production of CF661 was beginning at 12 h and increased to 2.37 U/ml at 52 h.

The effects of pH values at the range of 5.0 to 8.0 on CF661 production were studied. Time courses of CF661 fermentation at the different pH are shown in Figure 2. At pH lower than 5.0 and pH higher than 8.0, little cell growth occurred, so their time courses of CF661 fermentation were not listed in Figure 2. At pH 5.0 DCW and CF661 activity reached maxima of 2.582 g/L and 1.553 U/ml at 46 and 36 h, respectively. However, the lag time of cell growth was very long, almost lasting 12 h. Moreover, the cells grew slowly during the whole CF661 fermentation, so it indicated that pH 5.0 was not appropriate for CF661 production. Time course of CF661 production at pH 6.0 was different from that at pH 5.0, the lag time of cell growth was the shortest than all tested pH value, DCW reached the maximum value of 3.999 g/L at 32 h, while the maximum activity of CF661 was obtained at 48 h. At pH 7.0 and pH 8.0, the lag time was longer than that of pH 6.0, the maximum of DCW was 4.615 g/L at 12 and 24 h, respectively. Time course of CF661 production at pH 7.0 and 8.0 were similar, the difference was that the maximum activity (8.166 U/ml) of CF661 at pH 8.0 is higher than that at pH 7.0 (6.996 U/ml).

According to the above results, it is clear that pH plays an important role in the process of CF661 fermentation. At pH lower than 5.0 and higher than 8.0, the lag time of cell growth was longer, CF661 formation rate was enhanced by increasing of pH value.

The specific growth rate was determined from the slope of the semi-logarithmic plot of cell density versus

fermentation time, $\mu = \frac{1}{x} \frac{dx}{dt} = \frac{1}{x} \lim_{\Delta t \rightarrow 0} \frac{\Delta x}{\Delta t}$. μ_{cell} at definite time

was obtained through computing cell density by interpolating calculating of experimental data with Origin 7.0 and Microsoft Excel. Time courses of the specific cell growth rate at different pH are shown in Figure 3. As shown in Figure 3, profiles of μ_{cell} at different pH had similar tendencies, μ_{cell} was the highest at pH 7.0 before 12 h, but the highest at pH 8.0 after 12 h. The μ_{max} at different pH showed that the optimum pH for cell growth was 6.0 and μ_{max} reached to 0.759 h⁻¹. It is indicated that, at early phase of CF661 fermentation (before 12 h), pH should be controlled at 6.0 in order to shorten the lag phase of cell growth and thus total fermentation time and to move up the time of CF661 formation.

The specific CF661 formation rate (q_p) was determined from reciprocal of cell density and the slope of CF661

activity versus fermentation time, $q_p = \frac{1}{x} \frac{dp}{dt} = \frac{1}{x} \lim_{\Delta t \rightarrow 0} \frac{\Delta p}{\Delta t}$

$q_p = \frac{1}{x} \frac{dp}{dt} = \frac{1}{x} \lim_{\Delta t \rightarrow 0} \frac{\Delta p}{\Delta t}$. q_p was obtained with the method

similar to μ_{cell} . The duration of reaching maximum q_p was 32 h, and same at different pH. Although the highest q_p could be achieved at pH 8.0, fastest increasing rate was observed at pH 6.0. At pH 5.0, the CF661 formation rate was significantly low compared to that at other tested pH value and duration of CF661 formation was very short. Therefore, at early phase of CF661 fermentation, it is appropriate to control pH at low value (e.g. pH 6.0) to maximize q_p , while at mid- and later-fermentation phase, higher pH (e.g. pH 8.0) was more proper in order to maintain high q_p .

pH control strategy of batch CF661 production by *B. cepacia* CF-66

From the results discussed above, relative lower pH at earlier fermentation stage not only made the lag phase of cell growth shorter but also was advantageous to cell growth and CF661 formation. At later fermentation stage, properly increasing pH value can strengthen cell growth and CF661 formation, thus, increase CF661 fermentation level. During the whole process, the suitable pH for cell growth and CF661 formation are different, it is favorable to use a three-stage pH control process instead of constant pH process. As suggested from the results shown in Figures 2 and 3, an optimal pH-shift strategy was developed as following: pH was controlled at 6.0 during 0-12 h and then raised naturally to pH 8.0 without control for the second 12 h, maintained the pH value at 8.0 till the end of the fermentation.

Time course of pH-shift strategy is shown Figure 4. When pH was controlled at 6.0 at the early phase, the lag

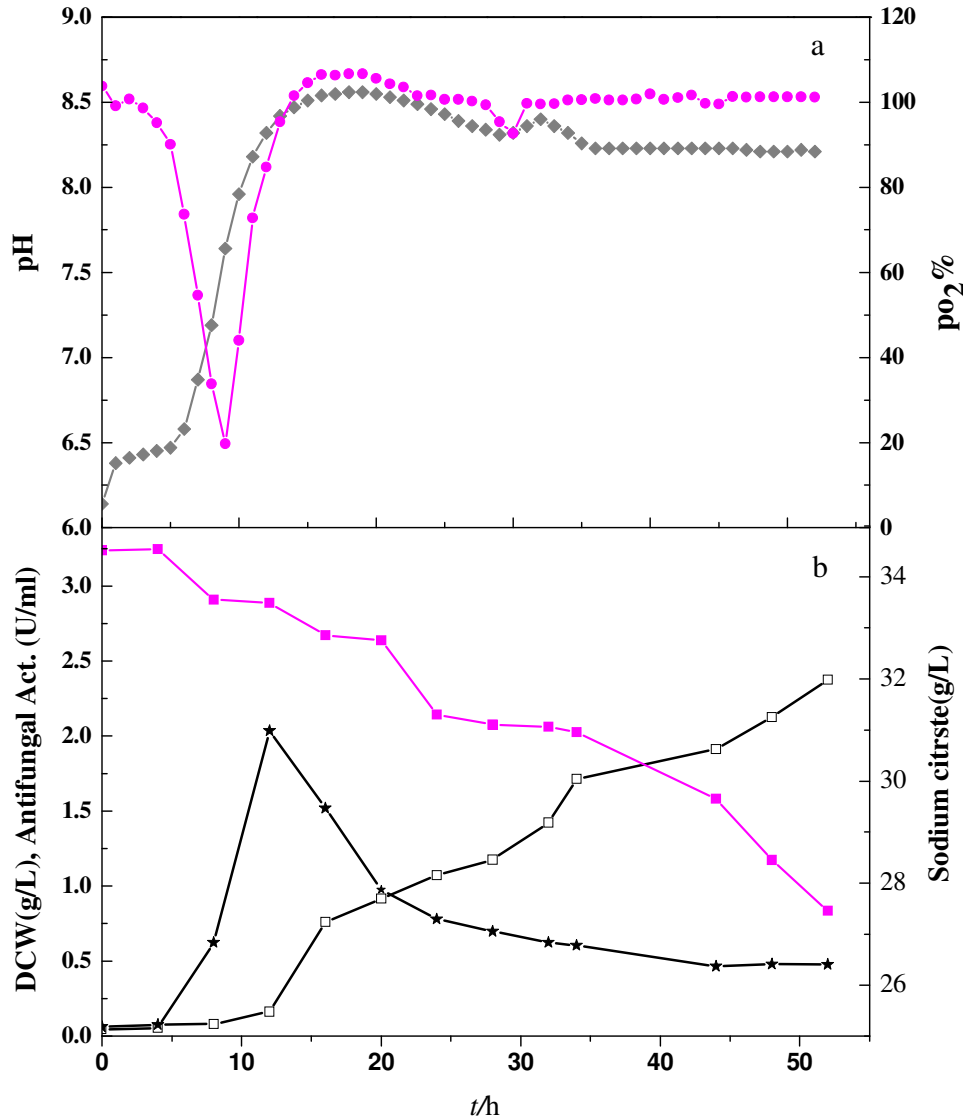


Figure 1. Time profile of the batch cultivation at natural pH value. (a) pH and PO₂%; (b) cell growth, antifungal activity and substrate consume. Symbols: -◆- pH; -●- PO₂%; -■- sodium citrate; -○- antifungal activity; -*-* cell growth.

time was short, cell growth rate was high than that of at pH 8.0. After pH was shifted to 8.0 at 24 h, cell growth rate continued keeping at high levels and CF66I production was on exponential phase. The CF66I production was kept maximum after 48 h, CF66I activity was reached to 8.599 U/ml. CF66I yield and CF66I productivity were increased by 15.4 and 5.3%, respectively, compared with the best results of pH process. It is concluded that the proposed three-step pH-shift strategy can obviously improve CF66I fermentation level.

Effects of temperature on cell growth and CF66I production

The effects of temperature on cell growth and CF66I

production of *B. cepacia* Cf-66 is shown in Figure 5. Maximum CF66I activity was obtained at 25°C, but cell growth was more rapid at 30°C (Figure 5a). Temperature which is higher than 33°C or lowers than 25°C is not suitable for cell growth. When the temperature control to 33°C, although lag phase was short, but cell growth very quickly achieve a stationary phase. At 25°C, CF66I activity was increased rapidly than others temperatures and reached maximum at the end of fermentation though CF66I production appears late (Figure 5b).

As shown in Figure 5c, along with the temperature increase, the consumption rate of sodium citrate and cell growth go fast evident and the time of entering into stationary phase is short significantly. Sodium citrate was nearly exhausted after 20 h at 33°C and the concentration

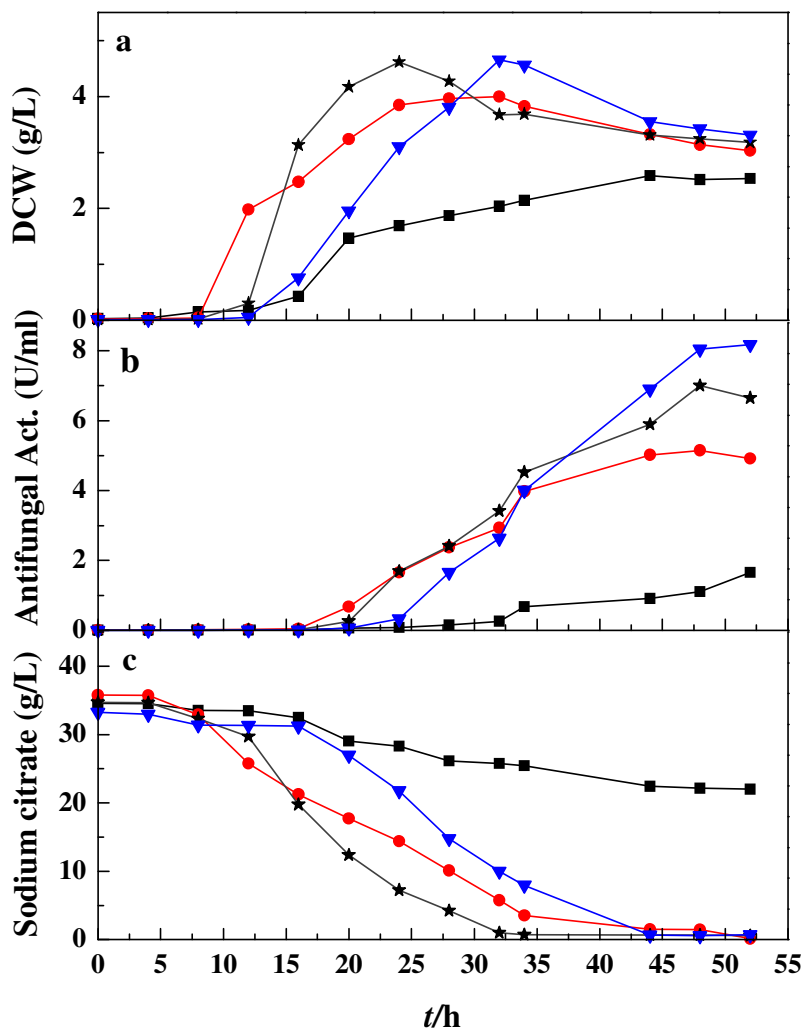


Figure 2. Effects of pH on biomass (a), antifungal activity (b), and substrate consumed (c). Symbols: ■- pH 5.0; ●- pH 6.0; * - pH 7.0, and ▼- pH 8.0.

of residual substrate was 1.89 g/L. At 30°C, sodium citrate was consumed mostly after 28 h and the concentration of substrate was 0.11 g/L. However, at 23°C, the substrate remained high concentration of 19.96 g/L until the end of fermentation process. Therefore, adopting higher temperature (e.g 30°C) at beginning of the fermentation is profit to shorten lag phase and fermentation time, it also can increase use ratio of substrate.

Temperature control strategy of batch CF66I production by *B. cepacia* CF-66

The optimum fermentation temperature generally mean that it not only suit for cell growth, but also profit for metabolite synthesis. However, in the production process, the optimum growth temperature is differing from the optimum production temperature. It is necessary that to unify promoting cell growth and increasing product

synthesis with controlling optimum temperature in different phases. When control fermentation temperature was constant, higher temperature at prophase can not only shorten cell growth lag phase, decrease total fermentation time and profit cell growth, but also enhance efficiency of CF66I synthesis. In the later phase of fermentation, degrading fermentation temperature which only can maintain normal cell metabolism of *B. cepacia* CF-66 is effective to raise CF66I synthesis rate and improve the total fermentation level. Thus, following control strategy was used: control temperature at 30°C before 20 h and 25°C after 20 h.

Figure 6 shows the fermentation course in 3.7 L-jar fermentor using above two-stage temperature control strategy. It is indicated that cell growth lag phase was shorten, cell growth rate was faster and antifungal activity was reached to 11.349 U/L at 48 h. Table 1 shows the comparison of key parameter under different temperature. CF66I's yield was raised obviously by two-stage tempe-

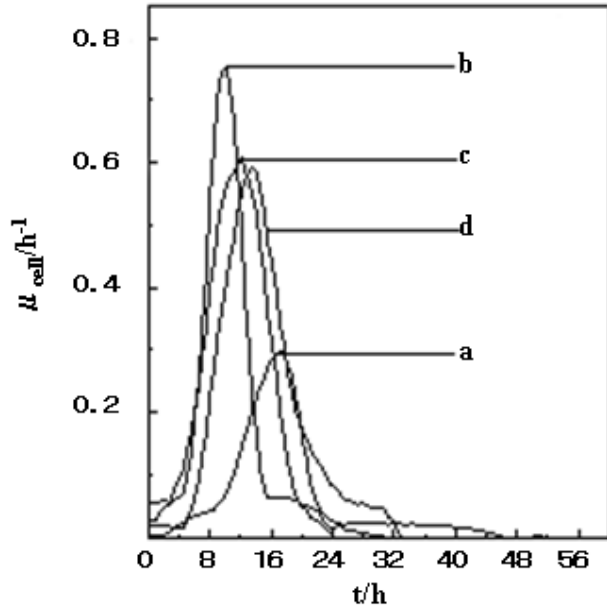


Figure 3. Time courses of specific CF66I formation rate under different pH; (a) 5.0 (b) 6.0 (c) 7.0 and (d) 8.0.

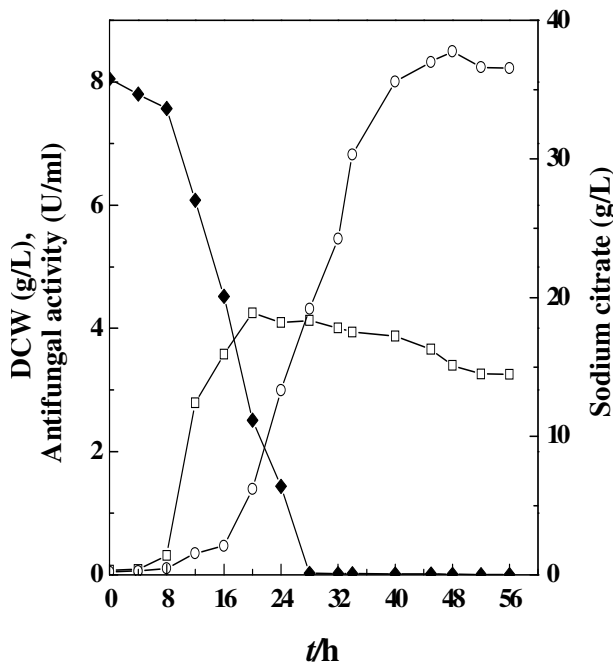


Figure 4. Time profile of CF66I production using a three-stage pH control strategy. Symbols: -♦- sodium citrate; -○- antifungal activity; and -□- cell growth.

perature control strategy and the productivity was also enhanced markedly. The maximum antifungal activity was reached to 218.28 U/L which was enhanced to 26.1% than single temperature controlled and average specific CF66I production rate was 40.12U/L/h.

DISCUSSION

B. cepacia consists of genotypically distinct but phenotypically similar groups of strains and closely related species, which have been collectively designated as the *B. cepacia* complex. Recent taxonomic analyses have demonstrated that the complex actually comprise a cluster of nine species and genomovars referred to as *B. cepacia* genomovar (I) *B. multivorans* (II) *B. cenocepacia* (III) *B. stabilis* (IV) *B. vietnamiensis* (V) *B. dolosa* (VI) *B. ambifaria*(VII) *B. anthina* (VIII) and *B. pyrrocinia* (IX) (Whitby et al., 2000; Payne et al., 2005; Ramette et al., 2005; Coenye et al., 2001a–c; Vandamme et al., 2002; Tabacchioni et al., 2002; LiPuma et al., 1999). Preliminary studies also indicated that most epidemic *B. cepacia* species isolated are genomovar II and III and that this species is associated with great morbidity and mortality than other members of the *B. cepacia* complex (LiPuma et al., 1999; Tabacchioni et al., 2002). *B. cepacia* genomovar V and VII have higher plant growth promoting activity and lower frequency recovery from cystic fibrosis than other members. The strain CF-66 with strong antifungal activity, which was isolated from compost, was identified as *B. cepacia* complex by morphological and biochemical tests and 16S rDNA sequence and species-specific PCR showed that this strain may belong to *B. cepacia* genomovar V, VI, or IX and the highest probability is *B. cepacia* genomovar V. To avoid the risks caused by using *B. Cepacia* in biological control process, it is better to use the antimicrobial compounds extracted from the fermentation broth of *B. Cepacia*. This paper investigated the effect of pH value and temperature on fermentation process and the characteristics of fermentation kinetic by batch fermentation experiment with 3.7-L fermentor. The control parameters got from the experiment can be used for broad scale industrialization.

The pH of the culture medium is a very important parameter in the process of batch fermentation. It is the comprehend indicator of cell metabolic activity in certain environmental conditions. Not only the substrate, but also the protein molecule’s ionization state changes with pH value of the broth. Especially for the enzyme, its ionization state is fairly sensitive to pH value. The growth and output production of the microorganism need many kinds of enzyme catalytic reactions, so pH value is the most important environmental factor of cell growth. The pH value will change with the accumulation of organic acids and amino acids, because the bacteria utilize the carbon and nitrogen source in the culture medium in a certain temperature and venting condition. The cell density of strain CF-66 maximize at 12 h and then will go to decline phase with no marked lag or stationary phase, if the pH value is unregulated. On the other hand, the plot of pH value suggests that pH have a fast upgrade (from 6.4 to 8.5) as the cells go into log phase and the biomass achieved maximum and then declined and autolyzed with no marked change of pH value. The pH value has a marked effect on cell growth and production of CF66I. The

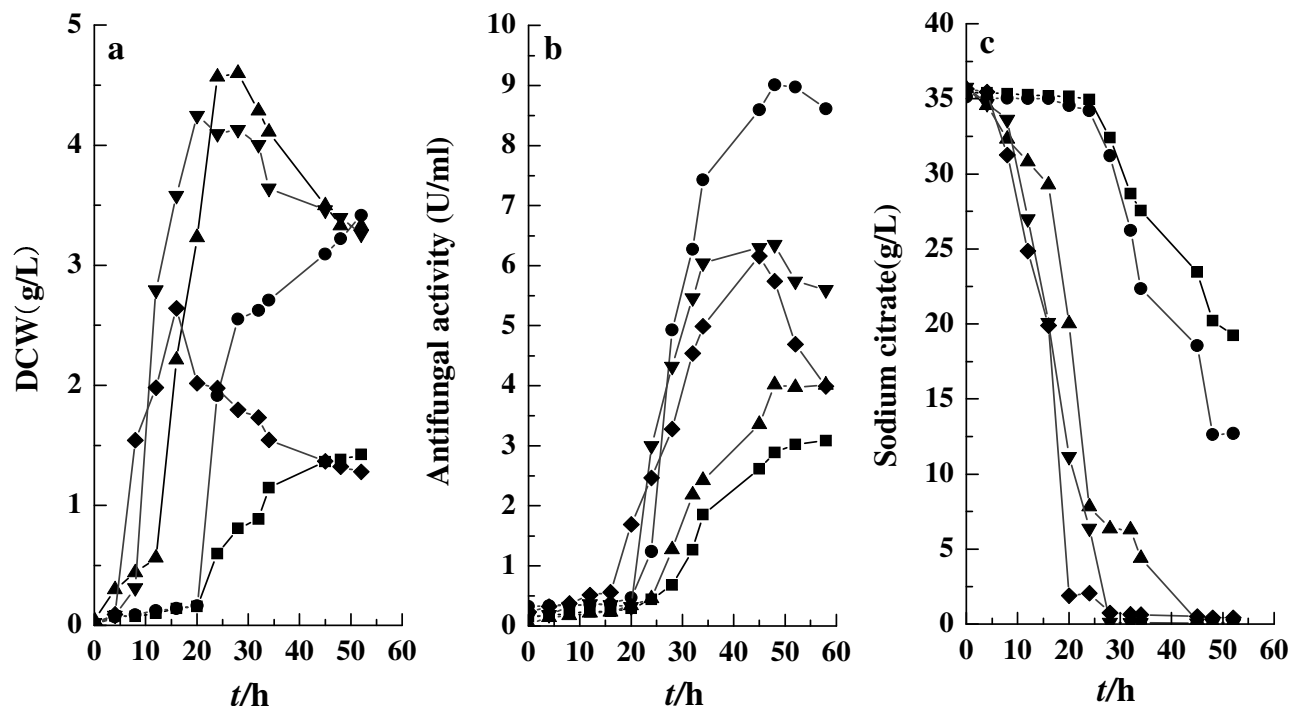


Figure 5. Effects of temperature on biomass (a), antifungal activity (b), and Substrate consumed (c). Symbols: -◆- 33°C; -▼- 30°C; -▲- 28°C; -●- 25°C; and -□- 23°C.

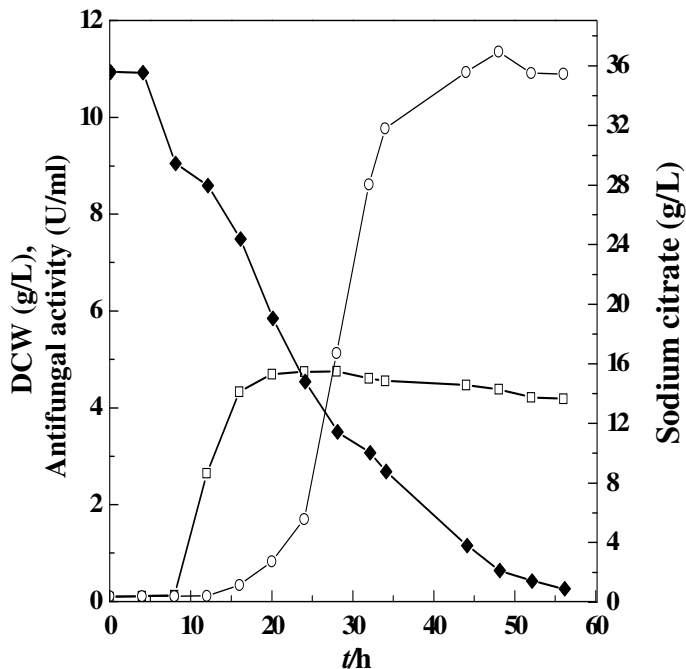


Figure 6. Effects of optimized pH control strategy on antifungal activity (-○-), cell growth (-□-) and substrate consumed (-◆-).

lag phase will be much longer when pH set lower (e.g. 5.0) or higher (e.g. 8.0). In the fermentation process, the

higher pH during the late phase will be in favor of the production of CF66I. But for earlier phase pH 6.0 will be better, because the lag phase can be shortened and the whole fermentation phase will be shorter and then quickly goes into CF66I production phase. According to the effects of pH on cell growth and CF66I production, pH value of fermentation broth go up to 8.0 spontaneously with the metabolic activity of the cells from 12 to 24 h, therefore, the pH value is not necessary to control before 24 h. Then sulfuric acid was need to be added to hold the pH value and prolong the stationary phase to prevent cell autolysis which often happened when pH higher then 8.0.

The growth of microorganism and the synthesis of the products need a series of enzyme catalysis reaction and temperature is the important terms. For enzyme kinetics, the rate of reaction, metabolism will step up and production phase will be ahead of schedule if set up the temperature higher. However, higher temperature can weaken the activity of the enzyme and the weaken rate corresponds to the temperature. It is reflected by aging of the cells, short of fermentation cycle and decrease of the production. For the above, it is essential to keep the temperature stable and suitable. In general, the best suitable temperature of cell growth and product synthesis are different and different stages need different temperature. Beside of the rate of reaction, the temperature can also affect the product synthesis by the physical property change of the broth. For example, the temperature can affect the rate of dissolve and delivery of oxygen in the substrate and the rate of disassociation and

Table 1. Comparison of key parameters under different temperatures.

Parameter	T (°C)					
	23	25	28	30	33	30-25*
CF66I productivity (U L ⁻¹ h ⁻¹)	58.05	173.13	77.22	122.04	118.32	208.48
Average μ_{cell} (h ⁻¹)	0.056	0.074	0.047	0.073	0.052	0.050
Average q_p (U g ⁻¹ h ⁻¹)	82.80	110.40	28.14	44.40	54.90	121.56
Average Y_{cell} (g g ⁻¹)	0.087	0.152	0.155	0.172	0.167	0.153
Average Y_{CF66I} (U g ⁻¹)	186.63	340.11	114.72	177.54	174.57	360.93

*Using two-stage temperature control strategy.

absorption of the substrate. Beside of, the temperature can also affect the biosynthetic directions.

This paper has investigated the contribution of different temperature made to cell growth and CF66I synthesis. The result indicated that lower temperature (below 25°C) can induce a longer lag phase and higher (up 30°C) can make a shorter lag phase and the shorter time needed by DCW going up to maximum and the peak activity of product. Different temperature made different effect on cell yield, specific growth rate, CF66I yield, and specific synthesis rate. In the prophase of fermentation, it is better to set higher temperature (such as 30°C) to make the cell growth maximizing as soon as possible. However in mid-anaphase, lower temperature is set to shorten the fermentation time, reduce heating energy and the cost. The final temperature controlling strategy of this paper is : 0~12 h, pH 6.0, 30°C; 12~20 h, pH to rise naturally, 30°C; 20~24 h, pH to rise naturally, 25°C; after 24 h pH 8.0, 25°C, until the end. By this control strategy production strength was increased to 72.76 U/L/h, the maximum activity was raised to 26.1% than single temperature control and the average of CF66I synthetic rate reached 40.12 U/g/h.

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