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Optimal pH control of batch processes for production of curdlan by *Agrobacterium* species

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We sought an optimal pH profile to maximize curdlan production in a batch fermentation of *Agrobacterium* species. The optimal pH profile was calculated using a gradient iteration algorithm based on the minimum principle of Pontryagin. The model equations describing cell growth and curdlan production were developed as functions of pH, sucrose concentration, and ammonium concentration, since the specific rates of cell growth and curdlan production were highly influenced by those parameters. The pH profile provided the strategy to shift the culture pH from the optimal growth condition (pH 7.0) to the optimal production one (pH 5.5) at the time of ammonium exhaustion. By applying the optimal pH profile in the batch process, we obtained significant improvement in curdlan production (64 g L^{-1}) compared to that of constant pH operation (36 g L^{-1}).

Keywords: curdlan production; optimal pH profile; batch process

Introduction

Curdlan is a water-insoluble polysaccharide composed exclusively of β-1,3-linked glucose residues, and is synthesized by *Agrobacterium* species and *Alcaligenes faecalis* under nitrogen-limiting conditions [4,7,11,13]. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties. A great deal of work has made it possible to use curdlan in food products such as jelly, noodles, edible fibers, and new calorie-reduced products [3,5,18,22]. It is also being used as an admixture to enhance the fluidity of concrete and has recently been commercialized by Takeda Chemical Industries Ltd, Japan [1]. In addition, since drug release can be sustained and diffusion-controlled by curdlan gel, it offers the possibility of a drug delivery polymer [6]. Furthermore, researchers have developed curdlan sulfate as an antiviral agent able to inhibit the infection of human immunodeficiency virus (HIV)-1 [21]. Thus, the potential of curdlan has been recognized in industries as diverse as foods, admixtures, immobilizing supports, and drugs, provoking strong interest in reducing the manufacturing cost.

Since Harada *et al* found curdlan in 1961 and investigated its properties [4,5], Lawford *et al* [7–10,16] have placed a major effort in developing a process for curdlan production, especially with respect to reactor design. With a low-shear system using an axial-flow marine-type propeller, they obtained curdlan production of 46 g L^{-1} with a product yield of approximately 0.5 g curdlan g^{-1} glucose [10]. We [11] recently reported a production of curdlan (60 g L^{-1}) by *Agrobacterium* species using sucrose as the carbon source in a two-step fed-batch operation technique. We sought the optimal timing of nitrogen limitation in order to maximize curdlan production since curdlan is produced under nitrogen-limiting conditions. In addition, the authors [12] also achieved a high production of curdlan (58 g L^{-1}) in a batch fermentation by optimizing the agitation speed and the aeration rate. However, there are many other factors that should be considered in the optimization of curdlan production. The pH of the culture is one of the most important factors because it significantly influences cell growth and product formation rates. Previously, we [11] employed a pH-shift strategy in a two-step fed-batch operation where the culture pH was shifted from the neutral pH, 7,0, at the cell growing step to the slightly acidic pH, 6.5. There might be an advantage to enhance curdlan production by lowering the culture viscosity since curdlan is insoluble at acidic pHs. However, detailed studies have not been reported.

Moreover, there seems to be more than one single optimal pH since fermentation of the culture for curdlan production is divided into two phases, the cell growth phase and the curdlan production phase. Therefore, we sought to find the optimal pH profile to maximise curdlan production. The minimum principle has been widely used for calculating optimal profiles for bioreactor control [2,15,17,19]. The objective of this work is to compute the precise optimal pH profile using an algorithm based on the minimum principle. Eventually, by applying the optimal pH profile, a high production of curdlan was also attempted in a batch fermentation process.

Materials and methods

Microorganism and culture conditions

Agrobacterium sp ATCC 31750 (formerly *Alcaligenes faecalis* subsp *myxogenes*) was used. The seed culture medium contained 20 g L^{-1} sucrose, 5 g L^{-1} yeast extract, and 5 g L^{-1} peptone, pH 7.0. The basal fermentation medium contained (per liter): 140 g sucrose, 3 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 10 ml

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of a trace element solution, unless otherwise specified. The composition of the trace element solution was: 5 g $FeSO_4 \cdot 7H_2O$, 2 g $MnSO_4 \cdot H_2O$, 1 g $CoCl_2 \cdot 6H_2O$, and 1 g $ZnCl_2$ per liter of 0.1 N HCl. Various concentrations of sucrose and ammonium were tried in order to find their effects on cell growth and curdlan production. Fermentation was carried out in a 5-L jar fermentor (Korea Fermentor Co, Incheon, Korea) equipped with a dissolved oxygen analyzer and a pH controller. The seed culture (300 ml), grown at 30°C for 17 h in shake flasks, was transferred to the fermentor containing 2.7 L of the fermentation medium. Culture temperature was controlled at 30°C. Agitation speed and aeration rate were maintained at 600 rpm and 0.5 vvm, respectively during cultivation. The culture pH was controlled at either the constant values or the optimal profiles.

Analytical methods

For measurement of the dry cell mass, 10 ml of sample was mixed with 15 ml of 0.5 N NaOH solution. The supernatant was removed by centrifugation. The aliquot was washed with distilled water twice and the dry cell mass was measured after drying overnight at 80°C. Sucrose concentration was measured with a modified dinitrosalicylic acid (DNS) method [14]. One milliliter of sample was mixed with 20 μl of 3 N HCl and the mixture was hydrolyzed at 100°C for 15 min. After cooling the mixture, 3 ml of DNS solution was added and it was boiled again at 100°C for 10 min. Sucrose concentrations of the samples were determined by measuring absorbance at 570 nm. For the analysis of curdlan, 1 ml of appropriately diluted sample was mixed with 15 ml of 3 N NaOH solution, then the mixture was incubated at room temperature for 30 min to dissolve the curdlan. After centrifuging the mixture at $5000 \times g$ for 15 min, the curdlan present in the supernatant was precipitated under acidic conditions by adding 15 ml of 3 N HCl. Precipitated curdlan was harvested by centrifuging at $5000 \times g$ for 15 min, and washed three times with distilled water to remove salts. The concentration of curdlan was determined by measuring the dry weight after drying overnight at 80°C. The ammonium concentration was determined using the indophenol method [20].

Results and discussion

Batch fermentation at pH 7.0

A batch fermentation was performed at pH 7.0 to examine the time profiles of cell growth and curdlan production. Figure 1 shows the time courses of concentrations of cells, curdlan, ammonium, and sucrose consumption during the cultivation of *Agrobacterium* species. The cell grew to a concentration of 6.2 g L^{-1} in 20 h while consuming all the nitrogen initially present, and ceased to grow after the ammonium was depleted. On the other hand, curdlan production was started from the outset of nitrogen limitation, and maximum curdlan concentration (36 g L^{-1}) was obtained in 120 h cultivation. Sucrose was consumed gradually during cultivation, and the curdlan yield from sucrose was 0.48 g curdlan g^{-1} sucrose. The lines in Figure 1 represent the simulated values obtained with the parameters in the next section.

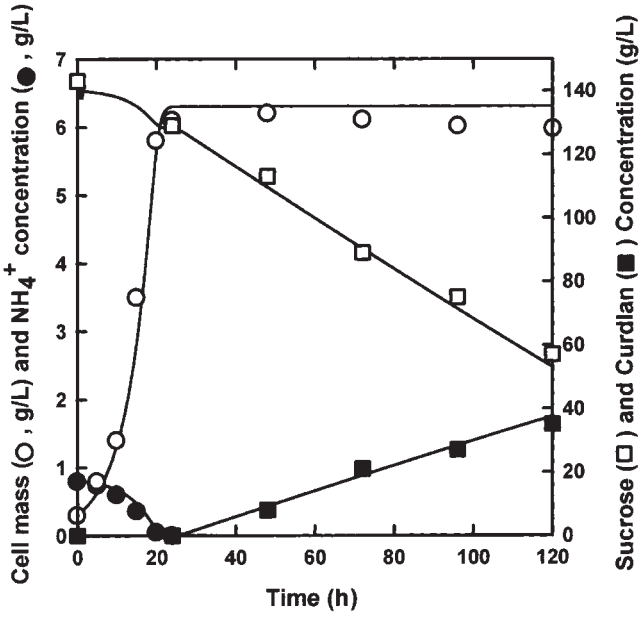

Figure 1 The batch profile of cell growth and curdlan production at constant pH(7.0). The initial concentrations of sucrose and ammonium were 140 and 0.8 g L^{-1}, respectively. The lines were simulated with the estimated parameters.

Effects on cell growth of pH, sucrose, and ammonium

Batch fermentations were carried out under different conditions in order to determine the effects of pH, sucrose, and ammonium on cell growth, and to form model equations. The changes of the specific growth rate were determined by cultivating the cells at different culture pHs. The cell growth rate was at its maximum at pH 7.0. The model parameters were estimated with nonlinear regression analysis. The regression result below is shown in Figure 2(a).

$$\mu_{pH} = \frac{4.41 \times 10^{-4}\,[pH]}{3.45 - [pH] + 0.076\,[pH]^2} \tag{1}$$

In a batch operation, the initial sucrose concentration should be high enough to support a large amount of curdlan production. Previously, we found that the *Agrobacterium* strain used in this study is tolerant of high concentrations of sucrose, making batch operation possible. When cells were grown at the initial sucrose concentration of more than 100 g L^{-1} with the fixed amount of nitrogen source (0.80 g L^{-1} of NH_4^+), the sucrose concentration did not change much. As shown in Figure 2(b), the specific growth rate was almost constant in the sucrose concentration range of 80–160 g L^{-1}. The specific cell growth rate for low sucrose concentrations was not measured in this experiment. Simple Monod growth kinetics were adopted for the high sucrose concentrations tested. It is noted that, however, μ_S is dimensionless because μ is defined as the combined form of $\mu_{pH}\mu_S\mu_N$ (see Eqn 11) and only μ_{pH} has dimension (h^{-1}).

$$\mu_S = \frac{S}{12.35 + S} \tag{2}$$

In addition, the cell growth rate was examined at the

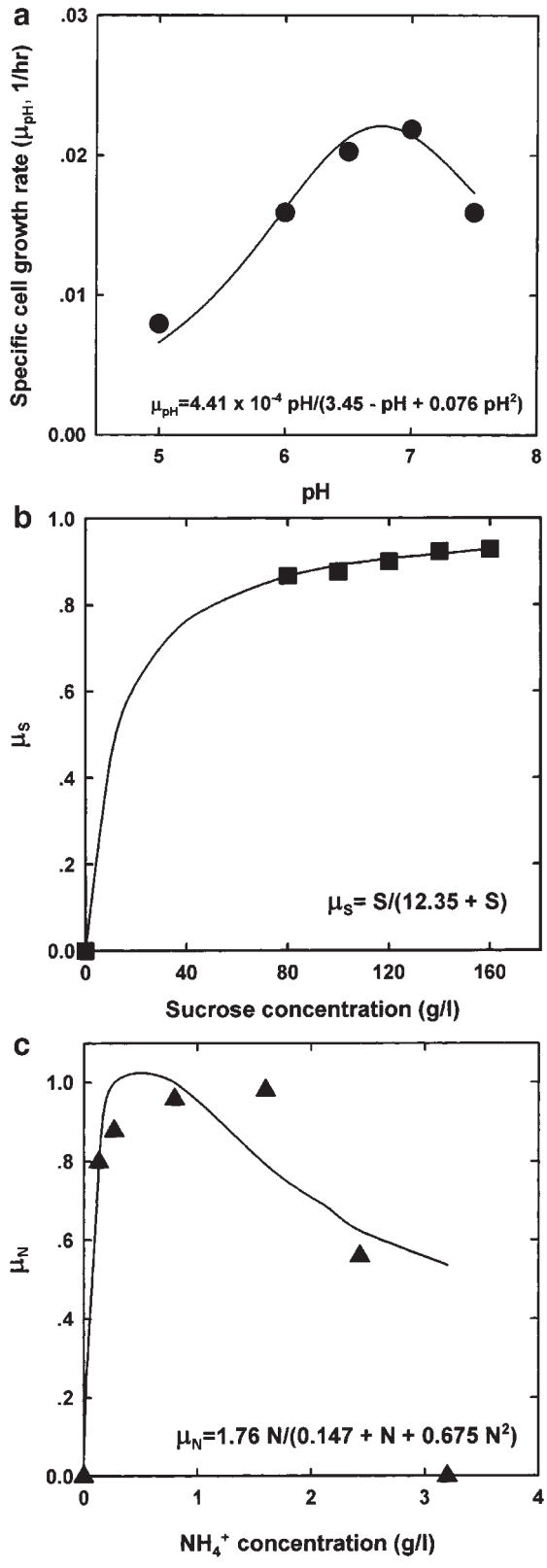

Figure 2 Variation of the specific growth rate with (a) pH; (b) sucrose concentration; and (c) ammonium concentration. The equations in the figures were calculated by the nonlinear regression method. Unless otherwise specified, the culture pH was 7.0, the initial sucrose concentration 140 g L^{-1}, and the initial ammonium concentration 0.8 g L^{-1}.

different initial ammonium concentrations at pH 7.0 and sucrose concentration of 140 g L^{-1}. Figure 2(c) shows that at high initial ammonium concentrations (>2.4 g L^{-1}), the cell growth rate was significantly inhibited by the ammonium concentration, and there is an optimal initial ammonium concentration for cell growth. From these results the ammonium effect can be expressed in a mathematical form and is shown in Eqn (3). μ_N is also dimensionless, like μ_S.

$$\mu_N = \frac{1.76N}{0.147 + N + 0.675N^2} \tag{3}$$

Effects on curdlan production of pH, sucrose, and ammonium

The effect of pH on the curdlan production was also determined at different pHs. After the cells were grown at pH 7, the pH was shifted to various pHs to examine its effect on curdlan production. As shown in Figure 3(a), the curdlan production rate changed with changes in pH. The optimal pH was 5.5 for the maximum curdlan production rate. The model parameters were estimated from the experimental results with the nonlinear regression method. The regression result is shown below.

$$\pi_{pH} = \frac{0.51[pH]}{2.92 - [pH] + 0.094[pH]^2} \tag{4}$$

The effect of sucrose on the curdlan production is shown in Figure 3(b). The effect of sucrose on the curdlan production rate was negligible when sucrose concentrations were higher than 40 g L^{-1}. Simple production kinetics were employed to simulate the sucrose effect on the curdlan production rate, and are expressed below. It should also be noted that π_S is dimensionless because π is defined as the combined form of $\pi_{pH}\pi_S\pi_N$ (see Eqn 12) and only π_{pH} has dimension (h^{-1}).

$$\pi_S = \frac{S}{9.98 + S} \tag{5}$$

The effect of ammonium concentration on curdlan production was also examined. As shown in Figure 3(c), curdlan production was completely inhibited in the presence of ammonium, and no curdlan was formed. Thus, the ammonium inhibition model was proposed to simulate experimental results. The inhibition constant was estimated with the regression method. π_N is also dimensionless, like π_S.

$$\pi_N = \exp(-161N) \tag{6}$$

Optimal pH profile and its application for curdlan production

The effects of pH, sucrose and ammonium are included in the following equations. The equations we developed were used for the optimization of the fermentation process for the maximal production of curdlan.

$$\dot{X} = \mu X \qquad X_0 = 0.5 \tag{7}$$

$$\dot{S} = -\sigma X \qquad S_0 = 140.0 \tag{8}$$

$$\dot{N} = -\delta X \qquad N_0 = 0.8 \tag{9}$$

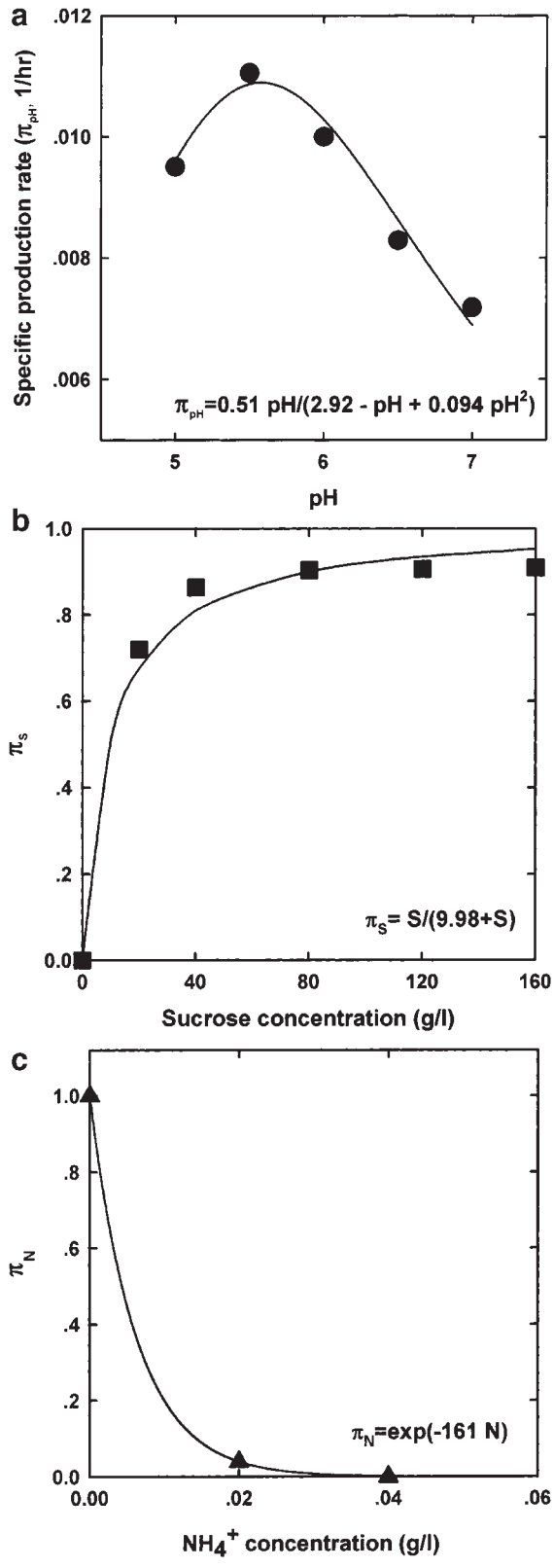

Figure 3 Variation of the specific production rate with (a) pH; (b) sucrose concentration; and (c) ammonium concentration. The equations in the figures were calculated by the nonlinear regression method. To examine the pH effect, cells were grown at pH 7.0 in the growth phase, and then the pH was shifted to the predetermined value after the ammonium was exhausted.

$$\dot{P} = \pi X \qquad P_0 = 0.0 \tag{10}$$

where X is cell concentration, S is sucrose concentration, N is ammonium concentration, and P is curdlan concentration. The specific rates of cell growth (μ), substrate consumption (σ for sucrose and δ for ammonium), and curdlan production (π) are functions of pH, sucrose, and ammonium concentration, and expressed in the following equations.

$$\mu = \mu_{\mathrm{pH}}\mu_{\mathrm{S}}\mu_{N} \tag{11}$$

$$\pi = \pi_{\mathrm{pH}}\pi_{\mathrm{S}}\pi_{\mathrm{N}} \tag{12}$$

$$\sigma = \frac{\mu_{\mathrm{pH}}\mu_{\mathrm{S}}\mu_{\mathrm{N}}}{Y_{\mathrm{X/S}}} + \frac{\pi_{\mathrm{pH}}\pi_{\mathrm{S}}\pi_{\mathrm{N}}}{Y_{\mathrm{P/S}}} \tag{13}$$

$$\delta = \frac{\mu_{\mathrm{pH}}\mu_{\mathrm{S}}\mu_{\mathrm{N}}}{Y_{X/N}} \tag{14}$$

where $Y_{\mathrm{X/S}}$, $Y_{\mathrm{P/S}}$, and $Y_{\mathrm{X/N}}$ are yield coefficients and were estimated from experimental data as 0.5, 0.5 and 7.5, respectively. Since the proposed equations were developed as constant yield values, sucrose and ammonium concentrations can be expressed as analytical functions of cell mass and curdlan.

$$S = S_0 - (X - X_0)/Y_{\mathrm{X/S}} - (P - P_0)/Y_{\mathrm{P/S}} \tag{15}$$

$$N = N_0 - (X - X_0)/Y_{\mathrm{X/N}} \tag{16}$$

The system equations can be reduced to two differential equations and two analytical equations.

$$\dot{X} = \mu X \qquad X_0 = 0.5 \tag{17}$$

$$\dot{P} = \pi X \qquad P_0 = 0.0 \tag{18}$$

The object of this fermentation is to maximize the curdlan concentration by controlling pH and is expressed in the following equation.

$$\underset{[\mathrm{pH}]}{\text{Minimize}}\ [PI = -P(t_{\mathrm{f}}),\ t_{\mathrm{f}} \text{ is fixed}] \tag{19}$$

The optimal profile of the system can be calculated with a minimum principle of Pontryagin [17]. The Hamiltonian of the system is formulated as:

$$H = \lambda_1\mu X + \lambda_2\pi X \tag{20}$$

The adjoint variable of the system is calculated by the partial derivative of the Hamiltonian with respect to state variables. The adjoint variables of the system were calculated. The final conditions of the adjoint variable were calculated with the transversality condition.

$$-\dot{\lambda}_1 = \lambda_1\mu + \lambda_2\pi \qquad \lambda_1(t_{\mathrm{f}}) = 0 \tag{21}$$

$$-\dot{\lambda}_2 = (\lambda_1\mu' + \lambda_2\pi')X \qquad \lambda_2(t_f) = -1 \tag{22}$$

where $'$ represents the partial derivative of function with respect to product concentration ($\partial/\partial P$).

Optimal control is achieved by making the partial derivative of the Hamiltonian zero with respect to the control variable.

$$\frac{\partial H}{\partial [pH]} = (\lambda_1\bar{\mu} + \lambda_2\bar{\pi})X = 0 \tag{23}$$

where the overbar (-) denotes the derivative with respect to the control variable [pH].

Since the proposed model is nonlinear and it is difficult to calculate the optimal profile with the analytical method, we are proposing the following numerical procedure to solve the optimal control profile with the gradient iteration algorithm.

(1) Guess the optimal pH profiles.
(2) Integrate the state variables by forward integration.
(3) Integrate the adjoint variable by backward integration.
(4) Calculate the derivative of the Hamiltonian and check the errors.
(5) Update the optimal control profiles:

$$[pH]^{i+1} = [pH]^{i} + \varepsilon \left[\frac{\partial H}{\partial [pH]}\right]^{i} \quad (24)$$

(6) Iterate steps 1–5 until it converges.

The gradient iteration algorithm based on the minimum principle was used for the calculation of the optimal pH control profile. The optimal pH profile shown as the solid line at the top of Figure 4 provided the strategy on how to shift the culture pH by simply switching from the optimal growth condition (pH 7.0) to the optimal production one (pH 5.5). Also, the timing for switching has to be the time when the ammonium concentration falls to zero. It is practical to change the culture pH quickly from 7.0 to 5.5 without any transient period, since curdlan production is strongly inhibited by even the least amount of ammonium. From the minimum principle, the concentrations of cells and curdlan were estimated to be 6.2 and 58 g L^{-1}, respectively, in 120 h cultivation.

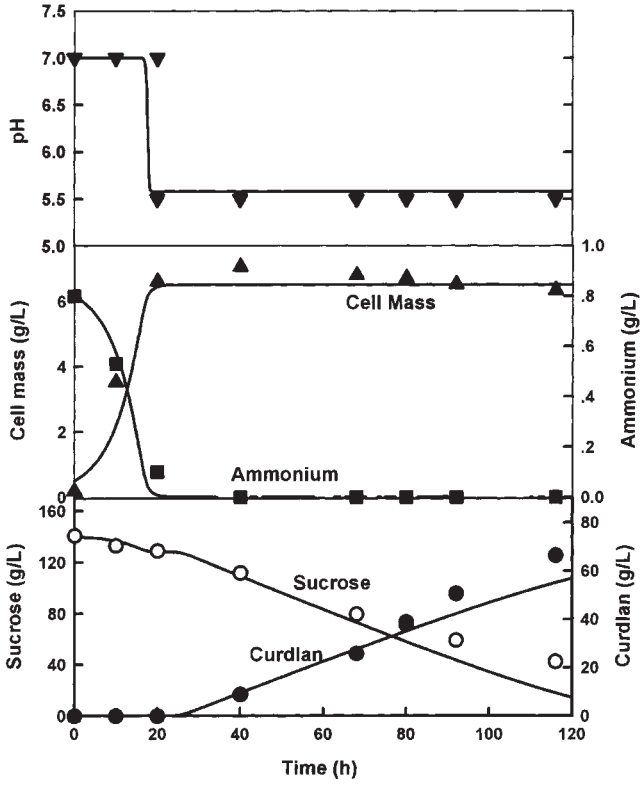

Figure 4 Curdlan production by batch fermentation with optimal pH control strategy. The lines in the figure represent optimal pH profile and estimated values by the minimum principle, and data points were obtained by a batch fermentation with the optimal pH control strategy.

We applied the calculated optimal pH profiles to the batch process for maximal production of curdlan. When the ammonium concentration decreased to zero at 20 h, the pH was shifted to the optimal production pH, 5.5. Figure 4 shows the data points of concentrations of cells, curdlan, ammonium, and sucrose consumption. The cell concentration reached 6.0 g L^{-1} in 20 h when the ammonium in the culture broth was depleted. The concentrations of cells and ammonium fit well with those estimated from the optimal profile. The maximum curdlan concentration (64.0 g L^{-1}) was obtained in 120 h fermentation, showing a 70% improvement in curdlan production compared to that of a constant pH operation (pH 7.0 in Figure 1). The maximum curdlan concentration achieved by this fermentation was a little higher than the value estimated by the minimum principle. Previously, we [11] reported high production of curdlan (60 g L^{-1}) with the productivity of 0.5 g L^{-1} h^{-1} by a fed-batch operation. In this study, the same amount of curdlan production was achieved in a batch operation by applying the optimal pH profile, which is economically more feasible than the fed-batch fermentation process.

Nomenclature

H	Hamiltonian
N	ammonium concentration (g L^{-1})
P	curdlan concentration (g L^{-1})
S	sucrose concentration (g L^{-1})
t	time (h)
t_f	final time (h)
X	cell concentration (g L^{-1})

Greek letters

μ	specific growth rate (h^{-1})
π	specific product formation rate (h^{-1})
δ	specific ammonium consumption rate (h^{-1})
σ	specific substrate consumption rate (h^{-1})
λ	vector of adjoint variable

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