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Residual phosphate concentration under nitrogen-limiting conditions regulates curdlan production in *Agrobacterium* species

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We investigated the influence of inorganic phosphate concentration on the production of curdlan by *Agrobacterium* species. A two-step culture method was employed where cells were first cultured, followed by curdlan production under nitrogen-limiting conditions. In the curdlan production step, cells did not grow but metabolized sugar into curdlan. Shake-flask experiments showed that the optimal phosphate concentration for curdlan production was in the range of 0.1–0.3 g l^{-1}. As the cell concentration increased from 0.42 to 1.68 g l^{-1} in shake-flask cultures, curdlan production increased from 0.44 to 2.80 g l^{-1}. However, the optimal phosphate concentration range was not dependent upon cell concentration. The specific production rate was about 70 mg curdlan g-cell^{-1} h^{-1} irrespective of cell concentration. When the phosphate concentration was maintained at 0.5 g l^{-1} under nitrogen-limiting conditions, as high as 65 g l^{-1} of curdlan was obtained in 120 h. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 180–183.

Keywords: curdlan; production; phosphate concentration; *Agrobacterium* sp.

Introduction

Curdlan is a water-insoluble extracellular polysaccharide composed exclusively of β-1,3-linked glucose residues. It is synthesized by *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium radiobacter* under nitrogen-limiting conditions [6,9,12,14]. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties. Curdlan is used in food products such as jelly, noodles, edible fiber, and new calorie-reduced products [5,15,19]. It is also being used to enhance the fluidity of concrete while increasing its segregational stability [1,20]. In addition, curdlan might be used as a drug-delivery polymer since curdlan gel can hold and control diffusion of the drug [8]. Furthermore, curdlan sulfate was developed as an antiviral agent able to inhibit infections by the human immunodeficiency virus [18]. Thus, there is strong interest in reducing the manufacturing cost of curdlan.

We reported the production of curdlan by *Agrobacterium* species using sucrose as the carbon source in a two-step fed-batch technique, where cells were initially grown to substantial biomass, followed by optimizing conditions for curdlan production [9]. A maximum titre of 64 g curdlan l^{-1} was obtained by regulating N limitation, agitation speed and pH profile [10,11].

Phosphate concentration must also be considered because it significantly influences cell growth and product formation. Production of rhamnose-containing polysaccharide by a *Klebsiella* strain was enhanced by a reduction in the phosphate content of the medium [4]. The production of alginic acid by *Azotobacter vinelandii* drastically increases with a decrease in phosphate concentration [7]. In contrast, a sufficient supply of phosphate produced good yields of alginate in *Pseudomonas* strains showing growth-associated production [3]. Other examples are a high production of polysaccharides by a *Pseudomonas* strain [13] and maximum production of xanthan by *Xanthomonas campestris* at a high phosphate concentration [16]. Thus, the effect of phosphate on the production of polysaccharides is different from one strain to another.

In this study, we studied the influence of inorganic phosphate concentration on curdlan production by *Agrobacterium* species. We examined the relationship between curdlan production and residual phosphate concentration by varying cell concentrations under nitrogen-limiting conditions. We also attempted to maximize the production of curdlan in a 5-l fermentor by using the optimal phosphate concentration.

Materials and methods

Microorganism and culture medium

Agrobacterium sp. ATCC 31750 (formerly *A. faecalis* subsp. *myxogenes*) was used. The seed culture medium (YP medium) contained 20 g sucrose, 5 g yeast extract, and 5 g bacto-peptone, pH 7.0, per liter of distilled water. The nitrogen-free medium for flask cultures contained (per liter): 20 g sucrose, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2SO_4, 0.6 g $CaCO_3$, 10 ml of a trace element solution, and varying amounts of $Na_2HPO_4 \cdot 12H_2O$ to give different concentrations of phosphate. The composition of the trace element solution was 5 g $FeSO_4 \cdot 7H_2O$, 2 g $MnSO_4 \cdot H_2O$, 1 g $CoCl_2 \cdot 6H_2O$, 1 g $ZnCl_2$ per liter of 0.1 M HCl. Sucrose, $MgSO_4 \cdot 7H_2O$, and the trace element solution were sterilized separately. Initial medium pH was adjusted to 6.0. Five-liter jar fermentation medium contained (per liter): 140 g sucrose, 4.42 g

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NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, varying amounts of KH_2PO_4 and 20 ml of the trace element solution.

Flask culture

A two-step culture technique was used to examine the effect of phosphate on curdlan production. Cells were first grown at 30°C for 17 h in 500-ml baffled flasks containing 100 ml of YP medium on a rotary shaker at 150 rpm. Then, the appropriate amount of cells (42–168 mg dry weight harvested by centrifuging at 5000×*g* for 15 min) was transferred to 100 ml of the nitrogen-free fermentation medium. Further cultivation was done at 30°C on a rotary shaker at 150 rpm.

Five-liter fermentor cultures

Curdlan production was studied in a 5-l jar fermentor (Korea Fermentor Co., Incheon, Korea) equipped with a dissolved oxygen analyzer and a pH controller. The inoculum (300 ml grown for 17 h in shake-flask culture as described previously) was transferred into the fermentor containing 2.7 l of the fermentation medium. Culture temperature was controlled at 30°C. Agitation speed was maintained at 600 rpm, and the aeration rate was 0.5 vvm. The pH was controlled at 7.0 with 3 M NaOH during the cell-growth step, and then shifted to 5.5 with 3 M HCl at the time of nitrogen limitation.

Analytical methods

For measurement of dry cell mass, 10 ml of sample was mixed with 15 ml of 0.5 N NaOH. The supernatant was removed by centrifugation. The aliquot was washed with distilled water twice, and the dry cell mass was measured after drying it overnight at 80°C. For the analysis of curdlan, 1 ml of sample was mixed with 15 ml of 3 N NaOH solution, and incubated at room temperature for 30 min to dissolve the curdlan. After centrifuging the mixture at 5000×*g* for 15 min, the curdlan in the supernatant was precipitated by adding 15 ml of 3 N HCl. The precipitated curdlan was harvested by centrifuging the suspension at 5000×*g* for 15 min, and it was then washed three times with distilled water to remove salts. Curdlan concentrations were determined by measuring the dry weight after drying overnight at 80°C. Sucrose concentration was measured with a modified dinitrosalicylic acid method [11]. Ammonium concentrations were determined using the indophenol method [17]. The phosphate concentration was determined with the ascorbic acid method [2].

Results

Effect of extracellular phosphate concentration on curdlan production

A two-step culture technique was used to examine the effect of phosphate on curdlan production. Cells grown in a nutrient-rich medium (YP medium) were transferred to the nitrogen-free fermentation medium containing various concentrations of phosphate. As shown in Figure 1, curdlan production was highest (3.1 g l^{-1}) at a phosphate concentration of 0.1 g l^{-1}, increasing significantly as phosphate concentration increased from 0 to 0.1 g l^{-1}. Curdlan production gradually decreased with further increases in phosphate concentrations. Sucrose consumption showed the same pattern as curdlan production. Thus, the curdlan production yield from sucrose was around 0.5 g curdlan g^{-1} sucrose over the phosphate concentration range examined, except in the absence of phosphate. Phosphate consumption was negligible because cell growth is highly restricted under nitrogen-limiting conditions. Even though the cells did not grow, they remained viable as evidenced by continuing curdlan production.

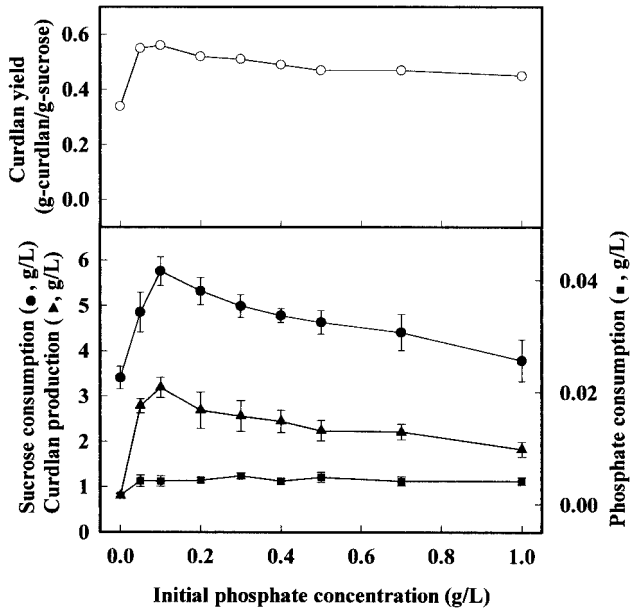

Figure 1 Effect of phosphate concentration on curdlan production in nitrogen-free medium. A two-step culture technique was employed. Cells were cultivated in the YP medium for 17 h at 30°C. Then, cells (84 mg dry weight) harvested by centrifugation at 5000×*g* for 15 min were suspended in 100 ml of the nitrogen-free medium containing different concentrations of phosphate. Further cultivation was performed for 24 h at 30°C. Experiments were done in triplicate.

Relationship between cell concentration and optimal phosphate concentration

To examine the relationship between cell density and the optimal phosphate concentration in terms of curdlan production, we repeated the experiments described in Figure 1 at different cell concentrations. Different amounts of cells (42, 84, 126, and 168 mg of cells, on a dry weight basis) grown in YP medium were added to 100 ml of the nitrogen-free fermentation medium containing different phosphate levels and further cultured. The optimal phosphate concentration proved to be constant, within the range of 0.1–0.3 g l^{-1}, which demonstrated that it was independent of cell density (Table 1). Curdlan production correspondingly increased from 0.64 to 2.86 g l^{-1}, being directly proportional to cell concentration.

Batch fermentation of curdlan at different concentrations of phosphate

To examine the pattern of curdlan production at different phosphate concentrations in more detail, batch fermentation was carried out in a 5-l jar fermentor. The conditions used during the batch fermentation were different, in that the nitrogen-limited condition was allowed to develop as the cells consumed the nitrogen source, whereas in flask cultures, a nitrogen-free medium was used in the second step. It is important to determine the initial concentration of

Table 1 Optimal phosphate concentration range for curdlan production by *Agrobacterium* species at different cell concentrations[a]

Cell concentration[b] (g/l)	Optimal phosphate concentration[c] (g/l)	Curdlan production (g/l)	Specific production rate (mg curdlan/g cell/h)
0.42	0.1–0.2	0.64–0.72	63–71
0.84	0.1–0.3	1.48–1.56	73–77
1.26	0.1–0.3	1.85–2.26	61–75
1.68	0.1–0.3	2.80–2.86	69–70

[a]Experimental conditions are the same as described in Figure 1 except for the culture time (12 h). The predetermined amount of cells was transferred to flasks containing the nitrogen-free medium plus the different concentrations of phosphate ranging from 0.0 to 1.0 g/l.
[b]Cell concentration was determined by dry weight. One gram of cells represents 9×10^{11} cells.
[c]Optimal phosphate concentration indicates the concentration range where curdlan production was the highest.

the nitrogen source because it is the limiting factor for cell growth. In this regard, we examined cell growth at various ammonium concentrations using a Bioscreen analyzer system. Cell growth rate decreased as the ammonium concentration increased (Figure 2). However, since higher cell concentration produced more curdlan, as shown in Table 1, we chose an ammonium concentration of 1.6 g l^{-1} to provide an appropriate cell concentration whilst minimizing the inhibitory effect of the ammonium ion.

Once the optimum ammonium level was decided, batch fermentation was carried out at different phosphate levels. Cell and ammonium concentrations, phosphate consumption, and curdlan production levels during the batch fermentation are illustrated in Figure 3. Cell growth was proportional to ammonium consumption, and independent of phosphate concentration, which indicated that phosphate does not inhibit cell growth. When the cell concentration was at a maximum (12 g l^{-1}), about 0.5 g l^{-1} of phosphate was consumed, which was equivalent to a cell yield of 24 g of cells per gram of phosphate. Curdlan production began when the ammonium concentration had been significantly depleted. When the initial phosphate concentration was 0.5 g l^{-1}, it decreased to negligible levels, and curdlan production was as low as 15 g l^{-1} at the end of the fermentation. On raising the initial phosphate concentration from 0.5 to 1.0 g l^{-1}, curdlan production increased, reaching a maximum of 65 g l^{-1} in 120 h. Residual phosphate fell to 0.50 g l^{-1} over this period. At a phosphate

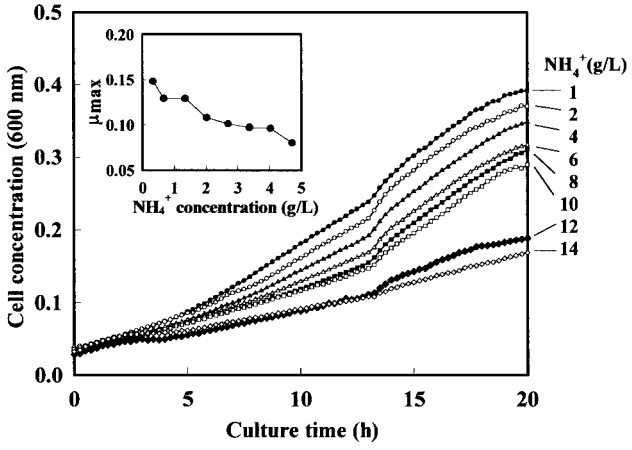

Figure 2 Effect of ammonium concentration on cell growth. Cell growth was measured by monitoring the optical density of the culture broth at 600 nm by using a Bioscreen analysing system (Labsystems, Helsinki, Finland). Cells were cultured for 20 h at 30°C. The basal medium compositions was (per liter): 20 g sucrose, 3.77 g Na_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g K_2SO_4, 10 ml of a trace element solution, and different concentrations of NH_4Cl. Inset in the figure indicates the variation of the maximum specific growth rate (μ_{max}).

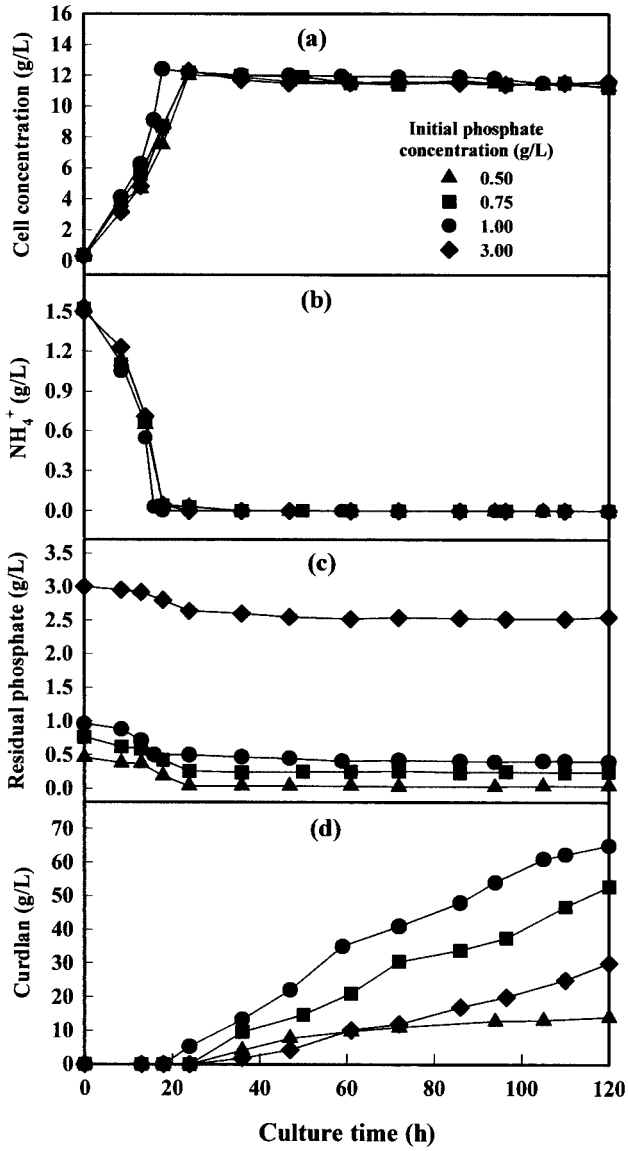

Figure 3 Batch fermentation profiles with different phosphate concentrations in a 5-l jar fermentor. (a) Cell growth, (b) ammonium concentration, (c) phosphate consumption, and (d) curdlan production. Details are described in Materials and Methods.

concentration of 2.0 g l^{-1}, curdlan production was 54 g l^{-1} (data not shown), but at 3.0 g l^{-1}, curdlan production was significantly reduced to 30 g l^{-1}.

Discussion

Several published reports have quoted initial phosphate concentration in the medium when dealing with phosphate optimization for polysaccharide production [4,7,13,16]. However, because phosphate concentrations naturally fall as cells grow, it is difficult to determine the intrinsic optimum phosphate concentration. This study suggests that curdlan production is highly dependent upon the residual extracellular phosphate concentration. Under nitrogen-free conditions where curdlan is produced, the phosphate concentration remains constant without being further utilized for cell growth. The optimal residual extracellular phosphate concentration for curdlan production was in the range of 0.1–0.3 g l^{-1} in flask cultures, although the optimal phosphate concentration (0.5 g l^{-1}) in the jar fermentation was a little higher than that in flask cultures for unknown reasons. Relatively low concentrations appeared to be optimal for curdlan production although without phosphate, curdlan production was extremely low. In addition, when phosphate was depleted in the medium during jar fermentation, the medium foamed.

To obtain a high level of curdlan, residual phosphate concentration, which is dependent upon the amount of ammonium used for cell growth, needs to be defined. From the yield data (the cell growth yield from ammonium is 8.0, and from phosphate 24 g cells g^{-1} substrate), it can be estimated that an initial ammonium concentration of 1.6 g l^{-1} can yield 12 g l^{-1} of cells, thus requiring 0.5 g l^{-1} of phosphate for cell growth. Thus, the initial phosphate concentration should be 0.5 g l^{-1} higher than the optimal residual phosphate concentration to support 12 g l^{-1} of cells. With 1.0 g l^{-1} of initial phosphate concentration that rendered 0.5 g l^{-1} under nitrogen-limiting conditions, as high as 65 g l^{-1} of curdlan in 120 h of batch culture was obtained. This optimal strategy can be used for the production of desired products under limiting conditions of certain essential nutrients such as nitrogen.

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