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Production of curdlan using sucrose or sugar cane molasses by two-step fed-batch cultivation of *Agrobacterium* species

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Maltose and sucrose were efficient carbon sources for the production of curdlan by a strain of *Agrobacterium* sp. A two-step, fed-batch operation was designed in which biomass was first produced, followed by curdlan production which was stimulated by nitrogen limitation. There exists an optimal timing for nitrogen limitation for curdlan production in the two-step, fed-batch operation. Maximum curdlan production (60 g L^{-1}) was obtained from sucrose with a productivity of 0.2 g L^{-1} h^{-1} when nitrogen was limited at a cell concentration of 16.0 g L^{-1}. It was also noted that the curdlan yield from sucrose was as high as 0.45 g curdlan g^{-1} sucrose, and the highest specific production rate was 1.0 g curdlan g^{-1} cells h^{-1} right after nitrogen limitation. Of particular importance was the use of molasses as a cheap carbon source to produce curdlan in the two-step, fed-batch cultivation. As high as 42 g L^{-1} of curdlan with a yield of 0.35 g curdlan g^{-1} total sugar was obtained after 120 h of fed-batch cultivation.

Keywords: curdlan production; *Agrobacterium* sp; nitrogen limitation

Introduction

Curdlan is a water-insoluble polysaccharide composed exclusively of β-1,3-linked glucose residues, and synthesized mostly by *Agrobacterium* sp and *Alcaligenes faecalis* under nitrogen-limiting conditions [4,10]. Since Harada *et al* found curdlan in 1961 [3], its production has drawn attention because of its unique rheological and thermal gelling properties, which allow the potential use of curdlan in food products such as jelly-like foods, edible fibers and films, and immobilizing supports [4,13]. Furthermore, since curdlan is not readily degraded by digestive enzymes, it offers the possibility of new calorie-reduced products. In addition, it was used recently as an admixture to enhance fluidity and has been commercialized by Takeda Chemical Industries Ltd, Japan [1]. Thus, curdlan has great potential if it can be produced less expensively.

Harada *et al* [3,4] and Lawford *et al* [7–10,14,15], conducted pioneering investigations for process development of curdlan production. The amount of curdlan produced by fermentation was 46 g L^{-1} with a product yield of approximately 0.5 g curdlan g^{-1} glucose [8,14]. Maximum productivity was also reported as 0.2 g L^{-1} h^{-1}. However, glucose is the only carbon source used for the production of curdlan to date [3,4,7–10,14,15]. The cost of raw materials, especially carbon sources, seems to govern the production cost, since curdlan can be readily isolated from the fermentation broth without using organic solvents which have often been found to be burdensome in the production of other exopolysaccharides. Thus, high productivity by use of a cheap carbon source is important for the industrial production of curdlan. Sucrose is a less expensive substrate than glucose, and sugar beet or sugar cane molasses, cheap by-products widely available from the sugar industry, are very attractive carbon sources from an economic point of view.

The purpose of this study was to optimize culture conditions to produce curdlan by cultivation of a strain of *Agrobacterium* sp using sucrose and sugar cane molasses. A high production of curdlan was also attempted in jar fermentation to test economic feasibility under optimal conditions.

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Materials and methods

Microorganism and culture media

Agrobacterium sp ATCC 31750 (formerly *Alcaligenes faecalis* subsp *myxogenes*) was used in this study. 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 fermentation medium contained (per liter): 100 g sucrose, 2.3 g $(NH_4)_2HPO_4$, 1.0 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, and 10 ml of a trace element solution (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 N HCl). Feed solution for the fed-batch operations contained (per liter): 700 g sucrose, 2.8 g $MgSO_4 \cdot 7H_2O$, and 30 ml of the trace element solution. Sugar cane molasses from Thailand was also used as an alternative fermentation medium. To clarify the sugar cane molasses, the pH of an appropriately diluted medium was adjusted to 2.5 with sulfuric acid and incubated overnight at room temperature. Precipitates were removed by centrifugation, the pH was neutralized by NaOH, and the molasses solution was sterilized by autoclaving it.

Culture conditions

For flask cultures, the cells (5 ml) grown at 30°C for 17 h in 100 ml of seed medium were inoculated into the fermentation medium containing 0.3% (w/v) calcium carbonate and cultivated at 30°C. Fermentation was carried out in a 5-L jar fermenter (Korea Fermenter Co, Incheon, Korea) equipped with a dissolved oxygen (DO) analyzer and a pH controller. Seed culture (100 ml), cultivated at 30°C for 17 h in shake flasks, was transferred to the fermenter containing 1.9 L of the fermentation medium. The pH was controlled at 7.0 with 4 N NaOH/KOH. For fed-batch operations, the feed solution consisting of either sucrose solution or molasses medium was continuously fed into the fermenter in order to maintain the sucrose concentration in the culture broth above 20 g L^{-1}. A two-step, fed-batch operation technique was employed to stimulate curdlan production. During the first step, ammonia water (28%) was supplied as the nitrogen source along with the control of the culture pH. DO was maintained at above 10% of air saturation by controlling both the agitation speed and the aeration rate up to 1000 rpm and 2.0 vvm, respectively. After the cell concentration increased to a predetermined level, the feed solution for the pH control was replaced with 4 N NaOH, and pH was controlled at 6.5. The agitation speed and aeration rate were maintained at 700 rpm and 1.0 vvm, respectively. Thus, nitrogen and DO in the culture broth were limited in the second step.

Analytical methods

The concentrations of cells and curdlan were determined by measuring the dry weight. A suitably diluted sample was centrifuged at $8000 \times g$ at 4°C for 30 min. The pellet consisted of cells and curdlan was washed with 0.01 N HCl, and harvested by recentrifugation. Then, 0.5 N NaOH was added to solubilize curdlan for 1 h. Cells were separated by centrifugation at $8000 \times g$ for 30 min. Curdlan present in the supernatant phase was precipitated under acidic conditions by adding an appropriate volume of 2.0 N HCl. Either cells or curdlan were washed and then dried to a constant weight. Total sugar was determined by the dinitrosalicylic acid method [12] after hydrolyzing samples at 100°C for 15 min. Glucose, fructose, and sucrose were determined by using a high performance liquid chromatograph (Waters, Milford, MA, USA) with distilled water as a mobile phase, and ammonia was determined by the indophenol method [16].

Results

Effect of carbon source

To find a suitable carbon source for the production of curdlan, *Agrobacterium* sp ATCC 31750 was cultivated in a medium containing various carbon sources such as glucose, fructose, galactose, maltose, sucrose, lactose, or raffinose. When the cells were grown in the maltose-containing medium, curdlan production (48 g L^{-1}) was the highest among those tested (Figure 1). The curdlan concentration produced (47 g L^{-1}) from sucrose was almost identical to that from maltose. Glucose also yielded a considerable amount of curdlan production (40 g L^{-1}). However, the curdlan production from the other carbon sources was much lower than that from maltose. Considering that sucrose is a cheaper carbon substrate than glucose and maltose, sucrose was used as the carbon source in subsequent experiments.

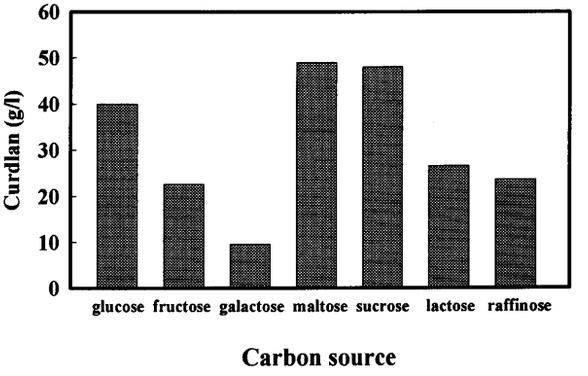

Figure 1 Effect of carbon source on curdlan production by *Agrobacterium* sp ATCC 31750. Cells (5 ml) grown in the seed medium were transferred to 50 ml of the fermentation medium in a 250-ml flask and cultivated at 30°C for 3 days. Initial concentration of the carbon source was 100 g L^{-1}.

Batch fermentation using sucrose

To examine the pattern of curdlan production in detail, batch fermentation was carried out in a jar fermenter. Figure 2 shows the time courses of concentrations of cells, curdlan, ammonium, sucrose consumption, curdlan yield, and the specific production rate of curdlan (q_P) during the cultivation. The nitrogen source added initially was completely consumed in 15 h and the cell concentration increased to 5.0 g L^{-1} resulting in the cell yield from ammonium of 8.0 g dry cell weight (DCW) per g ammonium. The q_P rose in the absence of ammonium limitation and then was maintained at about 0.07 g curdlan g^{-1} DCW h^{-1}. Thus, curdlan production was stimulated by ammonium limitation. Maximum curdlan concentration (32 g L^{-1}) was obtained after 95 h cultivation and the curdlan yield from sucrose was 0.51 g curdlan g^{-1} sucrose.

Fed-batch fermentation using sucrose

Results obtained from the batch cultivation led us to carry out a fed-batch fermentation to promote curdlan production by raising the cell concentration. A two-step, fed-batch operation technique was employed in which a biomass was first produced, followed by curdlan production. During the first step, cells were grown by supplying ammonia water (28%) as the nitrogen source; the pH was controlled. Production of curdlan was stimulated under a nitrogen-limiting condition by replacing ammonia water with 4 N NaOH. Figures 3 and 4 show the time courses of concentrations of cells, curdlan, ammonium, sucrose consumption, curdlan yield, and q_P during the fed-batch cultivations. When nitrogen was limited at a cell concentration of 16 g L^{-1}, the curdlan concentration continued to increase from the onset of limitation, and the maximum concentration of curdlan obtained after 115 h cultivation was 60 g L^{-1} (Figure 3). The conversion yield of curdlan from sucrose was 0.45 g curdlan g^{-1} sucrose. The q_P after limitation of nitrogen was as high as 0.10 g curdlan g^{-1} DCW h^{-1}, and decreased

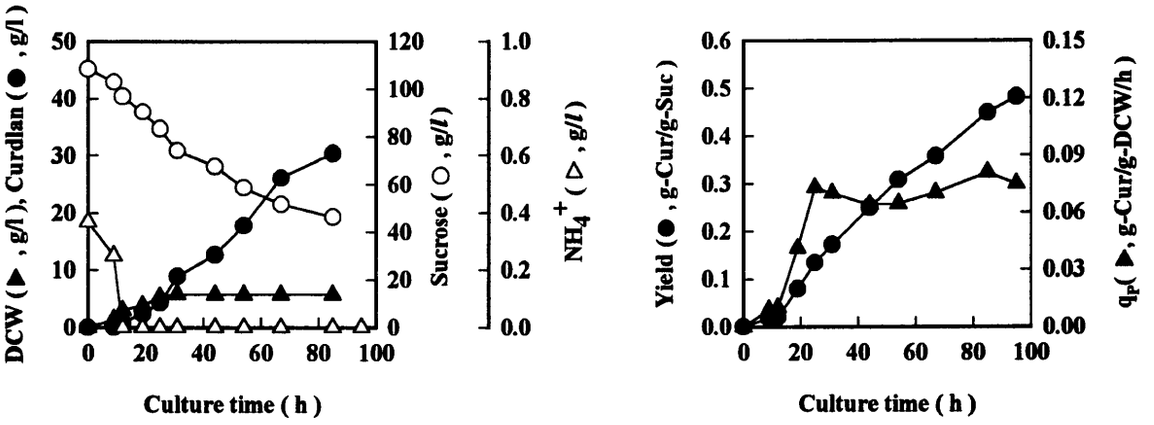

Figure 2 Production of curdlan in batch culture by *Agrobacterium* sp ATCC 31750 with sucrose as carbon source.

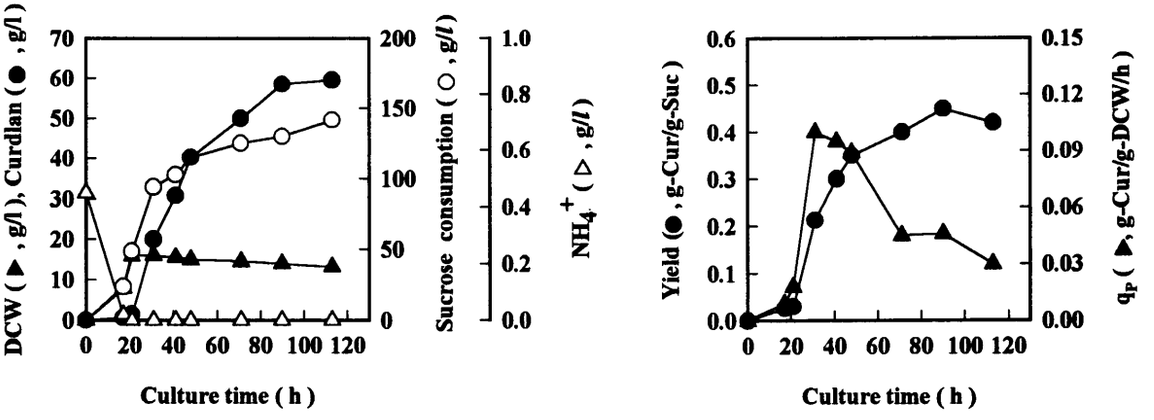

Figure 3 Production of curdlan from sucrose by a two-step, fed-batch cultivation. Ammonium water was replaced with NaOH to limit nitrogen after 18 h cultivation.

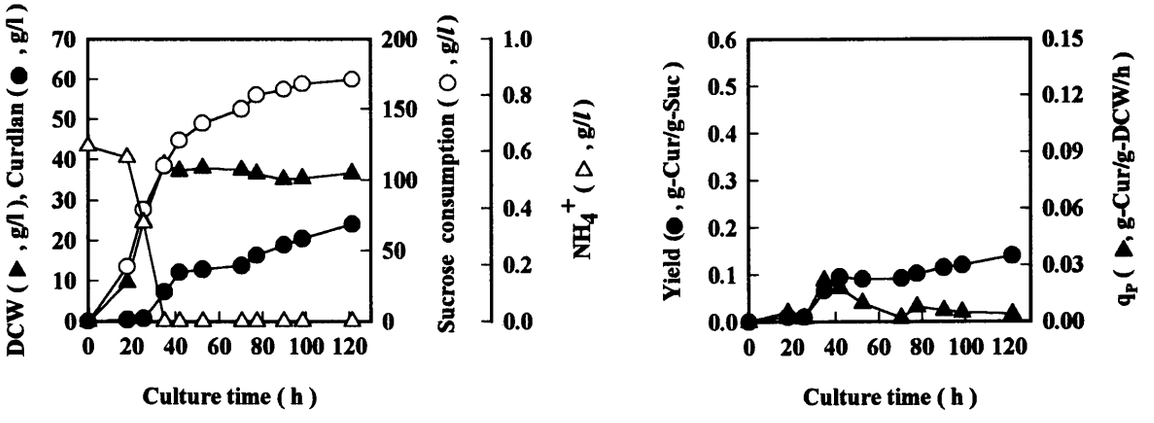

Figure 4 Production of curdlan from sucrose by a two-step, fed-batch cultivation. Ammonium water was replaced with NaOH to limit nitrogen after 35 h cultivation.

gradually to 0.030 at the end of fed-batch operation. We also examined the maximum potential amount of curdlan that could be produced by a higher cell concentration (Figure 4). Cells were grown to a concentration of 38 g L^{-1} by supplying ammonia water for a 35-h cultivation period. Maximum curdlan concentration was 25 g L^{-1} in spite of the higher cell concentration, while keeping q_P below 0.030 g curdlan g^{-1} cells h^{-1}. Curdlan yield was also less than 0.15 g curdlan g^{-1} sucrose, indicating that the carbon source was mainly used for cell growth and its maintenance.

Table 1 Comparison of molasses treatment for their composition and curdlan production

Composition	Concentration (g L^{-1})	
	No treatment	H_2SO_4 treatment[a]
Sucrose	99.2	89.2
Glucose	22.4	25.8
Fructose	33.0	34.6
Total sugars	166.6	163.2
NH_4^+	0.1	0.1
PO_4^{3-}	1.5	1.7
Na^+	1.2	5.2
K^+	7.7	7.3
Mg^{2+}	0.6	0.6
Ca^{2+}	2.5	0.4
Fe^{2+}	0.1	0.1
Curdlan production[b]	16.6	22.1

[a] Sugar cane molasses is diluted five-fold during the treatments.
[b] Cells were cultivated in molasses medium containing 2.0 g L^{-1} of $(NH_4)_2HPO_4$ at 30°C for 4 days on a rotary shaker.

Curdlan production from sugar cane molasses

Since sucrose was a good substrate for curdlan production, molasses, which contains a great amount of sucrose, could be the substrate of choice. In addition, molasses is a very cheap by-product widely available from the sugar industry. To examine the effect of pretreatment of molasses on curdlan production, sugar cane molasses was prepared by two different techniques. One method involved removal of solid matter by centrifugation of five-fold diluted molasses. In the second method, the pH of appropriately diluted molasses was adjusted to 2.5 by adding H_2SO_4, the solution was incubated at room temperature for 20–30 h, followed by centrifugation. The supernatant phase was then neutralized with NaOH. Table 1 shows the composition of molasses and the results of curdlan production. When cells were cultivated in the H_2SO_4-treated molasses medium, curdlan production was enhanced about 30% compared to that without treatment. It was noteworthy that only Ca^{2+} concentration was significantly reduced in the molasses medium by treatment with H_2SO_4.

A fed-batch fermentation was carried out to obtain high curdlan productivity by using molasses only (Figure 5). Cells were grown to a concentration of 10.0 g L^{-1} and growth was stopped by limiting nitrogen sources. Curdlan production was gradually increased from the onset of nitrogen limitation, and showed the maximum concentration of 42 g L^{-1} after 120 h cultivation. The highest q_P was 0.08 at the beginning of the curdlan production step, which was similar to the value obtained from sucrose. The curdlan yield from the total sugars was about 0.35 g curdlan g^{-1} total sugar consumed.

Discussion

Sucrose was an efficient carbon source for the production of curdlan, although glucose has been the most preferred carbon source in the production of homopolymers composed of glucose subunits [2,4,5,7]. Kai *et al* [6] reported the biosynthetic pathway of curdlan using ^{13}C-labeled glucose. From the analysis of the labeled product, the biosynthesis of curdlan was interpreted as involving five routes: direct synthesis from glucose, rearrangement, isomerization of cleaved trioses, from fructose-6-phosphate, and from fructose fragments produced in various pathways of glycolysis. In our preliminary experiments, sucrase activity from the *Agrobacterium* sp was not found in the culture broth and cell suspensions, thus it seemed likely that sucrose can be directly transported into the cell via group translocation or by facilitated transport. In addition, a mixture of glucose and fructose was not sufficient to support curdlan production comparable to that from sucrose only (unpublished data). From these observations, an enhanced production of curdlan from sucrose seemed to result from higher cellular metabolic fluxes occurring not only from cellular glucose but also from the fructose moiety. For this reason, the molasses medium treated with H_2SO_4 was neutralized before sterilization to avoid hydrolysis of sucrose into glucose and fructose.

Nitrogen limitation was the most efficient means to stimulate production of curdlan. This can be generally explained in that isoprenoid lipids, which play a crucial

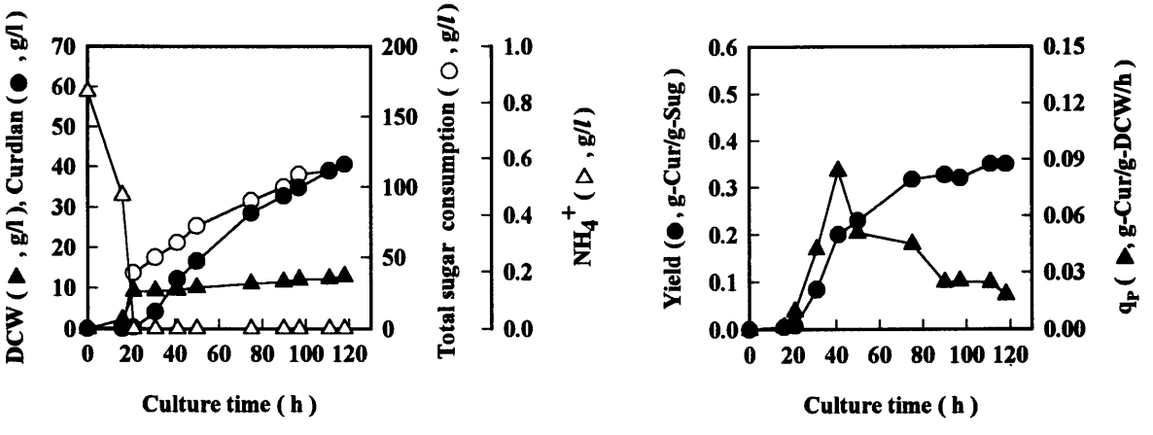

Figure 5 Production of curdlan using molasses by a two-step, fed-batch cultivation. Ammonium water was replaced with NaOH to limit nitrogen after 18 h cultivation. Five-fold diluted molasses treated with H_2SO_4 containing 3 g L^{-1} $(NH_4)_2HPO_4$ was used as the initial medium, and the feed medium consisted of three-fold diluted molasses.

role in carrying cellular oligosaccharides, would be more available for the synthesis of exopolysaccharides instead of the synthesis of cellular lipopolysaccharide under nitrogen-limiting conditions [17]. However, for a high production of curdlan, achievement of enough cell concentration, requiring a sufficient supply of nitrogen source, should be considered. Thus, a two-step, fed-batch operation, in which biomass production was followed by curdlan production, was designed for the production of curdlan. Feeding of ammonium hydroxide for pH control yielded a nitrogen-sufficient condition, resulting in efficient cell growth. In the second step, curdlan production was stimulated under nitrogen-limiting conditions obtained by replacing ammonium hydroxide with sodium hydroxide. In addition, the culture pH during curdlan production in the two-step, fed-batch operation was controlled at 6.5 while it was at 7.0 during the cell growth step. Since the solubility of curdlan increases with culture pH in the alkaline pH range, a high viscosity of culture broth, which is often a critical problem in polysaccharide production [11], can be obviated by operating the fermentation at a slightly acidic pH. Another aspect concerns the optimal timing of nitrogen limitation for curdlan production in the two-step, fed-batch cultivation. The highest productivity of curdlan was obtained at a cell concentration of 16 g L^{-1}, while less curdlan was produced at a cell concentration of 38 g L^{-1}. It seemed likely that a low productivity and a low yield at the higher cell concentration resulted from a higher oxygen demand and a higher energy requirement for cellular maintenance.

By optimization of the fermentation conditions, a high concentration of curdlan (60 g L^{-1}) was obtained using sucrose in a 120-h cultivation of *Agrobacterium* sp, resulting in curdlan productivity of 0.2 g L^{-1} h^{-1}. The curdlan yield from sucrose was as high as 0.45 g curdlan g^{-1} sucrose and the highest q_P was 1.0 g g^{-1} h^{-1}. These values are quite comparable to those obtained from glucose in the low shear system, which used a turbine impeller [10]. Of particular importance is the use of an inexpensive carbon source, which would reduce the production cost. In addition, a much cheaper carbon source such as molasses could be used for curdlan production by this strain. Thus, this process having a high curdlan productivity from cheap carbon sources is very attractive from an economic point of view.

Acknowledgements

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