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Improved Production of Curdlan with Concentrated Cells of *Agrobacterium* sp.

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Abstract The addition of a limited concentration of yeast extract to a minimal salt medium (MSM) enhanced cell growth and increased the production of curdlan whereas nitrogen-limitation was found to be essential for the higher production of curdlan by *Agrobacterium* sp. ATCC 31749. As the amount of the inoculum increased, the cell growth as well as the production of curdlan also increased in the MSM without a nitrogen source. The cell growth and production of curdlan increased as the initial pH of the medium decreased as low as 5.0. The conversion rate and concentration of curdlan from 2% (w/v) glucose in the MSM with concentrated cells under nitrogen deletion was 67% and 13.4 g/L, respectively. The highest conversion rate of curdlan under the conditions optimized in this study was 71% when the glucose concentration was 1% (w/v).

Keywords: curdlan, *Agrobacterium* sp., nitrogen depletion, concentrated cells, inoculum size

INTRODUCTION

Agrobacterium sp. ATCC 31749 produces an extracellular unbranched homo-β-(1→3)-glucan called curdlan, which is water-insoluble at a neutral pH [1,2]. Curdlan forms a firm gel when heated in a suspension at or above 54°C [3], and has been produced on an industrial scale by the fermentation of *Agrobacterium* sp. for use as a the gelling agent in the chemical and food industries.

A complex medium (CM) for the production of curdlan-type polysaccharides was previously developed including yeast extract as the best nitrogen source [1,4]. Yeast extract as a source of nutrients for the cultivation of microorganisms has been used since the 1920's [5]. A mineral salt medium (MSM) containing mineral salts and a phosphate buffer to maintain a suitable pH during culture has also been developed [6]. This MSM can be used in a continuous culture or a two-stage continuous process for improved productivity [7,8].

Some microorganisms produce extracellular polysaccharides at a maximal rate during exponential growth, whereas other strains produce them after growth has ceased and exhibit the maximal rate of production under nitrogen depletion [9-11]. The production of curdlan is associated with the poststationary phase of a nitrogen depleted aerobic culture [12]. The conversion rate of curdlan from glucose is about 50% if the cultivation pH is maintained at 6 [13]. In this study, the physiological factors that affect the production of curdlan were investigated and a new process using concentrated cells of *Agrobacterium* sp. ATCC 31749 is presented to improve the productivity of curdlan.

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MATERIALS AND METHODS

Microorganism and Media

The *Agrobacterium* sp. ATCC 31749 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained on a complex medium (CM) with a 1.5% (w/v) agar. The CM contained 4% (w/v) glucose, 0.5% (w/v) yeast extract, and 1.0% (w/v) calcium carbonate [4]. This strain has already been described in patents for the production of gelable exopolysaccharide and curdlan [14,15]. A mineral salt medium (MSM) was used for the production of curdlan. The MSM contained the following components (g/L): KH_2PO_4, 1.74; K_2HPO_4, 0.49; $Na_2SO_4 \cdot 10H_2O$, 3.7; $MgCl_2 \cdot 6H_2O$, 0.25; $FeCl_3 \cdot 6H_2O$, 0.024; $CaCl_2 \cdot 2H_2O$, 0.015; $MnCl_2 \cdot 4H_2O$, 0.01; citrate, 0.21; NH_4Cl, 1.5; and a 2% (w/v) carbon source [8]. The initial pH of the medium before sterilization was adjusted to 6.5. The carbon source was autoclaved separately for 20 min at 121°C and added to the media under aseptic conditions.

Production of Curdlan

The seed culture was prepared by transferring cells from the CM agar slants to 50 mL of the MSM with 2%

(w/v) glucose in a 250-mL Erlenmeyer flask. This culture was incubated for 72 h at 30°C and 180 rpm and then used as a 5% (v/v) inoculum for 150 mL of the MSM with a 2% (w/v) carbon source in a 500 mL Erlenmeyer flask. This culture was then incubated for 5 days with glucose as the sole carbon source under the same conditions as for the starter culture.

The purification of curdlan was carried out exactly as described elsewhere [16,17]. The culture was centrifuged at 12,000 × *g* for 30 min. The pellet was added to an equivalent volume of 0.5 N sodium hydroxide at 4°C, the mixture was stirred for 10 min, and then left to stand for 3 h at the same temperature. The resulting viscous solution was centrifuged at 12,000 × *g* for 40 min, and the curdlan in the clear supernatant was precipitated by neutralization with 10% acetic acid and repeatedly washed with water, acetone, and ether.

Preparation of Inoculum with Concentrated Cells

The seed culture for the concentrated cells of *Agrobacterium* sp. ATCC 31749 was prepared under the same conditions as described above. The culture was incubated for 72 h at 30°C and 180 rpm and then used as a 5% (v/v) inoculum for 150 mL of the MSM with 0.5% (w/v) yeast extract in a 500-mL Erlenmeyer flask. This culture was then incubated for 48 h under the same conditions as for the seed culture. The optical density of the culture at 600 nm was about 15.0. The culture was centrifuged at 12,000 × *g* for 30 min and the pellet was added to one fifth volume of the MSM without a carbon source. The mixture was gently stirred for 10 min at 4°C to prepared the concentrated cells and then used as an inoculum.

Analytical Methods

The dry cell weight (DCW) was determined by the direct weighing of the cell fraction after drying to a constant weight at 100-105°C. The yield of the curdlan after washing with water, acetone, and ether was determined by the same procedure.

A gas chromatographic analysis after the methanolysis and subsequent trimethyl-silylation of the curdlan purified from the culture of *Agrobacterium* sp. ATCC 31749 was used to determine the composition of the carbohydrates in the exopolymer [18]. The preparation of the samples for the GC analysis of the curdlan was carried out exactly as described in previous reports [16, 17]. The gas chromatographic analyses were performed on a Hewlett Packard (HP) gas chromatograph, model 5890 Series II, equipped with a flame-ionization detector and HP model 7673 injector. The column was 30 m × 0.32 mm I.D. fused silica with a cross-linked 0.25 mm 5% (v/v) phenylmethyl silicone liquid phase (Supelco, PA, USA). Dry oxygen-free nitrogen (2.9 mL/ min flow rate) was used as the carrier gas at 10 psi head pressure, using a temperature program (140°C for 2 min, then increasing at 8°C per min up to 260°C). The injector was purged for 0.8 min after injection.

Table 1. Effect of yeast extract added to minimal salt medium on cell growth and production of curdlan by *Agrobacterium* sp.[1)]

Yeast extract (%)	pH[2)]	DCW[3)] (g/L)	Curdlan (g/L)	Productivity		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0.000	4.10	0.56	0.29	0.01	0.03	0.52
0.125	4.23	0.82	0.40	0.02	0.04	0.49
0.250	4.22	1.61	0.54	0.03	0.08	0.34
0.375	4.05	2.14	0.68	0.03	0.11	0.32
0.500	4.05	2.72	0.76	0.04	0.14	0.28
0.750	4.75	4.01	0.63	0.03	0.20	0.16
1.000	6.80	4.41	0.63	0.03	0.22	0.14

[1)] 5 day culture at 30°C, [2)] final pH of culture, [3)] dry cell weight

RESULTS AND DISCUSSION

Addition of Yeast Extract to the Minimal Salt Medium

The effect of the addition of yeast extract to the MSM on cell growth and the production of curdlan was investigated (Table 1). The addition of yeast extract to the MSM, in which ammonium chloride is the sole nitrogen, resulted in an enhanced cell growth. It also increased the production of curdlan with a limited concentration of yeast extract. The highest production of curdlan was 0.76 g/L when 0.5% (w/v) yeast extract was added to the MSM. The utilization rate of the substrate (glucose) for cell growth and the production of curdlan ($Y_{p/s} + Y_{x/s}$) with 0.5% (w/v) yeast extract was 0.18.

Yeast extract is a complex mixture of amino acids, peptides, and proteins as well as a good source of vitamin B and certain mineral salts [19,20]. The addition of yeast extract into the MSM resulted in higher cell growth yet did not increase the production of curdlan as much as described in previous reports [12,13]. Although some of the nutrients in the yeast extract enhanced the cell growth of *Agrobacterium* sp., major components in yeast extract acted as a nitrogen source thereby prohibiting the production of curdlan, which is known to be stimulated by nitrogen depletion [14].

Ammonium Chloride as the Sole Nitrogen Source

The effect of the addition of ammonium chloride in the MSM on cell growth and the production of curdlan was also examined (Table 2). Ammonium chloride is the sole nitrogen source in the MSM for the production of curdlan. As the concentration of ammonium chloride in the MSM increased, the cell growth also increased yet the production of curdlan decreased. The highest production of curdlan and specific yield of curdlan from 2% (w/v) glucose as the carbon source were 0.91 g/L and 2.68, respectively, when ammonium chloride was not added to the MSM. The utilization rate of the substrate for cell growth and the production of curdlan ($Y_{p/s} + Y_{x/s}$) without ammonium chloride in the MSM was as low as 0.07.

Table 2. Effect of ammonium chloride on cell growth and production of curdlan by *Agrobacterium* sp.

Ammonium chloride (%)	pH	DCW (g/L)	Curdlan (g/L)	Productivity		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0.00	6.2	0.34	0.91	0.05	0.02	2.68
0.05	5.4	0.45	0.80	0.04	0.02	1.78
0.10	4.7	0.55	0.72	0.04	0.03	1.31
0.15	4.4	0.52	0.55	0.03	0.03	1.06
0.20	4.3	0.53	0.32	0.02	0.03	0.60
0.25	4.3	0.52	0.30	0.02	0.03	0.58

Nitrogen depletion was found to be essential for a higher production of exopolymers, even though the inoculum size was relatively large and higher cell growth occurred [9]. The production of curdlan exhibited a positive relationship with the intercellular levels of AMP and UMP [21]. Accordingly, as the concentration of nitrogen source decreased, the intercellular levels of AMP and UMP increased and the production of curdlan followed. This shows that nitrogen depletion is essential for the higher production of curdlan by *Agrobacterium* sp. ATCC 31749.

Inoculum Size with Concentrated Cells

To investigate the effect of the inoculum size on cell growth and the production of curdlan, cells concentrated 5 times were prepared as described in Materials and Methods. The initial OD value and DCW of the culture with inoculum size of 50% (v/v) was about 7.5 at 600 nm and 4.4 mg/mL, respectively. As the amount of the inoculum increased, the cell growth as well as the production of curdlan also increased in the MSM without a nitrogen source (Table 3). The highest conversion rate and concentration of curdlan from 2% (w/v) glucose were 50% and 10.0 g/L, respectively. The utilization rate of the substrate for cell growth and the production of curdlan ($Y_{p/s}$ + $Y_{x/s}$) increased up to 0.58. The specific yield of curdlan ($Y_{p/x}$, g curdlan per g cell mass) at a conversion rate of 50% was 6.45.

It would appear that the amount of inoculum was related to production of curdlan, particularly, under nitrogen depletion. A higher inoculum size with concentrated cells and nitrogen depletion resulted in a higher production of curdlan. A conversion rate of 50% from glucose was previously reported in the production of curdlan when the cultivation pH was maintained at 6 [13]. Accordingly, a higher production of curdlan would seem to occur with a sufficient amount of cells under nitrogen depletion. The cultivation pH also appears to affect the production of curdlan.

Initial pH of Medium

The effect of the initial pH of the MSM without a nitrogen source on cell growth and the production of curdlan was investigated (Table 4). The initial pH of the MSM ranged from 7.0 to 4.2 and the inoculum size

Table 3. Effect of inoculum size on cell growth and production of curdlan by *Agrobacterium* sp. under nitrogen depletion

Inoculum size (%)	pH	DCW (g/L)	Curdlan (g/L)	Productivity		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0	6.5	0.00	0.00	0.00	0.00	–
5	6.5	0.40	0.83	0.04	0.02	2.08
10	6.4	0.73	2.00	0.10	0.04	2.74
15	6.4	0.88	2.88	0.14	0.04	3.27
20	6.3	1.23	4.30	0.22	0.06	3.50
25	6.3	1.10	4.98	0.25	0.06	4.53
30	6.3	1.48	9.44	0.47	0.07	6.38
40	6.3	1.55	10.00	0.50	0.08	6.45
50	6.3	1.52	9.88	0.49	0.08	6.50

Table 4. Effect of initial pH of medium on cell growth and production of curdlan with concentrated cells of *Agrobacterium* sp. under nitrogen depletion

Initial pH	pH	DCW (g/L)	Curdlan (g/L)	Productivity		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
4.2	5.7	2.68	11.30	0.57	0.13	4.22
4.6	5.8	2.60	11.86	0.59	0.13	4.56
5.0	5.9	2.52	13.38	0.67	0.13	5.31
5.4	6.0	1.92	12.14	0.61	0.10	6.32
5.8	6.1	1.80	12.14	0.61	0.09	7.59
6.2	6.3	1.60	10.74	0.54	0.08	6.71
6.6	6.6	1.56	10.90	0.55	0.08	6.99
7.0	6.9	1.68	10.54	0.53	0.08	6.27

with concentrated cells was 40% (v/v). The cell growth and production of curdlan increased as the initial pH of the medium decreased as low as 5.0. The highest conversion rate and concentration of curdlan from 2% (w/v) glucose were 67% and 13.4 g/L, respectively. The utilization rate of the substrate for cell growth and the production of curdlan ($Y_{p/s}$ + $Y_{x/s}$) increased up to 0.80. The specific yield of curdlan ($Y_{p/x}$) at a conversion rate of 67% was 5.31.

The optimal cultivation pH for the production of curdlan by *Agrobacterium* sp. (formerly *Alcaligenens faecalis*) was reported to be 6 [13]. A significant improvement in the production of curdlan was previously obtained by shifting the culture pH from 7.0 to 5.5 during a batch fermentation of *Agrobacterium* sp. [17]. The highest production of curdlan in this study occurred when the initial pH of the medium without a nitrogen source was 5.0 and the final pH of culture was about 6. These differences in the pH for the maximal production of curdlan may have been due to the fact that the later study was cultured on a flask scale, whereas former was cultured in a fermentor with pH control. On the basis of these two different experiments, it would appear that the optimal pH of the medium for the production of curdlan is about 6 or less than 6.

Glucose as the Sole Carbon Source

The effect of glucose as the sole carbon source in the

Table 5. Effect of glucose concentration on cell growth and production of curdlan with concentrated cells of *Agrobacterium* sp under nitrogen depletion

Initial pH	pH	DCW (g/L)	Curdlan (g/L)	Productivity		
				$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0.0	6.1	1.52	0.06	–	–	0.04
1.0	6.1	1.60	7.10	0.71	0.16	4.44
2.0	6.0	2.70	12.35	0.62	0.14	4.57
3.0	5.7	3.75	15.30	0.51	0.13	4.08
4.0	5.7	4.20	15.15	0.38	0.11	3.61
5.0	5.5	4.45	14.90	0.30	0.09	3.35
7.5	5.4	4.30	16.35	0.22	0.06	3.80
10.0	5.4	4.15	15.70	0.16	0.04	3.78

MSM without a nitrogen source on cell growth and the production of curdlan was investigated (Table 5). The initial pH of the MSM without a nitrogen source was 5.0, and the inoculum size with concentrated cells was 40% (v/v). The production of curdlan increased as the concentration of glucose increased, whereas the conversion rate of curdlan from glucose decreased. The highest conversion rate of curdlan under this condition was 71% when the glucose concentration was 1% (w/v). The utilization rate of the substrate for cell growth and the production of curdlan ($Y_{p/s}$ + $Y_{x/s}$) increased up to 0.87. The specific yield of curdlan ($Y_{p/x}$) at a conversion rate of 71% was 4.44.

The production of curdlan did not increase yet the conversion rate did dramatically decrease with more than 3% (w/v) glucose as the sole carbon source (Fig. 1). It would seem that *Agrobacterium* sp. ATCC 31749, in the production of curdlan from glucose, exhibits a catabolite repression against the glucose found in other species [22-25]. This means that another process must be developed to overcome this catabolite repression to enhance the production of curdlan under the physiological conditions optimized in this study.

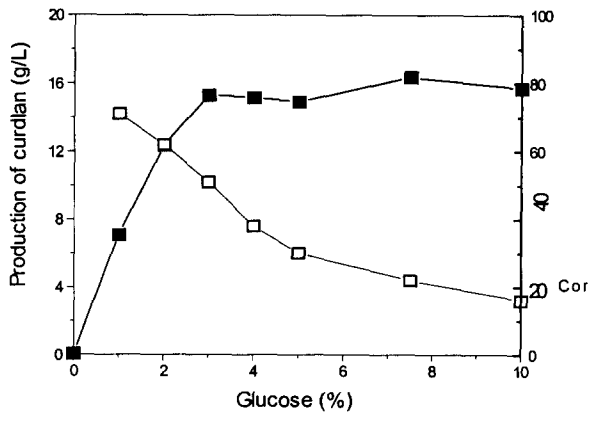

Fig. 1. Effect of glucose concentration on production (■) and conversion rate (□) of curdlan from glucose.

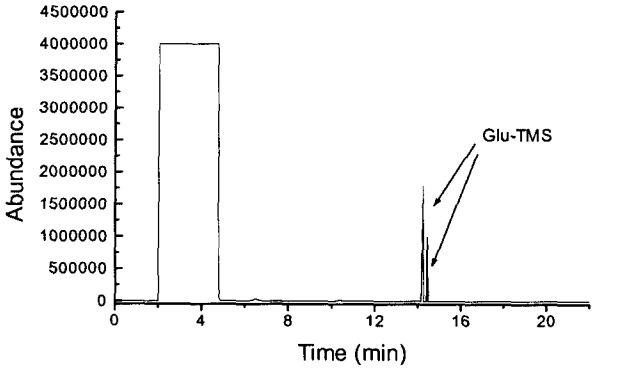

Fig. 2. Gas chromatogram of trimethylsilylated (TMS) sugar components of curdlan purified from culture grown in MSM with 1% (w/v) glucose under nitrogen depletion. Glu is an abbreviation of glucose.

Structural Analysis

The purified curdlan from cultures of *Agrobacterium* sp. ATCC 31749 under the conditions optimized in this study was confirmed with gas chromatography (Fig. 2). The gas chromatogram showed the major component to be glucose as identified by the peak retention times for the α and β anomer (14.22 and 14.44 min, respectively) and the peak area ratio of the α to the β peak (2.4 to 1.0). This showed that the purified curdlan only consisted of glucose [16-18].

Agrobacterium sp. ATCC 31749 produces three different types of exopolymers [16]. The major product is curdlan and the minor products are water-soluble non-curdlan-type exopolymer-A and B (WSNCE-A and B) [2]. WSNCE-A consists of glucose and galactose with rhamnose as a minor component. The major components of WSNCE-B are glucose and mannose with rhamnose as the minor component. The ratio of these components in the product varies with the physiological conditions of the cultivation such as the initial pH of the medium and the nitrogen source [17]. Based on the fact that the gas chromatogram of the product from *Agrobacterium sp.* ATCC 31749 only showed glucose, the culture conditions optimized in this study can be applied to enhance the production of curdlan as well as increase the ratio of curdlan in the exopolymers produced by *Agrobacterium* sp.

CONCLUSION

Many physiological factors affect the production of curdlan by *Agrobacterium* sp. [9,13,15,21,26]. In this study some of these factors were optimized and a process for concentrating cells was developed for the production of curdlan. *Agrobacterium* sp. ATCC 31749 was found to exhibit a catabolite repression against glucose (Fig. 1). In order to improve the total productivity of curdlan, mutants, which can overcome the catabolite repression against glucose, should be isolated. Another way to overcome catabolite repression is with a fed-batch culture with the addition of glucose or a continu-

ous culture with a limited concentration of glucose [9]. The best way to improve the production of curdlan would appear to be a two-stage culture on the basis of the result that higher cell growth was obtained with the addition of yeast extract into the medium and nitrogen depletion was essential for the production of curdlan. The enough amount of cells used for the inoculum of the next stage could not be obtained with a previously reported two-stage culture, in which the MSM was used for the culture of *Agrobacterium* sp. without any additional nutrients[8]. The first stage involves the preparation of a higher amount of cells through the addition of yeast extract and the second stage is the production of curdlan without any nitrogen sources.

On the basis of the results obtained in this study, further work will focus on the isolation of mutants which can overcome the catabolite repression against glucose and the development of new processes, such as a fed-batch culture with the addition of glucose during the culture and a two-stage culture.

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