Production of Extracellular Water Insoluble β-1,3-Glucan (Curdlan) from *Bacillus* sp. SNC07

Sathyanarayana N. Gummadi* and Kislay Kumar

Department of Biotechnology, Indian Institute of Technology-Madras, Chennai 600 036, India

Abstract β-1,3-Glucan (curdlan) is a water-insoluble polysaccharide composed exclusively of β-1,3 linked glucose residues. Extracellular curdlan was mostly synthesized by *Agrobacterium* species and *Alcaligenes faecalis* under nitrogen-limiting conditions. In this study, we screened the microorganisms capable of producing extracellular curdlan from soil samples. For the first time, we reported Gram-positive bacterium *Bacillus* sp. SNC 107 capable of producing extracellular curdlan in appreciable amounts. The effect of different carbon sources on curdlan production was studied and found that the yield of curdlan was more when glucose was used as carbon source. It was also found that maximum production was achieved when the initial concentration of ammonium and phosphate in the medium was 0.5 and 1.9 g/L respectively. In this study the curdlan production was increased from 3 to 7 g/L in shake flask cultures.

Keywords: extracellular polysaccharide, β-1,3-D-glucan, curdlan, *Bacillus*, nitrogen limitation

INTRODUCTION

Curdlan is a water-insoluble polysaccharide composed exclusively of β-1,3-linked glucose residues. β-(1-3)-D-glucans are normally present in cell walls and membranes of fungi, yeasts, algae, bacteria and higher plants, however only in the case of bacteria this polymer is produced extracellularly under nutrient deficient conditions. However, until now only bacteria belonging to the *Alcaligenes* and *Agrobacterium* species have been reported to produce the linear β-1,3-glucan type of homopolymer [1-3]. Harada and coworkers discovered this biopolymer and named it curdlan because it forms curdle type product after heating [4]. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties. One of the unique features of curdlan is that its aqueous suspensions do not come to liquid state once being heated [4]. Curdlan has potential applications in manufacture of food products such as jelly, noodles, edible fibers, and new calorie-reduced products [5,6]. In 1996, US launched curdlan into market (Pureglucan™) after obtaining approval from Food and Drugs Administration [6]. Curdlan has also been used as drug delivery polymer, since it can sustain and control the diffusion of drugs [7]. Furthermore, researchers developed curdlan sulfate as an antiviral agent to inhibit the infection of human immunodeficiency virus [8]. Curdlan and its derivatives have growing potential applications in pharmaceutical industries [3]. Hence, polymer with different degree of polymerization and with improved physical properties will be required in future. In order to achieve this, curdlan from different microbial source is needed. In the present study, we screened bacteria capable of producing extracellular curdlan from soil samples and studied the important parameters affecting the production of curdlan by the isolate. For the first time, we reported *Bacillus* sp. (Gram-positive bacteria) producing extracellular curdlan whereas most of the reports are on *Agrobacterium* and *Alcaligenes* sp.

***Corresponding author**
Tel: +91-44-2257-4114 Fax: +91-44-2257-0509
e-mail: gummadi@iitm.ac.in

MATERIALS AND METHODS

Isolation of Microorganisms

Soil samples from ten different locations were collected from IIT-Madras campus in India. One gram of an air-dried soil samples was added to 10 mL of 0.9% (w/v) sterile saline and agitated at 100 rpm for 30 min. After centrifugation at 10,000 rpm for 10 min, 0.1 mL of the supernatant was added to 0.9 mL of sterile saline and a serial dilution (10^{-1} to 10^{-6}) was prepared. About 0.1 mL of each dilution was added and distributed on aniline blue agar medium containing 20 g/L sucrose, 5 g/L yeast extract, 20 g/L agar, 0.05 g/L aniline blue at pH 7 [9]. The plates were incubated for 48 h at 30°C and the different colonies showing intense blue color were picked and purified. The promising strains producing blue colonies on aniline blue agar plates were further examined for morphological and biochemical characteristics according to Bergey's manual of systematic bacteriology.

Culture Conditions

A loop of isolated strain was grown in seed medium

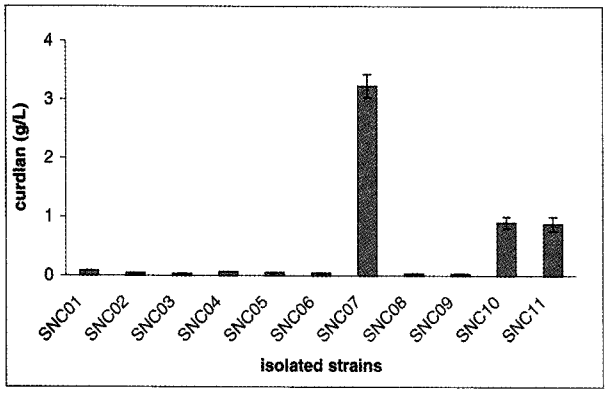

Fig. 1. Production of curdlan by eleven isolated strains. Fermentation was carried out at 30°C for 96 h on a rotatory shaker maintained at 180 rpm. The curdlan values reported are measured at 96 h and the experiments were performed in triplicates.

containing (g/L): sucrose 20; peptone 5; yeast extract 5 at pH 7. The incubation was performed on rotary shaker at 180 rpm for 20 h at 30°C. Approximately 10% (v/v) of seed culture was transferred to production medium containing (g/L): sucrose 100; $(NH_4)_2HPO_4$ 2.3; KH_2PO_4 1; $MgSO_4 \cdot 7H_2O$ 0.4, 1% (v/v) of 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 M HCl) and 0.3% (w/v) $CaCO_3$. Production of curdlan was carried out in 250 mL Erlenmeyer flasks containing 50 mL of production medium. The initial pH of the medium was adjusted to 6.5 and the pH was uncontrolled during fermentation. All the experiments were performed in triplicates and the values reported are mean within ± 5 to ± 10% standard error.

Estimation of Curdlan and Cell Dry Weight

The concentration of cells and curdlan was determined by measuring the dry weight. About 2 mL of sample was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet consisting of cells and curdlan was washed with 0.01 N HCl twice, and harvested by centrifugation. The curdlan was solubilized by adding 15 mL of 2 N NaOH. The cells were separated by centrifugation at 10,000 rpm for 10 min at 4°C. The curdlan present in the supernatant was precipitated under acidic conditions by the addition of an appropriate amount of 2.0 N HCl. Both cells and curdlan were washed three times and dried to a constant weight.

Analytical Methods

Glucose, fructose and galactose concentrations were determined by 3,5-dinitrosalicyclic acid method [10]. Sucrose in the medium was determined by hydrolyzing the sample with 3 N HCl at 100°C for 15 min and the reducing sugars were measured by the method of 3,5-dinitrosalicyclic acid method. Ammonium (NH_4^+) concentration was determined by the indophenol method [11]. Phosphate concentration was determined using ascorbic acid method [12].

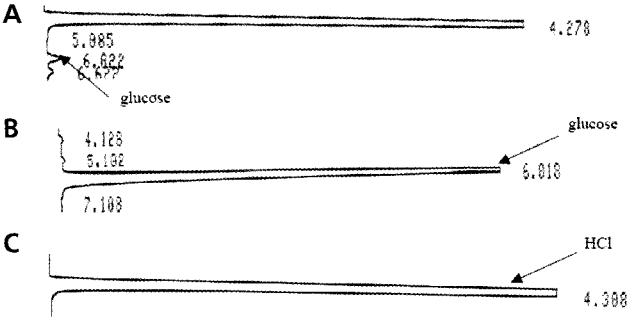

Fig. 2. HPLC analysis of product of hydrolysis of extracellular biopolymer produced by *Bacillus* sp. SNC07. (A) product of hydrolysis, (B) pure glucose (C) 3 N HCl.

Curdlan Hydrolysis

Curdlan was subjected to acid hydrolysis by adding appropriate amount of curdlan (in water) with 3 N HCl and incubated at 100°C for 2 h. The product of hydrolysis (glucose) was estimated using DNS method and also by HPLC. The hydrolyzed sample was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered. 25 μL of filtered sample was injected in Shimadzu analytical HPLC using SHIM PACK, SCR-101H column and mobile phase was water at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

The main objective of this work is (i) to isolate the microorganisms capable of producing extracellular curdlan and (ii) to study the different factors affecting the production of curdlan for isolated strain.

Screening and Isolation of Microorganisms

It has been reported that only curdlan producing microorganisms appeared blue on aniline blue agar plates and the intensity of colony color is proportional to concentration of curdlan [13]. Based on this, nearly 50 strains that are capable of producing extracellular curdlan were isolated to single colonies. To select stable strains, the strains were subcultured nearly 4 times and eleven strains with darker blue color were chosen. In order to study the ability of the strains to produce curdlan in extracellular fashion, shake flask experiments were performed (Fig. 1). It was found that only three out of eleven isolates produced curdlan in appreciable amount (SNC07, SNC10 and SNC11). All three strains were spore forming, Gram positive, rod shaped and identified as *Bacillus* sp. Among the three isolates, *Bacillus* sp. SNC07 was selected as the best producer of extracellular curdlan. The strain was positive for catalase and oxidase and negative for nitrite reduction, Indole, H_2S production and gelatin liquefaction. The isolate produced acid compounds when grown on glucose, fructose, mannitol and xylose. These morphological and biochemical data

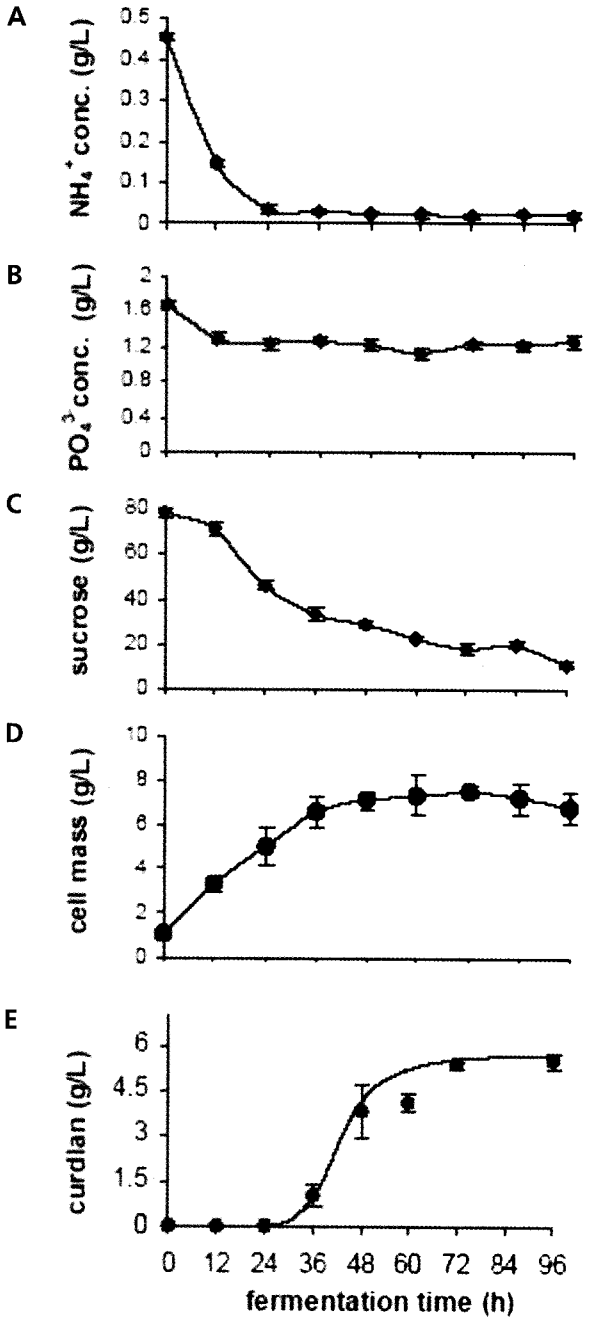

Fig. 3. Time course fermentation of the isolate *Bacillus* sp. SNC07. (A) NH_4^+ profile, (B) PO_4^{3-} profile, (C) sucrose profile, (D) cell dry weight profile and (E) curdlan profile. Fermentation was carried out at 30°C for 96 h on a rotatory shaker maintained at 180 rpm.

suggest that the isolate SNC07 is a *Bacillus* sp. The King's Institute, Chennai has also confirmed it as *Bacillus* sp. The major producers of curdlan are *Agrobacterium* and *Alcaligenes* species, both belonging to Gram-negative type bacteria [1,3]. For the first time, we reported Gram-positive *Bacillus* sp. produced 3 g/L of extracellular curdlan.

Curdlan Hydrolysis

In order to confirm whether the extracellular polymer produced by the isolate is a curdlan (a polymer of glucose); the polymer was subjected to acid hydrolysis as described in materials and methods. HPLC analysis of product of hydrolysis showed peak at 6.02 min and 4.3 min (Fig. 2A). The peak at 6.02 min corresponded to glucose (Fig. 2B) where as the peak at 4.3 min corresponded to HCl (Fig. 2C). These results also supported that the polymer produced by the isolate was curdlan.

Fermentation Profile for Isolate *Bacillus* sp. SNC07

Preliminary experiments were performed to study the detailed fermentation characteristics of the isolate. The time course profile of nutrient consumption, cell growth and curdlan production is shown in Fig. 3. The results clearly showed that the curdlan production started when the nitrogen source in the medium (NH_4^+) was depleted (Figs. 3A and 3E). Maximum production was obtained at 70~84 h of fermentation. This clearly indicated that the depletion of ammonium in the medium is crucial for the curdlan production. This observation is in agreement with the results obtained from *Agrobacterium* sp. [14]. Based on our results, it can be interpreted that the mechanism of curdlan biosynthesis in the isolated *Bacillus* sp. would be similar to that of reported Gram-negative bacteria (*Agrobacterium and Alcaligenes* sp.) [15]. It was found that the phosphate concentration in the medium initially decreased (till 24 h) and then reached a constant value (Fig. 3B). The cell concentration reached a constant value after the depletion of nitrogen source (Fig. 3D). Sucrose was consumed for both cell growth and curdlan production (Fig. 3C). Based on these results, it can be concluded that cell growth reaches a stationary phase, once the nitrogen source in the medium got depleted. Under nitrogen limiting conditions, the isolate SNC07 started synthesizing curdlan using the excess available sucrose in the medium. The phosphate concentration decreased up to 24 h and remained constant through out the fermentation. These results prompted us to study the effect of different carbon sources, effect of nitrogen source and phosphate concentration on the production of curdlan by *Bacillus* sp. SNC07.

Effect of Carbon Sources on Curdlan Production

The effect of different carbon sources on the production of curdlan by isolate SNC07 was studied. The fermentation was performed with different carbon sources (glucose, sucrose, galactose, and fructose) and other medium constituents were same as mentioned in the materials and methods. The initial concentration of sucrose, glucose, fructose, and galactose in the fermentation medium was 100 g/L. The yield of curdlan with respect to carbon source consumption was found to be higher in the case of glucose (0.16 g of curdlan per g of glucose) (Table 1). Ammonium consumption (95~99%) was found to be same when isolate SNC07 was grown in medium

Table 1. The effect of different carbon sources on curdlan production by *Bacillus* sp. SNC07 at 30°C. Fermentation was carried out for 96 h on a rotatory shaker maintained at 180 rpm. The values reported are measured at 96 h and the experiments were performed in triplicates.

Carbon source	$Y_{P/S}$ (g curdlan/g substrate)	Ammonium consumption (%)	Phosphate consumption (%)
Glucose	0.162 ± 0.02	92.3 ± 5.4	47.2 ± 8.0
Fructose	0.151 ± 0.03	84.8 ± 8.7	78.6 ± 6.0
Sucrose	0.110 ± 0.01	88.6 ± 9.5	63.6 ± 2.8
Galactose	0.120 ± 0.02	87.9 ± 6.0	47.6 ± 6.8

Table 2. The effect of different initial ammonium concentration on curdlan production by *Bacillus* sp. SNC07 at 30°C. Fermentation was carried out for 96 h on a rotatory shaker maintained at 180 rpm. The values reported are measured at 96 h and the experiments were performed in triplicates.

Initial ammonium concentration (g/L)	Ammonium consumption (%)	Phosphate consumption (%)	Curdlan (g/L)	Cell dry weight (g/L)
0.25	96.1 ± 1.5	61.8 ± 6.7	3.46 ± 0.12	5.82 ± 0.51
0.5	98.5 ± 1.1	77.3 ± 5.0	4.86 ± 0.20	6.14 ± 0.47
1.0	97.5 ± 0.8	38.2 ± 4.6	4.56 ± 0.08	6.46 ± 0.30
1.5	53.6 ± 2.6	19.2 ± 3.9	3.28 ± 0.10	7.74 ± 0.21

containing all tested carbon sources (Table 1). It was also observed that the final cell concentration did not vary much (result not shown). Phosphate consumption was low when glucose was used as the carbon source (Table 1). This observation suggested that residual phosphate concentration in the medium significantly influences the curdlan production. Hence in further studies, glucose was used as carbon source instead of sucrose.

Effect of Ammonium and Phosphate Concentration on Curdlan Production

In preliminary experiments, it was observed that curdlan production starts, only when ammonium concentration drops to very low level. Hence, it is very important to study the effect of initial ammonium concentration on curdlan production. For this purpose, the fermentation was carried out with glucose as carbon source (100 g/L) and with different initial ammonium concentrations (0.25, 0.5, 1.0, and 1.5 g/L). The maximum production was observed when initial ammonium concentration was 0.5 g/L (Table 2). Based on the experimental and published results it is clearly understood that nitrogen limitation is the most efficient means to stimulate curdlan biosynthesis [14]. It has been reported that isoprenoid lipid, which play a crucial role in carrying cellular oligosaccharides, would be more available for the synthesis of exopolysaccharides instead of the synthesis of cellular lipopolysaccharides under nitrogen-limiting conditions [16]. This could be the possible explanation for the synthesis of curdlan under nitrogen limiting conditions. At high initial ammonium concentration (1.5 g/L) it was found that NH_4^+ did not deplete completely, which in turn reflected by low curdlan production and high cell dry weight (Table 2). Even though the glucose consumption was found to be same in all experiments, high curdlan production was achieved when the initial NH_4^+ was 0.5 g/L. At higher concentrations of ammonium in the medium, the glucose was utilized for cell growth rather than curdlan production. However, even at lower concentration of ammonium in the medium (0.25 g/L initial concentration of ammonium), the curdlan production was lower than produced by isolate when grown at initial ammonium concentration of 0.5 g/L. This is probably due to the fact that at lower concentrations of nitrogen source, cell growth is low (Table 2) which in turn affected the curdlan production. Hence, maintaining appreciable cell growth and amount of nitrogen source in the medium is crucial for curdlan production.

Table 3. The effect of different initial phosphate concentration on curdlan production by *Bacillus* sp. SNC07 at 30°C. Fermentation was carried out for 96 h on a rotatory shaker maintained at 180 rpm. The values reported are measured at 96 h and the experiments were performed in triplicates.

Initial phosphate concentration (g/L)	Curdlan (g/L)
1.2	3.3 ± 0.15
1.9	5.0 ± 0.23
3.2	4.7 ± 0.12
4.5	3.1 ± 0.08

In order to study the effect of initial phosphate concentration on curdlan production, flask experiments were performed with different initial phosphate concentrations (1.23, 1.9, 3.2, and 4.5 g/L). The maximum production of curdlan was achieved when initial phosphate concentration was 1.9 g/L (Table 3). It was observed that curdlan was produced only when the ammonium concentration reaches low value. It was also found that the phosphate concentration remains constant during curdlan production (in all experiments). This observation is in agreement with published reports for *Agrobacterium* and *Alcaligenes* species [17,18].

Table 4. The effect of initial pH on curdlan production by *Bacillus* sp. SNC07 at 30°C. Fermentation was carried out for 96 h on a rotatory shaker maintained at 180 rpm. The values reported are measured at 96 h and the experiments were performed in triplicates.

Initial pH	Curdlan (g/L)	Cell dry weight (g/L)	Glucose consumption (%)
4.5	1.25 ± 0.10	2.45 ± 0.22	41.9 ± 0.44
5.5	3.79 ± 0.08	4.60 ± 0.40	42.9 ± 2.00
6.5	5.50 ± 0.16	5.22 ± 0.43	54.7 ± 1.60
7.5	7.13 ± 0.12	6.70 ± 0.20	59.3 ± 2.20
8.5	6.43 ± 0.11	6.10 ± 0.40	47.6 ± 3.10

Effect of pH on Curdlan Production

In order to study the effect of initial pH on curdlan production, experiments were performed with different initial pH varying between 4.5 and 8.5. It was found that maximum curdlan production was obtained when the initial pH was at 7.5 (Table 4). At pH 7.5, maximum curdlan production and cell growth was observed (Table 4). The glucose consumption was higher when initial pH of the fermentation was maintained at 7.5 suggesting that glucose was utilized for curdlan biosynthesis (Table 4). These results suggest that higher curdlan can be achieved by high cell density cultivation.

CONCLUSIONS

Three gram-positive *Bacillus* sp. capable of producing extracellular curdlan were isolated based on their ability to form blue colonies on aniline blue agar plate. Among the three strains, *Bacillus* sp. SNC07 was selected as the best producer of extracellular curdlan. This is first report on curdlan production by *Bacillius* sp. The yield of curdlan (0.18) with respect to substrate utilization was more for glucose. It was found that depletion of ammonium concentration in the fermentation medium triggers the curdlan production in isolate SNC07. Hence, it can be concluded that at nitrogen limiting conditions, *Bacillus* sp. utilized the available carbon source for the production of curdlan. The optimal pH for curdlan production was found to be 7.5. Under optimal conditions, the curdlan production was increased to 7 g/L (yield ~0.25). The low yields are possibly due to the utilization of glucose for cell maintenance under stress conditions (yield ~0.25). Further studies on optimization of process conditions in bioreactors will enhance the curdlan production by the new isolate *Bacillus* sp.

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