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Production of Extracellular Water-Insoluble Polysaccharide from *Pseudomonas* sp.

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ABSTRACT: Curdlan is a microbial polysaccharide composed exclusively of β-(1,3)-linked glucose residues. Until now only bacteria belonging to the *Alcaligenes* and *Agrobacterium* species have been reported to produce Curdlan. In this study, a bacterium capable of producing extracellular Curdlan, identified as *Pseudomonas* sp. on the basis of 16S rDNA gene sequencing, was isolated from soil samples. From the HPLC, permethylation linkage analysis, ^{13}C NMR, and FT-IR analytical data, the polysaccharide consisted exclusively of glucose; the most prominent sugar was 1,3-linked glucose, and most glycosidic bonds joining these sugar residues were of the β-type. This also supported that the exopolysaccharide produced by *Pseudomonas* sp. was actually Curdlan. In addition, the *Pseudomonas* sp. was studied for the production of Curdlan by conventional "one-factor-at-a-time technique" and response surface methodology (RSM). It was observed that glucose and yeast extract were the most suitable carbon source and nitrogen source for Curdlan production, respectively. By using RSM, Curdlan production was increased significantly by 188%, from 1.25 to 2.35 g/L, when the strain was cultivated in the optimal condition developed by RSM, and the highest Curdlan production rate of 0.81 g/(L h) was obtained. To the best of the authors' knowledge, this is the first report on Curdlan production by *Pseudomonas* sp.

KEYWORDS: *curdlan, insoluble polysaccharide, Pseudomonas, production, optimization*

INTRODUCTION

Curdlan is a water-insoluble polysaccharide composed exclusively of β-1,3-linked glucose residues. One of the unique features of Curdlan is that its aqueous suspensions can be thermally induced to produce high-set gels before aqueous, which will not return to the liquid state upon reheating;[1] there is a great interest in the production of Curdlan for its unique rheological and thermal gelling properties, which allow its use in food products such as jellies, noodles, edible fibers, and new calorie-reduced products.[2,3] In addition, its potential has also grown in the pharmaceutical industry because of its potent biological activities.[4,5] Curdlan has already been used as a drug delivery polymer for sustained drug and control drug diffusion,[6] and Curdlan sulfate was also developed as an antiviral agent to inhibit human immunodeficiency virus (HIV)-1 infections.[7,8] β-(1,3)-D-Glucans are normally present in cell walls and membranes of fungi, yeasts, algae, bacteria, and higher plants. Until now only bacteria belonging to *Agrobacterium* and *Alcaligenes* species have been reported to produce the linear β-1,3-D-glucan type of homopolymer.[9] Moreover, a variety of β-(1,3)-D-glucans from different sources have different physical properties. In particular, such biological activities seem to be dependent on specific features of the (1,3)-β-D-glucan molecules, such as their conformation, structure, and the presence or absence of branches.[10] Thus, to meet the great demand for Curdlan in different applications, Curdlan from different microbial sources is needed. Previous studies focused mainly on optimizing several of the key factors affecting the production of Curdlan by *Agrobacterium* and *Alcaligenes* species, such as carbon source, nitrogen limiting source, phosphate concentration, oxygen supply, and pH.[11−15] However, the new microbial source capable of producing Curdlan is scarce. In this study, we screened *Pseudomonas* sp. capable of producing extracellular Curdlan from soil samples, described some structural features of this polysaccharide, and applied response surface methodology (RSM) to optimize conditions for Curdlan production by *Pseudomonas* sp.

MATERIALS AND METHODS

Soil Sampling and Chemicals. Soil samples from 10 different locations were collected from corn fields in the suburbs of Shijiazhuang and Hebei University of Science and Technology campus in China during spring 2010. Samples were withdrawn at a depth of 10−15 cm below the surface, collected into sterile vials as described by Kole and Altosaar,[17] sieved through a 4 mm mesh sieve, and stored at field moisture content at 4 °C. Soil pH was measured. *Taq* enzyme was purchased from Takara Bio Co., Ltd. (Shiga, Japan). Aniline blue was purchased from Aladdin reagent database Inc. (Shanghai, China). All other chemicals were of analytical grade and commercially available.

Isolation. One gram of an air-dried soil sample was added to 10 mL of 0.9% (w/v) sterile saline and agitated for 30 min. After centrifugation at 10000*g* for 10 min, 0.1 mL of the supernatant was added to 0.9 mL of sterile saline, and a serial dilution (10^{-1}−10^{-6}) was prepared. About 0.1 mL of each dilution was added and distributed on

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aniline blue agar medium containing (g/L) 20 sucrose, 5 yeast extract, 20 agar, and 0.05 aniline blue at pH 7.0.[10] The plates were incubated for 48 h at 30 °C, and the different colonies showing intense blue color were picked. The promising strains producing blue colonies on aniline blue agar plates were further examined for morphological characteristics.

Medium and Culture Conditions. Seed medium contained the following per liter: sucrose, 20 g; peptone, 5 g; and yeast extract, 5 g, at pH 7. A synthetic medium (initial fermentation medium) contained the following per liter: sucrose, 140 g; NH_4Cl, 2.0 g; KH_2PO_4, 1.0 g; $MgSO_4{\cdot}7H_2O$, 0.4 g; 1% (v/v) trace elements solution (5 g of $FeSO_4{\cdot}7H_2O$, 2 g of $MnSO_4{\cdot}H_2O$, 1 g of $CoCl_2{\cdot}6H_2O$, and 1 g of $ZnCl_2$ per liter of 0.1 M HCl) at pH 7. A loop of isolated strain was added to seed medium (50 mL working volume in a 250 mL Erlenmeyer flask) and incubated on a rotary shaker at 30 °C and 200 rpm for 20 h. Subsequently, 5 mL of inoculum culture was added to 45 mL of fermentation medium in a 250 mL Erlenmeyer flask. The culture was incubated at 30 °C in a rotary set at 200 rpm for 3 days.

Morphological Characteristics. The cells from different isolates were cultured in aniline blue agar plates for 48 h at 30 °C. The features of colony morphologies on the plates, that is, color and texture, were observed. The resulting cells were then diluted and observed by a scanning electron micoscope (S-4800-I; Hitachi Research and Development Corp., Japan).

16S rDNA Gene Amplification. For molecular analysis, the isolated colonies were grown on aniline blue agar plates for 2 days at 30 °C. Crude template DNA was prepared as following: two or three colonies were suspended in 2 mL of extracting solution (100 mM Tris-HCl (pH 9.0), 100 mM NaCl, 40 mM EDTA, 20 g/L SDS). The mixture was agitated with a vortex during 2 min and then heated at 100 °C during 15 min and centrifuged to 10000*g* for 8 min.[18] The supernatant fluids were used for PCR amplification. The identification of the active strains was carried out by the sequencing of a fragment of the gene 16S rDNA.[19] The PCR amplification of the 16S rDNA gene was performed in a 50 μL reaction mixture containing 1 unit of Taq DNA polymerase, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 mM dNTPs, and 10 mM of each universal primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-CGGTTACCTTGTTACGACTT-3′)[20] purchased from Shanghai Sangon-Biological Technology and Science Co. Ltd. (Shanghai, China). For PCR, we used a temperature program consisting of one denaturation cycle (94 °C for 3 min) and 30 amplification cycles (94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s). The PCR session was terminated at 72 °C for 10 min, following by cooling at 4 °C. DNA amplification was checked by electrophoresis of 8 μL of each PCR product in a 1.5% (w/v) agarose gel, in TBE buffer (0.09 M Tris base, 0.09 M sodium borate, 2.5 mM EDTA, pH 8.3) for 1 h at 4 V/cm. Gels were stained in ethidium bromide for 15 min and thereafter washed for 5 min. DNA fragments were visualized with Gene Genius Bio Imaging System (Syngene, USA). QIAquick Kit (Qiagen, Valencia, CA, USA) was used to purify the DNA. Sequencing was carried out according to the technique of Sanger automated at SBS Genetech Co.. Ltd. (Beijing, China). Sequence identification was initially estimated by using the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). All available subsets of 16S rDNA gene sequences were selected, analyzed, and aligned with ClustalX 1.8, and the final phylogenetic tree was generated by DNAstar 5.

Estimation of Curdlan and Cell Dry Weight. The dry cell weight (DCW) and Curdlan were determined according to a modified method based on the protocol of Lee et al.[21] Samples collected from shake flasks were centrifuged at 10000*g* for 10 min. The pellet consisting of cells and crude Curdlan was washed twice with 0.01 M HCl and harvested by centrifugation. The Curdlan was solubilized by adding 10 mL of 2 M NaOH for 1 h. The cells were separated by centrifugation at 10000*g* for 15 min. The Curdlan present in the supernatant was precipitated by the addition of an appropriate amount of 2.0 M HCl. Both cells and Curdlan were washed three times with distilled water and dried to a constant weight at 80 °C.

Identification of Sugar Components of Polysaccharide. The composition of Curdlan was determined by the analysis of the monosaccharide using HPLC and GC-MS. Curdlan samples were completely hydrolyzed at 100 °C for 1 h following the addition of 3.0 M HCl to 3 mL of sample.[9] The hydrolyzed sample was centrifuged at 10000*g* for 10 min, and the supernatant was filtered. Ten microliters of filtered sample was injected in Agilent analytical HPLC (Agilent 1200, Agilent Technologies, USA). HPLC analysis was performed on a normal phase column (YMC-Pack NH2, 4.6 × 250 nm, YMC, Japan). Permethylation analysis of sugars is a useful technique for establishing the position of glycosidic linkages. To determine the position of glycosidic linkages, per-O-methylation of the Curdlan samples was performed by a sodium hydroxide−methylation protocol.[22,23] The methylation production was acid-hydrolyzed (4 M trifluoroacetic acid), converted to aldononitrile acetates, and analyzed as partial alditol acetates by GC-MS. GC-MS analysis of the derivatized samples employed an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP 7683 autoinjector interfaced with an HP 5973 series mass spectrometer configured in electron impact mode. Chromatography was accomplished with a capillary HP-1 column (2 × 250 nm) using helium as the carrier gas at a flow rate of 0.8 mL/min. The oven temperature was ramped over a linear gradient from 150 to 250 °C at 4 °C/min. Mass spectra were recorded in positive ion mode over the range m/z 50−500. For molecular structure, infrared spectra were obtained on a Paragon 500 Fourier transform-infrared (FT-IR) spectrophotometer (PerkinElmer, Boston, MA, USA) employing potassium bromide (KBr) disks. ^{13}C NMR data were obtained on a JEOL JNM-LA 400 spectrometer (JEOL Co. Ltd., Tokyo, Japan) operating at 100 MHz. The samples of the purified Curdlan were dissolved in dimethyl sulfoxide.[15,16]

Central Composite Design (CCD). The preliminary data showed that several major variables affected the performance of the culture in terms of Curdlan production by *Pseudomonas* sp.; they are the pH of the medium and the concentrations of carbon source (glucose) and nitrogen source (yeast extract). CCD was used to find the optimal concentrations of these three factors and to understand the relationship between the factors and Curdlan production. The carbon source glucose, pH, and the nitrogen source yeast extract were chosen as the independent variables shown in Table 1. Curdlan production (Y, g/L) was used as dependent output variable. A 2^3 full-factorial CCD for three independent variables at five levels was employed, and the total number of experiments was 20 (= $2^k + 2k + 6$), where k is the number of independent variables. Each variable was designated −1.68, −1, 0, 1, and 1.68, respectively. The interrelationships of the variables were determined by fitting the second-degree polynomial equation to data obtained from 20 experiments using mean values of the triplicates of each experiment conducted three times at different occasions. The maximum values of the yield were taken as the responses of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The minimum and maximum range of variables investigated and the full experimental plan with respect to their actual values are listed in Table 2. A multiple regression analysis of the data was carried out with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA), and the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_2, and X_3) was obtained:

Table 1. Process Variables Used Central Composite Design with Actual Factor Levels Corresponding to Coded Factor Levels

		coded level				
variable	symbol	−1.68	−1	0	1	1.68
glucose (g/L)	X_1	70	90	120	150	170
pH	X_2	5.3	6	7	8	8.7
yeast extract (g/L)	X_3	0.32	1	2	3	3.68

Table 2. Central Composite Design and Response Value

run	X_1 glucose	X_2 pH	X_3 yeast extract	Curdlan production (g/L)
1	−1	−1	−1	0.78
2	−1	−1	+1	6.56
3	−1	+1	−1	1.93
4	−1	1	+1	2.68
5	1	1	−1	0.85
6	+1	−1	+1	5.37
7	−1	1	−1	1.58
8	1	1	1	0.82
9	−1.68	0	0	2.71
10	+1.68	0	0	3.53
11	0	−1.68	0	4.12
12	0	+1.68	0	2.98
13	0	0	−1.68	1.96
14	0	0	+1.68	2.69
15	0	0	0	2.89
16	0	0	0	2.26
17	0	0	0	2.57
18	0	0	0	2.67
19	0	0	0	2.73
20	0	0	0	2.45

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X^2{}_1 + b_{22}X^2{}_2 + b_{33}X^2{}_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (1)$$

b_0 is a constant, b_1, b_2, and b_3 are linear coefficients, b_{11}, b_{22}, and b_{33} are squared coefficients, and b_{12}, b_{23}, and b_{13} are interaction coefficients. Combinations of factors (such as X_1X_2) represent an interaction between the individual factors in that term. Then the response is a function of the levels of factors.

RESULTS AND DISCUSSION

Screening and Morphology of the Isolates. Previous papers showed that the occurrence of Curdlan-type polysaccharides in microorganisms could be examined by using the water-soluble dye aniline blue,[9,23] with which Curdlan forms a blue complex. Furthermore, it was also shown that the rate of color complex formation was dependent on the polymer concentration and degree of polymerization (DP). These findings provided an excellent tool for the screening of Curdlan-producing microorganisms.[9,24] In particular, a variety of β-(1,3)-D-glucans from different microbial sources have different physical properties, such as conformation, molecular weight, and the presence or absence of branches, which resulted in different biological activities.[10] It is important to obtain new microbial sources capable of producing Curdlan. On this basis, nearly 30 strains that are capable of producing extracellular Curdlan were isolated to single colonies by using the aniline blue staining technique. To obtain stable strains, the strains were subcultured nearly three times, and eight blue-black strains were selected. Then, the ability of these strains to produce Curdlan was examined by shake flask experiments. The results showed that only one strain (Cur-4) produced Curdlan in appreciable amount. The colonies appeared moist, smooth, white, and round. The isolate Cur-4 was rod-shaped (Figure 1) and Gram-negative.

Molecular Identification. An approximately 1300 bp sized fragment of the 16S rDNA gene sequence of strain Cur-4 was amplified and sequenced. The fragment band was confirmed by electrophoresis after performance of PCR (data not shown). The alignments of the sequences were carried out by the ClustalX software to define the sequence consensus. The obtained sequences were subjected via Internet using the BLAST software for comparison with the homologous sequences contained in the data bank (GenBank). Phylogenetic relationships based on 16S rDNA gene sequences are described in Figure2. The 16S rDNA gene of the strain Cur-4 showed the closest match to *Pseudomonas* sp. with a homology of 98%. Therefore, it could be identified as a *Pseudomonas* sp. However, *Bacillus*, *Agrobacterium*, and *Alcaligenes* species (the major producers of Curdlan) are distant from strain Cur-4. In the past 30 years, different genera of bacteria capable of producing Curdlan were screened by using the aniline blue staining technique. Those strains, except *Alcaligenes* and *Agrobacterium* species, turned blue on agar plates containing aniline blue, and these have been used widely in the production of Curdlan-type polysaccharides. Some strains of *Bacillus* sp. also formed blue complexes with aniline blue.[9,24] In this study, a new strain capable of producing extracellular Curdlan-type polysaccharides from soil samples was screened by the aniline blue staining technique. Moreover, biochemical and 16S rDNA gene sequencing data suggest that the isolate Cur-4 is a *Pseudomonas* sp. For the first time, we report *Pseudomonas* sp. producing extracellular Curdlan-type polysaccharides, whereas most of the studies are on *Agrobacterium*, *Alcaligenes*, and *Bacillus* sp.

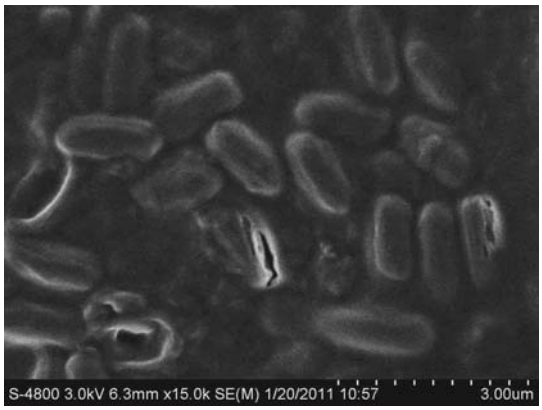

Figure 1. Scanning electron micrograph of the isolated strains (Cur-4).

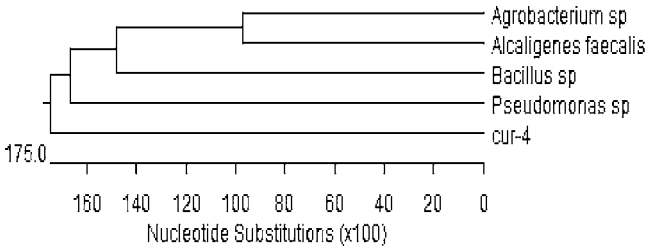

Figure 2. Phylogenetic tree derived from 16S rDNA sequence of Cur-4 strain.

Identification of Curdlan Produced by *Pseudomonas* sp. To determine whether the extracellular polymer produced by the isolate Cur-4 is actually Curdlan (a polymer of glucose), ^{13}C NMR and FT-IR were used to analyze the molecular structure. From the NMR spectrum, correlations with carbon chemical shifts at 103.4, 73.2, 86.4, 68.7, 76.8, and 60.6 ppm were assigned as C-1, C-2, C-3, C-4, C-5, and C-6, respectively, which represent the (1→3)-D-glucan backbone in the polymer chain. Moreover, evidence of other linkages was not seen in the

spectrum (Figure 3). Thus, it can be concluded that the extracellular polymer has a linear (1→3) linkage. In addition,

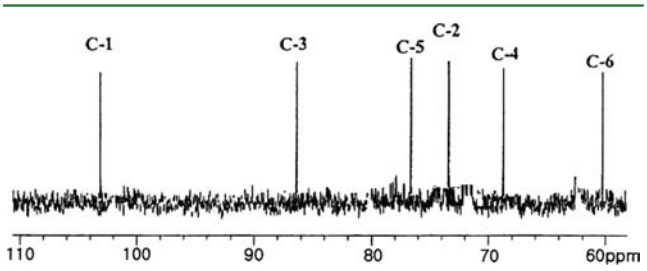

Figure 3. ^{13}CNMR spectrum of the purified Curdlan from Cur-4 strain.

the IR spectrum shows an absorption band at 890 cm^{-1}, indicating that D-glucopyranose has a β-configuration. It was also concluded that no α-configuration exists because there was no characteristic absorption band at 840 cm^{-1} (Figure 4). For

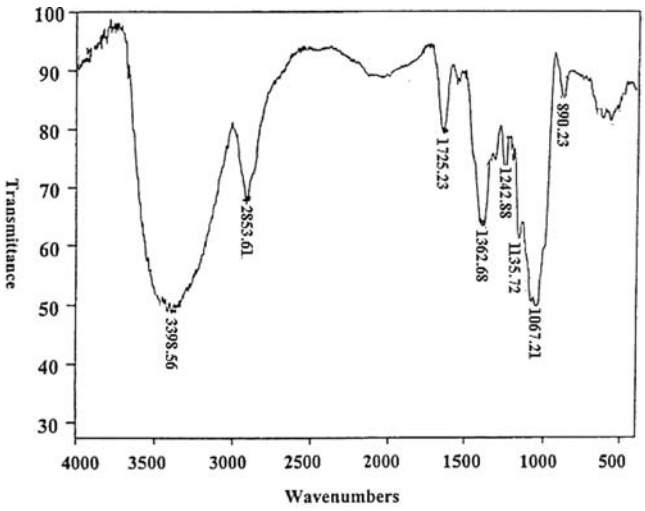

Figure 4. FT-IR spectrum of the purified Curdlan from Cur-4 strain.

the analysis of the monosaccharide, the HPLC profile shows that there were two peaks at 9.39 and 5.81 min. The peak at 9.39 min corresponds to glucose, whereas the other peak at 5.81 min corresponds to HCl (Figure 5). It was also indicated that the purified polysaccharide produced by the isolated Cur-4 strain consisted exclusively of glucose. In addition, to ascertain the positions of the under methylated hydroxyl group, the methylation production were acid-hydrolyzed, converted to aldononitrile acetates, and analyzed by GC-MS. Three peaks were observed for NaOH-catalyzed reaction corresponding to a 2,4,6-tri-Me-glucose peak, a 2,3,6-tri-Me-glucose peak, and a 2,3,4-tri-Me-glucose peak. Table 3 summarizes the methylation analysis of the purified polysaccharide. The results showed that the most prominent sugar was 1,3-linked glucose, followed in relative abundance by 1,4-linked glucose. The high content of 1,3-linked glucose residues suggested that the backbone chain of the polymer produced by *Pseudomonas* sp. may mainly contained the 1,3-linked glucosyl residues. All of the above results also supported that the polymer produced by the isolate was actually Curdlan.

Effect of pH, Carbon Sources, and Nitrogen Sources on Cell Growth and Curdlan Production. The effects of different carbon sources and nitrogen sources on cell growth

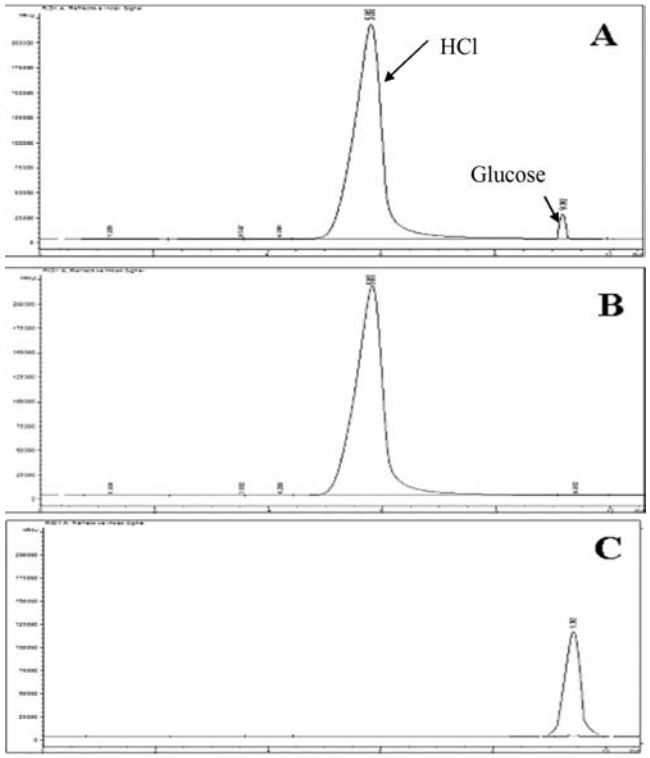

Figure 5. HPLC profile of the purified Curdlan treated with 3 M HCl (A) product of hydrolysis, (B) 3 N HCl, and (C) pure glucose.

Table 3. Methylation Analysis

methyl ether	relative retention time	area % (molar ratio)
2,4,6-tri-Me-glucose	1.02	88.7
2,3,6-tri-Me-glucose	1.06	5.3
2,3,4-tri-Me-glucose	1.11	3.6

and Curdlan production by isolate Cur-4 were studied. Cur-4 strain was cultivated in a medium containing various carbon sources (glucose, fructose, glycerol, sucrose, or lactose) and nitrogen sources (yeast extract, peptone, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, or KNO_3). The initial concentration of sucrose, glucose, fructose, glycerol, and lactose in the fermentation medium was 50 g/L. The results showed that Curdlan production was highest (1.25 g/L) when the cells were grown in the glucose-containing medium. Fructose also yielded a considerable amount of Curdlan production (data not shown). However, the Curdlan production from the other carbon sources was much lower than that from glucose. In contrast, maximum cell concentration was obtained when the cells were grown in the glycerol-containing medium. Previous studies showed that sucrose was the most suitable carbon source for Curdlan production by *Agrobacterium* sp.[12,25] In contrast, in this study, it was noted that glucose was the most suitable carbon source for Curdlan production by *Pseudomonas* sp,. which was beneficial for decreasing cost. In addition, in the study of the effect of glucose concentration on Curdlan production by *Pseudomonas* sp., it was found that the optimal glucose concentration was 120 g/L. In addition, as previously described, Curdlan production is associated with the poststationary phase of nitrogen depletion in strains of *Agrobacterium* and *Alcaligenes* sp.[13] Therefore, it is important to determine the nitrogen source for Curdlan production. The results showed that Curdlan production and cell concentration from organic

nitrogen source are higher than that of inorganic nitrogen source. Furthermore, the maximum production of Curdlan (1.28 g/L) was achieved when the cells were grown in the yeast extract-containing medium. Peptone also obtained a considerable amount of Curdlan production. However, the Curdlan production from the other nitrogen sources was much lower than that from yeast extract. Furthermore, Curdlan production and cell concentration from organic nitrogen source are superior to that of inorganic nitrogen source. In addition, only when yeast extract was used as nitrogen source did the biomass and Curdlan production increase exponentially after 16 h of fermentation. This is in accordance with various papers suggesting that the utilization of a carbon source depends on the type and concentration of nitrogen source used in the medium.[24] Some studies showed that the supplementation of yeast extract to the culture medium was capable of stimulating Curdlan production of *Agrobacterium* sp.[26] In the study of the effect of yeast extract concentration on the biomass and Curdlan production by *Pseudomonas* sp., it was found that the optimal yeast extract concentration was 2.0 g/L; beyond that concentration, the biomass and Curdlan production decreased. In addition, the effect of initial pH on Curdlan production was also studied; experiments were performed with different initial pH values varying between 4.0 and 8.0. It was found that maximum Curdlan production was obtained when the initial pH was 7.0 (Table 4). However, maximum cell growth was

Table 4. Effect of Initial pH on Cell Growth and Curdlan Production

	pH				
	4	5	6	7	8
DCW (g/L)	0.73	1.44	2.11	1.5	1.42
Curdlan production (g/L)	0.33	0.43	0.67	1.26	0.49

observed when the initial pH was 6.0. A previous paper showed that the cell growth in a batch fermentation of *Agrobacterium* species was maximum at pH 7.0, whereas Curdlan production was maximal at pH 5.5.[12] Our result differed from those of Lee et al.[12] The more likely explanation was that different pH culture changed the level of synthesis of the key metabolic enzymes involved in Curdlan biosynthesis and the nucleotide biosynthesis pathway by an unknown mechanism.[27] In addition, the time course profile of cell growth and Curdlan production for *Pseudomonas* sp. showed that the Curdlan production process could be divided into two stages based on the growth specialties of *Pseudomonas* sp., in other words, cell growth process and Curdlan accumulation process. During the cell growth process, *Pseudomonas* sp. grew quickly to obtain high biomass. The Curdlan accumulation process started when cell growth of *Pseudomonas* sp. entered the midexponential phase of cell growth (at around 12 h). Thus, it can be concluded that cell growth and Curdlan accumulation are not directly correlated.

Optimization of Curdlan Production by CCD and RSM. On the basis of the results obtained in preliminary experiments by the "one-factor-at-a-time" technique, the RSM using CCD was applied to determine the optimal levels of the three selected variables (glucose, pH, and yeast extract) that significantly influenced the Curdlan production. The results of CCD experiments studying the effects of three independent variables, namely, glucose, pH, and yeast extract, on the Curdlan production are presented in Table 2. The analysis of variance indicated that the model terms of X_2X_3 and X_3 were highly significant ("probe $>F$" < 0.01), X_2 was significant ("probe $>F$" < 0.05), and the interactive effect of X_2X_3 was highly significant ("probe $>F$" < 0.01). However, the interactive effects of X_1X_2 and X_1X_3 were not significant. This means that pH and yeast extract have important effects on Curdlan production and that the interactive effect of pH and yeast extract may be significant to some extent ("probe > F" < 0.01). Multiple regression analysis of the experimental data gave the second-order polynomial equation

$$Y\ (\mathrm{g/L}) = 2.61 - 0.143X_1 - 0.62X_2 + 0.843X_3 + 0.09X^2{}_1 - 0.136X_1X_2 - 0.346X_1X_3 + 0.242X^2{}_2 - 1.288X_2X_3 - 0.191X^2{}_3 \quad (2)$$

where Y is the response, that is, the Curdlan production, and X_1, X_2, and X_3 are the coded values of the test variables glucose, pH, and yeast extract, respectively. After the neglect of insignificant terms (on the basis of "probe > F" > 0.05), the model eq 2 was modified to reduced fitted model eq 3:

$$Y\ (\mathrm{g/L}) = 2.61 - 0.62X_2 + 0.843X_3 - 1.288X_2X_3 \quad (3)$$

The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.82. The model can explain 82% variation in the response. The model F value of 5.035 implied that the model was significant, and the P value was also very low (P < 0.01), indicating the significance of the model. From the statistical results obtained, it was shown that the above models were adequate to predict the Curdlan production within the range of variables studied. The 2D contour plots are generally the graphical representations of the regression equation, and 2D contour plots are presented in Figure 6. Each contour curve

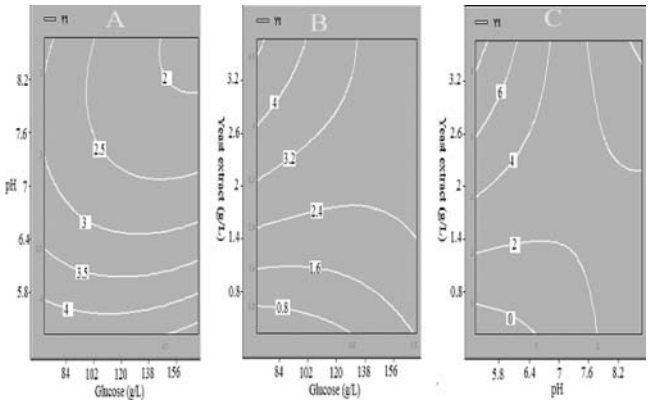

Figure 6. Contour plots: (A) effect of glucose (X_1) and pH (X_2); (B) effect of glucose (X_1) and yeast extract (X_3); and (C) effect of pH (X_2) and yeast extract (X_3) on Curdlan production.

plots represents an infinite number of combinations of two test variables with the other two maintained at their respective zero level. From the contour plots, it is easy and convenient to understand the interactions between two factors and also locate their optimum levels. The circular contour plots of response surfaces suggest that the interaction is negligible between the corresponding variables. An elliptical or saddle nature of the contour plots indicates the significance of the interactions between the corresponding variables. The contour plots in Figure 6C show that there was highly significant mutual interaction between pH and yeast extract. However, there was

almost no interaction between glucose and pH (Figure 6A) or between glucose and yeast extract (Figure 6B), as was evident from the relatively circular nature of the contour curves. The optimal conditions were extracted by Design Expert software with its optimization menus: $X_1 = -0.57$, $X_2 = -0.65$, $X_3 = -0.48$. The real values were glucose concentration at 103 g/L, pH 7.6, and yeast extract concentration at 1.52 g/L. The predicted maximum Curdlan production obtained by using the above optimized conditions of the variables is 2.21 g/L. The maximum Curdlan production obtained experimentally was found to be 2.35 g/L. This is obviously in close agreement with the model prediction. Under optimized condition, the Curdlan production was above 1.88-fold in comparison with that of original condition; the Curdlan production rate of 0.81 g/(L h) was obtained. In previous papers, some researchers have investigated Curdlan production rate during bacterial fermentation for Curdlan production. A high production rate (0.2 g/(L h)) was obtained from the batch culture of *Agrobacterium* sp. ATCC31750.[11] Furthermore, the Curdlan production rate was further increased to 0.5 g/(L h) under optimal pH by *Agrobacterium* sp. ATCC31750.[12] In the present study, a Curdlan production rate of 0.81 g/(L h) was obtained when *Pseudomonas* sp. was cultivated in the optimal conditions developed by statistical experimental method. Thus, in comparison with those by other bacteria species, Curdlan production by *Pseudomonas* sp. is more efficient. To the best of our knowledge, this was the highest Curdlan production rate by *Pseudomonas* sp. Further studies will be focused on the characterization of the detailed structure, function, and possible commercial benefit of this polysaccharide.

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Notes

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