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Cultural Condition for Biopolymer Production by *Pseudomonas delafieldii*

Yoo, Jin-Young* and Dong-Hyo Chung[1]

Microbiology Laboratory, Korea Food Research Institute, Kyonggido, Korea
[1]Department of Food Science and Technology, Chung Ang University, Seoul 156-756, Korea

Pseudomonas delafieldii 에 의한 Biopolymer 생산조건

유진영* · 정동효[1]

한국식품개발연구원 미생물연구실 [1]중앙대학교 식품가공학과

The cultural condition for polysaccharide production by *Pseudomonas delafieldii* was studied. The optimal medium contains the following composition per liter of distilled water: glucose (25 g/*l*), peptone (2.06 g/*l*), KH_2PO_4 (2 g/*l*), $MgSO_4 \cdot 7H_2O$ (2 g/*l*), yeast extract (0.5 g/*l*), $CaCO_3$ (2.5 g/*l*). The temperature and pH optimum were 30°C and 6.5. The agitation speed was 300 rpm. 5.91g of polysaccharide was produced at the condition in flask culture.

Hydrophillic colloid are very important in various industries as rheological additives and their application is well established (1, 2). The main source of these gums are from plant and seaweed (3). However, the increase of demand (4, 5) and the success of commercial production of dextran (6) and xanthan gum (7-9) highlighted the need of search for microbial polysaccharides. It has been known that *Pseudomonas* sp. produced extracellular polysaccharide in copious quantity (10, 11) and some of them are recently commercialized (1). In screening procedure for bacterium synthesizing biopolymer, we isolated a soil bacterium with potentiality and reported on the characteristics of the isolate in the previous paper (12).

In this paper, cultural condition for polysaccharide production will be discussed.

Materials and Methods

Microorganism and starter culture

Ps. delafieldii BT-4 was used in this study. Starter culture was prepared in YM broth (Difco) by incubating overnight at 30°C. The inoculum size was 5%.

Medium and cultivation

The basal medium contains glucose (25 g/*l*), peptone (2.06 g/*l*), KH_2PO_4 (1 g/*l*), $MgSO_4 \cdot 7H_2O$ (1.0 g/*l*), yeast extract (0.5 g/*l*) and $CaCO_3$ (2.5 g/*l*). Cultivation was performed in 250 m*l* Erlenmeyer flask (working volume: 50 m*l*, 120 stroke/min.) or in jar fermentor (working volume: 350 m*l* or 1500 m*l*, New Brunswick Sci. Co. U.S.A.) for 3 days.

Analysis

Analytical method was as described previously (12, 13). Viscosity was measured with Brabender viscotron (Mode 80241, measuring system: E-17).

Results and Discussion

Cultivation temperature

Temperature regulation has been reported to be a critical parameter in microbial polysaccharide production. Therefore, flask culture of microorganism was undertaken to find the optimum temperature for

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*Corresponding author

Table 1. Effect of temperature on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation in flask culture.

Temperature (°C)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)	pH
25	3.40	3.41	13.64	13.93	139.5	5.5
30	2.16	5.79	23.16	37.23	184.1	6.3
35	1.38	1.90	7.60	19.12	27.9	6.1
40	–	–	–	–	–	6.5

Viscosity was measured at the shear rate of 70 sec^{-1}, Medium: glucose 25g, peptone 2.06g, KH_2PO_4 1g, $MgSO_4 \cdot 7H_2O$ 1g, yeast extract 0.5g, $CaCO_3$ 2.5 g/l, initial pH 6.5, P: crude polysaccharide, Y: conversion yield, Q: overall polysaccharide productivity, η_{app}: culture vciscosity, DCW: dry cell weight

Table 2. Effect of sugar sources on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation in flask culture at 30°C.

Sugar source	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)	pH
Glucose	1.81	5.79	23.16	44.43	212.0	6.3
Lactose	0.23	–	–	–	–	–
Maltose	0.24	–	–	–	–	–
Sucrose	0.25	–	–	–	–	–
Fructose	1.80	2.36	9.44	18.21	290.2	6.9
Xylose	1.35	1.86	7.44	19.14	206.5	7.2

Sugar source: glucose, fructose, xylose 25g, lactose maltose, sucrose 23.75 g/l

polysaccharide production by *Ps. delafieldii* BT-4 in flask culture (Table 1).

Ps. delafieldii grew well at lower temperature. It did not grow at the temperature higher than 40°C. 3.40 g/l of cell was accumulated at 25°C. The temperature optimum for cell growth and polysaccharide production was not same. The temperature optimum for polysaccharide production was 30°C. At the temperature, 5.79 g/l of polysaccharide was produced. The conversion yield and overall polysaccharide productivity at the optimum temperature were 23.16% and 37.23 mg/g-cell/h. When grown at low temperature, the culture broth was very viscous. The viscosities of culture broths grown at 25 and 30°C were 139.5 and 184.1 mPa · s at 70 sec^{-1}, respectively.

It was commonly reported that the optimum temperature for cell growth was that for microbial polysaccharide production (14, 15) and most of polysaccharide producing microorganisms of commercial interests were mesophiles whose temperature optimum was 28-32°C (11, 16-18). However, *Pseudomonas* sp. was reported to have temperature optimum at 25°C (19). Reduced temeperature for cultivation could increase productivity of some enteric bacteria, whereas *Ps. aeruginosa* formed much more slime at 37°C (11).

Effect of carbon sources

Sugar sources are known to affect the formation and quality of polysaccharide for some microorganisms (14, 20).

The effect of carbon sources on the polysaccharide production by *Ps. delafieldii* BT-4 is shown in Table 2. *Ps. delafieldii* BT-4 had a very limited substrate specificity. The growth of *Ps. delafieldii* BT-4 in the medium containing lactose, maltose and sucrose was negligible. The microorganism grew well and produced polysaccharide only in the medium containing glucose, fructose and xylose. The dry cell weights (DCW) of the respective culture broth were 1.81, 1.80 and 1.35 g/l. Optimum carbon source for polysaccharide production by *Ps. delafieldii* BT-4 was found to be glucose. The conversion yield and overall polysaccharide productivity in the medium were 23.16% and 44.43 mg/g-cell/h. The culture viscosities from respective utilizable carbon source were 212.0, 290.2 and 206.5 mPa·s. However, the polysaccharide productions in fructose and xylose media were very small. The fact that the production was not consistent with the viscosity might be due to the possible change in the properties of the polysaccharide produced depending upon the carbohydrate used (14, 21).

Williams and Wimpenny (22) reported that higher yield could be obtained when using hexose rather than pentose for polysaccharide production. Commonly, glucose has been reported to be good substrate for polysaccharide production (22, 23) Sucrose was also known to be an efficient carbon source for the polysaccharide production by *Alcaligenes faecalis* (15), *Aeromonas hydrophila* (24), and *Xanthomonas campestris* (25). Sutherland (26) found that substitution of carbon sources could alter the level of functional groups such as acetyl or pyruate in xanthan gum without alteration of struc-

Table 3. Effect of glucose concentration on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation in flask culture at 30°C.

Concentration (g/l)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)
10	1.48	1.97	19.70	18.49	159.6
15	1.42	3.42	22.80	33.45	170.8
20	1.41	3.80	19.00	37.43	168.0
25	1.37	5.49	21.96	55.66	165.7
30	1.35	4.19	13.97	43.11	154.0
40	1.33	4.17	10.43	43.55	178.0

Table 4. Effect of nitrogen source on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in jar fermentor.

Nitrogen source	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)	pH
NH_4Cl	2.77	3.06	12.24	15.34	73.7	6.9
NH_4NO_3	1.65	4.11	16.44	34.60	205.3	7.0
$(NH_4)_2SO_4$	2.69	2.95	11.80	15.23	110.5	6.7
$(NH_4)_2HPO_4$	3.65	1.89	7.56	7.19	217.1	6.4
$(NH_2)_2CO$	1.06	4.35	17.40	57.00	331.5	6.8
Peptone	1.87	5.27	21.08	39.14	625.0	6.3

Working volume: 350 ml, aeration: 1 vvm, agitation: 300 rpm

tural backbone. However, the sugar component of the polysaccharide produced by *Bacillus* sp. was affected (14, 23). Genetic approach was also used to change substrate specificity of bacteria for utilizing industrial waste such as acid whey from dairy plant and thus cutting down the expense for polysaccharide production (27).

Effect of glucose concentration

The carbohydrate concentration was reported as a factor affecting the conversion efficiency of carbohydrate into polysaccharide (14, 20). Table 3 shows the effect of glucose concentration on the polysaccharide production by *Ps. delafieldii* BT-4. The cell growth was not much affected by glucose concentration. The polysaccharide production, however, increased as more glucose was added. The optimum glucose concentration was 25 g/l. The fact, that conversion efficiency is low when using higher glucose concentration, is similar to the result reported by Bender *et al.* (28). This kind of optimum concentration of carbohydrate for polysaccharide production is dependent upon the microorganisms used for polysaccharide production. Higher xanthan production was achieved when using 40 g/l of sucrose or glucose (25). Miura *et. al* (29) found the sucrose concentration to affect the molecular weight of the thus obtained pullulan.

Effect of nitrogen sources and concentration

Nitrogen source is very important for microbial growth and synthesis of enzyme for polysaccharide production (14). Therefore organic and inorganic nitrogen sources were tested for selecting optimum nitrogen source. Cultures were grown in 350 ml jar fermentor (Table 4). Ammonium chloride, ammonium sulfate and ammonium phosphate dibasic were good sources for the growth of *Ps. delafieldii* BT-4. Optimum nitrogen source for polysaccharide production by *Ps. delafieldii* BT-4 was peptone. The conversion yield and overall polysaccharide productivity were 21.08% and 39.14 mg/g-cell/h in the medium.. The culture viscosity was 625 mPa·s at 70 sec^{-1}. The recognizable change in pH did not occur.

Frequently, peptone (21, 30) and yeast extract (15, 31) were recommended as nitrogen source.

Although nitrogen source is necessary for

Table 5. Effect of C/N ratio on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in flask culture.

C/N ratio	5	10	20	30	50	100	200	300
P	2.63	2.21	2.89	5.34	4.40	4.52	4.59	3.45
Y	10.52	8.84	11.56	21.36	17.60	18.08	18.36	13.80
η_{app}	50.2	72.5	111.2	111.6	111.6	128.3	150.7	139.5
pH	7.6	6.4	6.2	6.9	7.1	7.0	7.1	7.1

Table 6. Effect of KH_2PO_4 concentration on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in flask culture.

Concentration (g/l)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)
1	1.50	5.04	21.01	46.67	182.5
2	1.74	6.17	24.68	49.25	202.0
3	1.69	3.34	13.36	27.45	137.3
4	1.58	3.72	14.88	32.70	188.6
5	1.59	3.75	15.00	32.76	176.3

Table 7. Effect of $MgSO_4$ concentration on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in flask culture.

Concentration (g/l)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)	pH
1	1.47	5.28	21.13	49.91	187.5	6.8
2	1.61	5.91	23.64	50.98	168.0	6.1
3	1.42	5.71	22.84	55.85	152.9	5.9
4	1.92	5.66	22.64	40.88	158.5	4.4
5	1.95	4.48	17.92	31.91	168.0	4.8

KH_2PO_4: 2 g/l

polysaccharide production, an excess of nitrogen source is known to reduce the conversion of carbohydrate substrate to exocellular polysaccharide (14, 32). Table 5 shows the effect of C/N ratio on the polysaccharide production in flask culture. The optimum C/N ratio was found to be 30. The viscosity was high when C/N ratio was higher than 20. This kind of effect was also reported by many workers (16, 19, 21) and recommended 10-40 of C/N ratio for polysaccharide production. It was reported that some bacteria such as *Pseudomonas* sp. (11, 33) and *Aerobacter* sp. (34) synthesized polysaccharide when the growth was limited by nitrogen sources, whereas *Xanthomonas* sp. (35) and *Azotobacter* sp. (33) produced also in carbon-limited culture as well as nitrogen-limited culture.

KH_2PO_4 concentration

Potassium and phosphorus are very important for microorganisms since they are involved in RNA, cell wall structure and function (36). Therefore, their concentration might be also related with polysaccharide synthesis.

Table 6 shows the effect of KH_2PO_4 concentration on the polysaccharide production from glucose by *Ps. delafieldii* BT-4 in flask culture. The cell growth was not much affected by KH_2PO_4 concentration. The biomass concentration was high at 2 g/l of potassium dihydrogen phosphate. The optimum concentration for polysaccharide production was 2 g/l. At the concentration, the conversion yield and overall polysaccharide productivity were 24.68% and 49.25 mg/g-cell/h. The culture viscosity was 202 mPa.s at 70 sec^{-1}. Alginate (37), shizophyllan (38) and xanthan (20) productions were reported to be affected by initial concentration of inorganic phosphate and phosphate-limited culture promoted polysaccharide production by *Azotobacter vinelandii* (11, 37). In contrast, Williams and Wimpenny (11) reported that phosphate-limitation negatively affected the polysaccharide production by *Pseudomonas* sp.

$MgSO_4$ concentration

Since Mg^{+2} plays an important role in controlling enzyme activity in extracellular synthesis and sulphur deficiency can help permeability of enzymes or reduce sulfur-containing essential components of the cell (coenzyme A, TPP, lipoic acid), the concentration of $MgSO_4$ was expected to affect the biosynthesis of polysaccharide (14, 38, 39).

Table 7 shows the effect of $MgSO_4$ concentration on the polysaccharide production from glucose by *Ps. delafieldii* BT-4. The cell growth was enhanced by increasing $MgSO_4$ concentration. However, polysaccharide production was sharply reduced when more $MgSO_4$ than 4 g/l was added. The optimum concentration was 2 g/l. At the condition, 23.64% of glucose added was converted to polysaccharide and overall polysaccharide productivity was 50.98 mg/g-cell/h. The pH of final culture broth was drastically low when more than 2 g/l of $MgSO_4$ was used. It was presumed that utilization of Mg^{+2} resulted in accumulation of SO_4^{-2} in culture broth, and thus caused the low pH which was detrimental to the synthesis of metabolite during fermentation (39, 40).

Effect of yeast extract concentration

Table 8 shows the effect of yeast extract concentration on the polysaccharide production from glucose by *Ps. delafieldii* BT-4. The cell growth was

Table 8. Effect of yeast extract concentration on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in flask culture.

Concentration (g/l)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)
0	1.41	1.54	6.16	15.18
0.1	1.61	2.70	10.80	23.28
0.3	1.92	4.28	17.13	30.98
0.5	2.23	5.28	21.13	32.90
0.7	2.01	2.44	9.75	16.86

KH_2PO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 1 g/l

Table 9. Time course of polysaccharide production by *Ps. delafieldii* BT-4 at 30°C in jar fermentor.

Elapsed fermentation time (hrs)	P (g/l)	Residual glucose (g/l)	K (mPa·s)	n
12	–	21.5	20	0.82
18	–	13.2	141	0.58
46	2.70	9.6	586	0.57
63	3.42	4.9	1943	0.42
135	4.01	–	4690	0.31

Working volume: 1500 ml, agitation: 200 rpm, aeration: 1 vvm, K: consistency coefficient (viscosity at the shear rate of 1 sec^{-1}), n: flow behaviour index, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$: 1 g/l

stimulated as the yeast extract concentration increased. More than 0.5 g/l was, however, not effective any more for promoting the cell growth. The polysaccharide production was affected by yeast extract. The amount of polysaccharide formed in the medium without yeast extract was 1.54 g/l. The optimum concentration was 0.5 g/l. Kikumoto *et al.* (38) and Iwamuro *et al.* (16) reported that yeast extract could enhance the polysaccharide production by *Shizophillum commune* and *Porodisculus pendulus*. However, they found that the addition of more than 4 g/l of yeast extract did not promote the polysaccharide production.

Fermentation profile of polysaccharide in jar fermentor

Fermentation pattern of polysaccharide production by *Ps. delafieldii* BT-4 in glucose-peptone medium was investigated (Table 9).

Glucose was completely consumed after 63 hours and polysaccharide production after 135 hours of fermentation was 4.01 g/l. The viscosity data were well fitted to Power law and fermentation broth behaved like pseudoplastic fluid. The consistency index (K-value) continuously increased to be 4,690 mPa.s after 135 hours. The flow behaviour index of culture broth after 12 hours was 0.82 and drastically dropped. The culture broth showed extremely high pseudoplasticity at the end of fermentation (n = 0.31).

Table 10. Effect of impeller tip speed on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation.

Tip speed (RPM)	(m/min)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)
50	7.85	0.98	1.33	5.32	18.85	46.9
100	15.70	1.37	3.69	14.76	37.41	178.6
150	23.55	1.86	3.93	15.72	29.35	195.3
200	31.40	1.89	3.98	15.92	29.25	251.1
300	47.10	1.87	5.27	21.08	39.14	625.0
400	62.80	1.65	3.98	15.92	33.50	535.7
500	78.50	1.92	4.45	17.80	32.19	491.0
600	94.20	1.54	4.07	16.28	36.71	518.9

Working volume: 350 ml, aeration: 1 vvm, KH_2PO_4: 1 g/l, $MgSO_4 \cdot 7H_2O$: 1 g/l

Effect of impeller speed and aeration rate

Fermentation process for the microbial polysaccharide production needs an adequate mixing and aeration to give the better oxygen transfer in the culture, since the nature of the culture broth is typified by highly viscous non-newtonian properties (32, 35, 39, 42). When growing *Ps. delafieldii* BT-4 in jar fermentor, it was noticed that the oxygen level fell off to zero from saturation level within 24 hours which might be due to the limitation of oxygen transfer caused by slime layer around the cell and hence, could restrict the oxygen availability for growth and polysaccharide production (35, 39, 43). To determine the proper agitation speed at the 1 vvm of aeration rate, culture was grown in small reactor with two disc turbine impeller by varying agitation speed (Table 10). The cell growth was positively affected by the increase of agitation speed. The biomass concentration of culture broth grown at 150 rpm was twice as high as that of 50 rpm. When the speed rose up over 300 rpm, the cell growth was rather reduced but not significant. The polysaccharide production was also enhanced by the increase of impeller

Table 11. Effect of aeration rate on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in jar fermentor.

Rate (vvm)	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)
1	2.37	5.54	22.16	32.47	475.0
2	2.46	2.99	11.96	16.88	462.0
3	2.64	2.52	10.08	13.26	420.0
4	2.80	2.06	8.24	10.22	428.0

Agitation: 300 rpm, working volume: 350 ml, KH_2PO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 1 g/l

speed. The amount of polysaccharide synthesized at 300 rpm was 4 times of that obtained at 50 rpm and somewhat reduced at the speed higher than 300 rpm which was optimum. The conversion yield and overall polysaccharide productivity at the optimum speed were 21.08% and 39.14 mg/g-cell/h. The viscosity of final culture broth was also higher when the fermentation was performed at the higher agitation speed and maintained at the high level when using the speed over 300 rpm.

Table 11 shows the effect of aeration rate the agitation of 300 rpm on the polysaccharide production. The cell growth was positively affected by increasing aeration rate. When increasing aeration rate to 4 vvm, 18% increase in biomass was observed. In contrast to this, polysaccharide production was reduced by increasing the rate. When the rate increased to 2 vvm, 46% reduction was recognized. This fact indicated that the important factor is not agitation speed and aeration rate itself but adequate combination and design of vessel, aeration and agitation system (35, 39). However, it was impossible in this experiment to maintain a certain level of D.O. concentration throughout the fermentation because of extremely thick property of broth at the later stage of fermentation. Therefore, it was concluded that a special fermentor system be designed.

Effect of pH

pH control is very important factor affecting the polysaccharide production especially when it is anionic polysaccharide. Lack of pH control or poor buffering of the culture media can cause reduction of polysaccharide and cessation of microbial growth (35, 39, 44).

In order to find optimum pH for polysaccharide production by *Ps. delafieldii* BT-4, pH of culture medium was controlled with pH controller during fermentation in reactor (Table 12). The growth was negligible at the pH below 5.5 and above 7.5. Polysaccharide was not detected at pH 4.5. The optimum pH for the polysaccharide production was 6.5-7.0. The overall polysaccharide productivity and culture viscosity was maintained at relatively high level around the pH optimum. This kind of pH optimum was reported by many workers for various microorganisms. Normally pH optimum for microbial polysaccharide production was near neutrality (30, 43, 45). However, the pH optimum for the polysaccharide production by *Rhodotorula glutinis* was found to be 2.8, very low value comparing with other microorganisms.

Table 12. Effect of pH on the polysaccharide production by *Ps. delafieldii* BT-4 after 72 hours of incubation at 30°C in jar fermentor.

pH	DCW (g/l)	P (g/l)	Y (%)	Q (mg/g/h)	η_{app} (mPa·s)
4.5	0.52	–	–	–	–
5.5	0.65	1.97	7.88	42.09	87.1
6.0	1.52	2.78	11.12	26.81	217.1
6.5	2.21	4.84	19.36	30.42	238.3
7.0	1.78	4.21	16.84	32.85	150.7
7.5	0.21	2.52	10.09	15.56	33.5

KH_2PO_4: 2 g/l, $MgSO_4 \cdot 7H_2O$: 1 g/l

요 약

*Pseudomonas delafieldii*의 다당류 생산을 위한 배양조건을 검토하였다. 최적 배지조성은 glucose 25g/l, peptone 2.06 g/l, yeast extract 0.5g/l, KH_2PO_4 2g/l, $MgSO_4$ 2g/l, $CaCO_3$ 2.5g/l이며 교반속도는 300 rpm, 온도는 30도, pH는 6.5이었다. 이 조건에서는 flask culture를 할 경우 5.91g/l가 생산되며 이 때 수율은 23.65%, overall productivity는 50.98 mg/g/h이었다.

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