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Determination of Medium Components in the Flocculating Activity and Production of Pestan Produced by *Pestalotiopsis* sp. by Using the Plackett-Burman Design

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Abstract Optimization for the production of Pestan was followed by the Plackett-Burman Design, using modified Czapek-dox medium as the starting point. At the flask level, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and aeration variables positively affected the Pestan production, DCW (dry cell weight), apparent viscosity, and flocculating activity response. KCl and $FeSO_4 \cdot 7H_2O$ negatively affected the Pestan production, DCW, apparent viscosity, and flocculating activity response. Aeration variable was shown to have a positive effect on only the flocculating activity response among Pestan production, DCW, and apparent viscosity responses. In comparison of the positive and negative variables media conditions, Pestan production and flocculating activity differed by about 9 and 125 times, respectively. In particular, at the jar fermentor level, the aeration variable was the most important factor of the all responses (Pestan production, DCW, apparent viscosity, flocculating activity, and anionic charge density). The flocculating activity and apparent viscosity of Pestan were closely related to the molecular chain length and charge density.

Key words: Bioflocculant, Plackett-Burman design, exopolysaccharide

Considerable work has been devoted recently to the search for microbial exopolysaccharides (EPS) with interesting characteristics. EPS produced by a few bacterial strains have already become commercially available [8].

In particular, xanthan gum is an EPS produced by bacteria of the genus *Xanthomonas* and is the most commonly used for production and application of EPS [1]. To produce EPS, microorganisms generally need sources of carbon, nitrogen, and other nutrients, including potassium, phosphorus, magnesium, iron, and calcium salts. Nutritional studies on EPS production were first carried out as experiments of continuous culture by Davidson [2] and batch culture by Souw [10]. Each was based on optimizing culture conditions by the classical method of investigating one factor at a time.

Recently, a nutritional study of *X. campestris* in the production of xanthan gum was carried out by factorial design and analysis. Garica-Ochoa *et al.* [3] found that the influence of nitrogen, phosphorus, and magnesium on the DCW, and the influence of nitrogen, phosphorus, and sulfur on xanthan gum production, were significant, although any variable influenced sucrose consumption. However, less nutritional studies of fungal EPS have been done than those of bacterial EPS.

In our previous papers [5, 6, 9], we reported that Pestan (fungal EPS) had higher flocculating activity than that of chemically synthesized flocculants (polyacrylamide) and better rheological properties (high apparent viscosity at the low concentration, pH and temperature stability, and high salt compatibility, etc) than those of xanthan gum. In this paper, we report findings on significant parameters of Pestan production increment, by carrying out experiments on the cultivation of *Pestalotiopsis* sp. in chemically defined medium using the Plackett-Burman experimental design.

MATERIALS AND METHODS

Medium, Inoculation, and Cultivation

The modified medium was already determined by the classical method (one factor at a time) based on Czapek-

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dox medium. The modified Czapek-dox medium used in the study as the basal medium consisted of sucrose 5%, KNO_3 0.0714%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.025%, KCl 0.075%, and $FeSO_4 \cdot 7H_2O$ 0.001%. The strain was cultured at 25°C and pH 7.0. The inoculum size was 2% mycelium suspension that contained NaCl 0.85%. The cultivation was performed at 25°C for 5 days with rotary shaking at 150 rpm in a 250-ml Erlenmeyer flask containing 50 and 100 ml of medium. All the chemicals were purchased from Showa and Junsei Chemical Co, Japan.

For the measurement of charge density and molecular weight distribution of Pestan, the strain was cultivated in a 5-l jar fermentol (KF-5, KFC, Korea) which contained 3 l medium and the culture broth was sampled at various culture times. All culture broth was used as supernatant after removal of cell or mycelium by centrifugation.

Measurement of Several Responses (Pestan Production, DCW, Apparent Viscosity, and Flocculating Activity)

The measurement of Pestan production, DCW, apparent viscosity, and flocculating activity were described in our previous papers [5, 6, 9].

Measurement of Charge Density of Pestan

The anionic charge titration solution used was potassium poly(vinyl) sulfate (PVSK) solution and the cationic charge titration solution used was poly(dially) dimethylammonium chloride (PDAC) solution. The indicator used was toludine blue with cationic charge. The measurement of charge density [7] was carried out using the following procedure performed in duplicate.

The cationic charge density measurement. Five ml supernatant was added to 45 ml dH_2O in a 100-ml Erlenmeyer flask and mixed at 300-400 rpm for 5 mins by a multi-stirrer. After addition of 300 µl of 0.1% toludine blue, the polymer solution was titrated with 0.0025 N PVSK solution. Addition of PVSK was stopped when the color changed from blue to red.

The anionic charge density measurement. Two ml supernatant was added to 48 ml dH_2O in a 100-ml Erlenmeyer flask and mixed at 300-400 rpm for 5 mins. Five ml of 0.0025 N PDAC solution was added and mixed at 300-400 rpm for 5 min by using a multi-stirrer. The other procedure was the same as described above.

The calculation of charge density. We calculated the charge density based on the following formula:

$$\text{Charge density (meq/g)} = 2.5 \times A \times (X - Y)/1{,}000 \times B \times C$$

A: Volume of Pestan
X: Volume of PVSK solution used for titration
B: Weight of Pestan
Y: Volume of PVSK solution used for basic experiment
C: Volume of Pestan solution

Measurement of Molecular Weight Distribution of Pestan

The molecular weight distribution of Pestan was measured by HPLC (Class-LC10, Shimadzu Co., Japan) with Ultrahydrogel™ linear 6-13 µm chromatography (7.8×300 mm GPC column, Waters Co., Japan) and RID-6A detector (Shimadzu Co., Japan). The mobile phase, flow rate, and temperature were 0.1 M $NaNO_3$, 0.5 ml/min, and 75°C, respectively.

Experimental Design

The Plackett-Burman design is a fraction of the two-factorial design and allows the investigation of up to N-1 variables in N experiments. Table 1 shows selected experimental variables and levels, and Table 2 is a design for conducting 12 experimental trials. In Table 2, each row represents a trial (number of fermentation medium) and each column represents an independent (medium components and culture condition) or dummy variable. At least three dummy variables were used to estimate the experimental error. The elements, + (high level) and - (low level), represent the two different levels of the independent variable under investigation.

Table 1. Selected experimental variables and levels.

Variables	High level	Low level
(A) Sucrose	56%	2%
(B) KNO_3	0.0714%	0.029%
(C) Dummy		
(D) K_2HPO_4	0.2%	0
(E) $MgSO_4 \cdot 7H_2O$	0.025%	0
(F) Dummy		
(G) KCl	0.075%	0
(H) $FeSO_4 \cdot 7H_2O$	0.001%	0
(I) Aeration	50 ml	100 ml
(J) Dummy		

Table 2. Matrix of Plackett-Burman design for 12 trials.

Trial	Variables									
	A	B	C	D	E	F	G	H	I	J
1	+	+	-	+	+	+	-	-	+	-
2	+	-	+	+	+	-	-	-	-	+
3	-	+	+	+	-	-	-	+	+	+
4	+	+	+	-	-	-	+	-	+	-
5	+	+	-	-	-	+	-	+	-	+
6	+	-	-	-	+	-	+	+	+	+
7	-	-	-	+	-	+	+	-	+	+
8	-	-	+	-	+	+	-	+	+	-
9	-	+	-	+	+	-	+	+	-	-
10	+	-	+	+	-	+	+	+	-	-
11	-	+	+	-	+	+	+	-	-	+
12	-	-	-	-	-	-	-	-	-	-

: Dummy variables; +, High level; -, Low level.

Statistical Analysis

The response of Pestan production, DCW, apparent viscosity, and flocculating activity were measured in each trial. The most important variables affecting these responses were identified according to the statistical analysis of Greasham and Inamine [4].

RESULTS AND DISCUSSIONS

The Plackett-Burman protocol was used for rapid identification of the most important variables affecting the Pestan production, DCW, apparent viscosity, and flocculating activity responses. Twelve experimental trials were carried out with three dummies at once.

The results of these dual experiments are summarized in Table 3. Each response of Pestan production, DCW, apparent viscosity, and flocculating activity showed at least 80% of significance at individual responses by statistical analysis. Only responses over 80% of significance were present to simplify the components of modified Czapek-dox medium which were already determined by using a classical method (one factor at a time, OFAT). The t-values of the three dummy variables in Table 3 showed some deviation from zero because experimental error was unavoidably generated by measurement of several responses.

In Pestan production response, the positive variables were sucrose, KNO_3, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$, and the K_2HPO_4 variable showed the major positive variable. The negative variables were KCl and $FeSO_4 \cdot 7H_2O$.

In DCW response, the positive variables were sucrose, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$ and the major positive variable among those variables was K_2HPO_4 as in the Pestan production response. $FeSO_4 \cdot 7H_2O$ exhibited a negative effect. It is already known that sucrose, KNO_3, and $MgSO_4 \cdot 7H_2O$ showed the more positive effect on Pestan production response than on DCW response, that KCl showed the more negative effect on Pestan production response than on DCW response, and that the $FeSO_4 \cdot 7H_2O$ variable differed from the effect of KCl variable showing a more negative effect on DCW response. These results were in accord with the general facts that the carbon source mainly functioned to produce polysaccharide, and phosphate and magnesium were required in metabolism of the organism or/and functioning as cofactors of polysaccharide biosynthetic enzymes. We could deduce that phosphate and magnesium functioned as the main elements of metabolic activity of *Pestalotiopsis* sp. and Pestan production. In particular, magnesium functioned more in Pestan biosynthesis than in the cell growth. This suggested that magnesium was a necessary element for Pestan production and as a cofactor of Pestan synthetic enzymes. KCl and $FeSO_4 \cdot 7H_2O$ variables showed the greatest negative effect on Pestan production and cell growth of *Pestalotiopsis* sp., which differed from the fact that one of the factors giving good succinoglucan production and cell growth of *Alcaligenes faecalis* var. *myxogenes* [11] may be ferric ion.

In the apparent viscosity response, sucrose, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$ showed a positive effect and K_2HPO_4 was the major positive variable. Because the apparent viscosity of culture broth depended on the production amount of Pestan, and Pestan production depended on the cell growth, the positive variables of apparent viscosity were similar to the positive variables for Pestan production and DCW responses. Therefore, the K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ variables showed commonly as positive effects on the Pestan production, DCW, and apparent viscosity responses.

The positive variables on the flocculating activity response were sucrose, KNO_3, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and aeration. The aeration variable showed a positive effect only for the flocculating activity response, and the other variables of flocculating activity response were similar to those of the Pestan production, DCW, and

Table 3. Cultural variables and their effects on the several responses at three dummies.

Variables	Responses							
	Pestan production		DCW		Apparent viscosity		Flocculating activity	
	T	S	T	S	T	S	T	S
Sucrose	3.48	92.65	2.72	88.72	1.95	80.95	3.25	91.68
KNO_3	1.97	81.21	-1.01		1.81		5.05	96.29
K_2HPO_4	10.14	99.04	11.85	99.29	4.78	95.90	17.51	99.68
$MgSO_4 \cdot 7H_2O$	4.09	94.50	1.90	80.22	1.93	80.66	3.83	93.8
KCl	-3.78	-93.67	-1.37		-1.83		-7.38	-98.21
$FeSO_4 \cdot 7H_2O$	-1.97	-81.21	-2.68	-88.47	-1.01		-4.27	-94.93
Aeration	-0.15		0.09		1.09		5.19	96.48
Dummy I	-0.15		-0.21		-0.66		-1.64	
Dummy II	-0.45		1.61		0.27		0.55	
Dummy III	-1.67		-0.60		-1.58		-0.09	

T: t-value, S: significance (%).

Table 4. Separate experimental design of positive and negative variables on the cultivation of *Pestalotiopsis* sp. according to the results derived from the Plackett-Burman design.

Parameter	Media for positive variables				Media for negative variables			
	P-I		P-II		N-I		N-II	
Culture condition	Sucrose	5%	Sucrose	5%	Sucrose	2%	Sucrose	2%
	KNO_3	0.0714%	KNO_3	0.0714%	KNO_3	0.029%	KNO_3	0.029%
	K_2HPO_4	0.2%	K_2HPO_4	0.2%	KCl	0.075%	KCl	0.075%
	$MgSO_4 \cdot 7H_2O$	0.025%	$MgSO_4 \cdot 7H_2O$	0.025%	$FeSO_4 \cdot 7H_2O$	0.001%	$FeSO_4 \cdot 7H_2O$	0.001%
		50 ml		100 ml		50 ml		100 ml

apparent viscosity responses. This suggested that the positive result of flocculating activity response according to the aeration variable was based on some changes of structure of produced Pestan (eg., molecular chain length, charge density of Pestan, etc.). The KCl variable showed a more negative effect than $FeSO_4 \cdot 7H_2O$ on the flocculating activity response.

To verify the results derived from the Plackett-Burman design, we carried out two separate cultivations based on Table 4 and the results are shown in Fig. 1.

Figure 1 confirmed the positive and negative variables of each response. Also, it confirmed that Pestan production was closely related to microbial growth by comparison of A (Pestan production) with B (DCW). In comparison of P-I with P-II in Fig. 1C (apparent viscosity) and D (flocculating activity), the apparent viscosity and flocculating activity in the P-I condition were higher than those in the P-II condition. These differences resulted from aeration. But, the Pestan production and DCW in P-I and P-II conditions were similar.

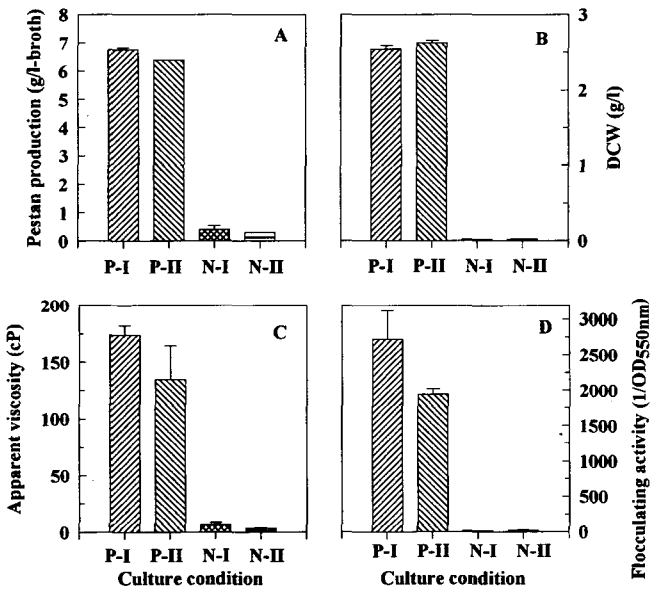

Fig. 1. Results of the positive and negative variables of media for several responses.

A: Pestan production; B, DCW; C, Apparent viscosity; D, Flocculating activity; P, positive variable; N, negative variable; I, aeration variable; II, nonaeration variable.

To more completely understand the effect of the aeration variable derived from the flask level, we tried a batch culture with a 5-l jar fermentor. Culture conditions, except for aeration, were the same in the P-I' and P-II' conditions. However, in the aeration condition, for P-I' condition only, air was supplied by 1 vvm and the P-II' condition was nonaerated.

As shown in Fig. 2, in the case of P-I' condition, Pestan production, DCW, apparent viscosity, and flocculating activity showed the similar tendency with increasing agitation, but the effect of agitation in the P-II' condition differed from that of agitation in the P-I' condition. At 120 h culture time, the Pestan production, DCW, apparent viscosity, and flocculating activity in the P-I' condition were higher by 11.4 times (Pestan production in the P-I' condition/Pestan production in the P-II' condition: [10.25 g/l]/[0.9 g/l]), 4 times ([3.52 g/l]/[0.892 g/l]), 24 times, and 6.6 times than those in the P-II' condition, respectively. Although the Pestan production of the P-I' condition increased about 1.7 times ([10.25 g/l]/[0.892 g/l]) than that of the starting point (modified Czapek-

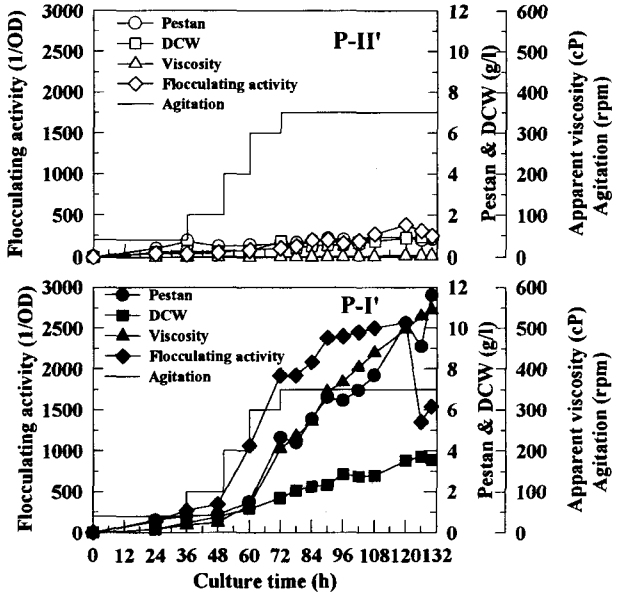

Fig. 2. Effect of aeration and nonaeration for the several responses according to culture time.

P-I': Aeration (1vvm); P-II': Nonaeration.

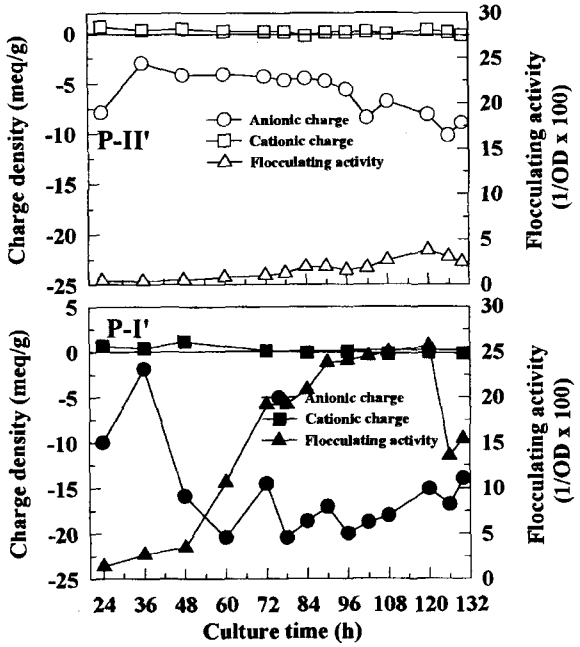

Fig. 3. Relation between charge density and flocculating activity for the culture condition based on the Plackett-Burman design.

P-I': Aeration; P-II': Nonaeration.

dox medium), DCW did not increase ([3.52 g/l]/[3.706 g/l]). This resulted from the effect on the removal of negative variables (KCl and $FeSO_4 \cdot 7H_2O$) in the DCW and Pestan production responses. Then, Pestan production increased extremely whereas DCW did not. This result could deduce that the chloride and ferric ions of the negative variables in Pestan production response inhibited some enzyme activity on the Pestan biosynthesis mechanism more than on the cell growth of *Pestalotiopsis* sp.

In comparing P-I with P-II in Fig. 1A and 1D, the flocculating activity of Pestan did not directly depend on the production amount of Pestan, but rather on some structural properties of the Pestan molecule. This result is explained in Figs. 3 and 4; the flocculating activity of Pestan was dependent on the charge density and molecular chain length of Pestan. According to the culture time, the anionic charge density of Pestan and the polymerization of Pestan molecule were increased gradually. Therefore, the larger the anionic charge density and molecular chain length of Pestan, the higher the flocculating activity. This result was related to the polymerization of Pestan molecule. The polymerization of Pestan was similar to Tamzerh's report [12] that as the production of biopolymer was mostly polymerized by oxidation reaction, the oxygen concentration dissolved in the fermentor was very important for biopolymer production by the microorganism, but, because the viscosity of culture broth in the fermentor was very high, the oxygen transport and maintenance of constant oxygen concentration was very important.

Based on these results, we concluded that aeration played the most important role in the maturation of

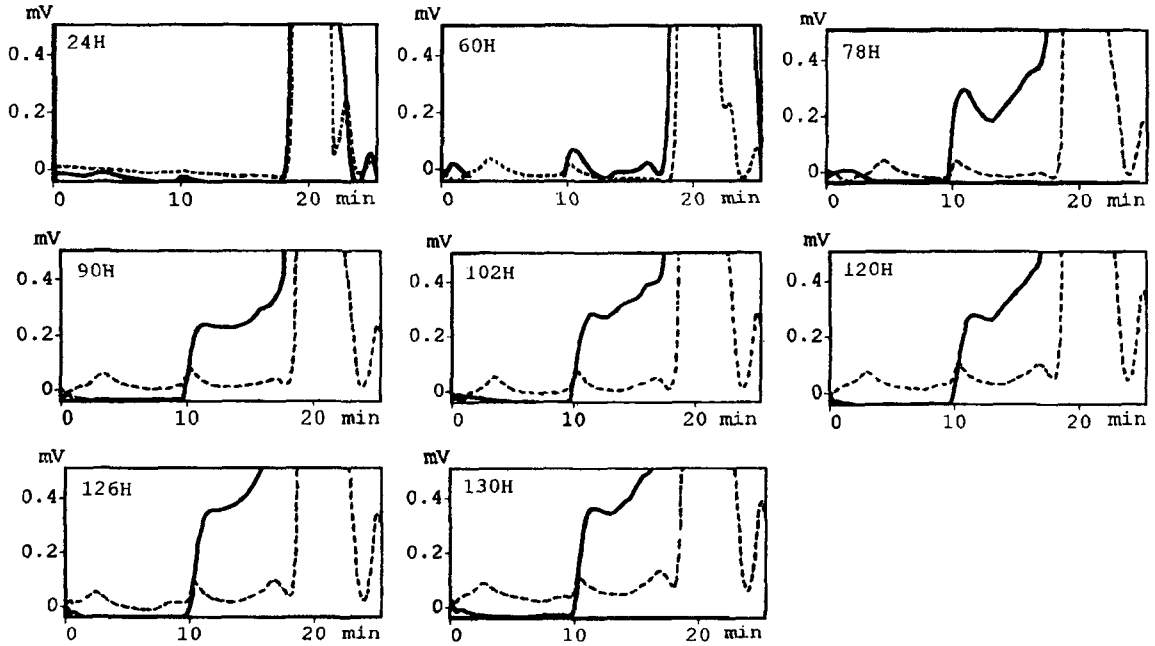

Fig. 4. Effect of aeration and nonaeration for the molecular weight distribution of Pestan in the culture condition based on the Plackett-Burman design.

—— Aeration (1 vvm); ····· Nonaeration.

Pestan molecule and these differences between P-I' and P-II' conditions resulted in the molecular chain length and charge density of Pestan. Therefore, we confirmed that the flocculating activity and apparent viscosity of Pestan were closely related to the molecular chain length and charge density of Pestan.

Among the various variables affected by each response (Pestan production, DCW, apparent viscosity, flocculating activity), the positive and negative variables were distinctly separate and indicated as overlap and/or common variables. We learned that each response was closely related to each other and some selected and/or improved variables directly affected the improvement of each responses.

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