

Selection of Medium Components by Plackett-Burman Design for Cell Growth of a Newly Isolated *Methylobacterium* sp. WJ4

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Abstract – We isolated a novel methylotrophic bacterium from soil collected in Dongducheon Environment Affairs Agency. The isolate was identified as *Methylobacterium* sp. WJ4 based on phylogenetic analysis. Plackett-Burman design was employed for screening eight parameters of nitrate mineral salts (NMS) medium for cell growth of a newly isolated *Methylobacterium* sp. WJ4 with experimental validation. Trace element solution and vitamin stock were found to affect cell growth, which can be further optimized for increased cell growth. This is the first report of screening parameters of NMS medium which affect cell growth of strain belonging to the genus *Methylobacterium* using Plackett-Burman design.

Key words: Cell growth, Isolation, *Methylobacterium*, NMS medium, Plackett-Burman design

1. Introduction

Currently, further methanol production facilities are being set up based on the abundance of resources for its production, such as natural gas. The advantages of methanol include its price stability, low price, complete water miscibility and restricted use by microorganisms. Methanol reduces the risk of contamination in fermentations due to its toxicity towards numerous microbial species. Thus, methanol has attracted much attention due to its many advantages as a raw material in the field of biotechnology [1-4].

Methylobacterium strains are Gram-negative, pink-pigmented, strictly aerobic, facultatively methylotrophic bacteria. They can utilize single-carbon compounds as their sole carbon and energy source, as well as a wide range of multi-carbon growth substrates [5]. They belong to the *Alphaproteobacteria* and have been isolated from soils, dust, lake sediments and plants, specifically from leaf surfaces [6,7]. Amino acids [8], various proteins [9, 10] and polyhydroxyalkanoates (PHA) [3] are products of *Methylobacterium* strains using methanol as a feedstock. This genus has appeared as premier biocatalysts for methanol-based biotechnology and been considered as a key model system for converting methanol into value-added products [11]. Thus, methanol and *Methylobacterium* strains have received considerable attention as a feedstock in the field of biotechnology.

An efficient way to screen for the most important growth factors among a large number of variables is the use of the Plackett-Burman design [12,13]. This technique generates different combinations of

the individual components with varying amounts. Medium components are simultaneously varied in one large experiment rather than in multiple separate experiments. Subsequently, statistical models are assigned to estimate the effect of varying one component either independently or in unison with other components. Thus, this approach can identify interactions between variables and use quantitative models to guide the optimization process [14-16]. In this study, significant growth factors of nitrate mineral salts (NMS) medium screened on the basis of the Plackett-Burman design for growth of *Methylobacterium* sp. WJ4 are reported.

2. Experimental

2-1. Culture medium

The cultivation was conducted in modified NMS medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/L, KNO_3 1 g/L, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.2 g/L, Fe-EDTA 0.0038 g/L, $\text{NaMo} \cdot 4\text{H}_2\text{O}$ 0.0005 g/L and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0017 g/L. Phosphate buffer (10 mL/L), Trace element solution (1 mL/L) and vitamin stock (0.1 mL/L) were added to the medium. Phosphate buffer, pH 7.0 contained KH_2PO_4 26 g/L and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 62 g/L. The trace element solution contained $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g/L, $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ 0.02 g/L, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.05 g/L, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.01 g/L, H_3BO_3 0.015 g/L and EDTA 0.25 g/L. Vitamin stock contained biotin 0.002 g/L, folic acid 0.002 g/L, thiamine HCl 0.005 g/L, Ca pantothenate 0.005 g/L, vitamin B_{12} 0.0001 g/L, riboflavin 0.005 g/L and nicotinamide 0.005 g/L. Methanol was added to give desired concentration [17,18].

2-2. Isolation of microorganism

Methylobacterium sp. WJ4 was isolated from the soil of the Dongducheon Environment Affairs Agency (Dongducheon city, Gyeonggi-do province, Republic of Korea). The soil sample was collected

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[‡]This article is dedicated to Prof. Choon Han on the occasion of his retirement from Kwangwoon University.

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in the facility. One gram of soil sample was suspended in 50 mL of liquid NMS medium containing 1% (v/v) methanol in a 250 mL flask and then incubated in a shaking incubator at 30 °C and 200 rpm for one week. Subsequently, 500 µL of the supernatant was transferred to 50 mL of same medium and incubated under the same conditions. This procedure was performed for four weeks and the supernatant was streaked onto modified NMS agar plates. The plates were incubated at 30 °C. After incubation, single colonies were picked up from plate with a loop and restreaked onto modified NMS agar plates until colonies derived from single colony were obtained. Isolates were checked for purity using 16S ribosomal RNA (16S rRNA) gene sequencing.

2-3. Identification of the isolate by 16S rRNA gene sequencing and phylogenetic analysis

For identification of the isolate, 16S rRNA gene was amplified by polymerase chain reaction (PCR) with universal primer pair 27F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1492R [5'-GGTTACCTGTTACGACTT-3']. Sequencing was done according to the Sanger dideoxy method [19]. The sequence was compared with those available in the GenBank database using Basic Local Alignment Search Tool (BLAST). To identify the isolate from the 16S rRNA gene sequence, phylogenetic and molecular evolutionary comparisons between the isolate and related methylotrophs were conducted using Clustal Omega and MEGA version 7.0.14 program by neighbor-joining method [20].

2-4. Plackett-Burman design

Plackett-Burman design is an efficient way to identify the most significant factors among a large number of variables [10]. In the present study, Plackett-Burman design experimental matrix was set to identify the important variables and estimate their significance in cell growth. The variables chosen were $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$,

$NaMo \cdot 4H_2O$, Fe-EDTA, trace element solution, vitamin stock, phosphate buffer and $CuCl_2 \cdot 2H_2O$. The experimental designs for the screening of the variables are shown in Table 2 and 3. Plackett-Burman design is based on the first order model:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i \quad (1)$$

where Y = response (cell growth), β_0 , β_i = constant coefficients, X_i = the coded independent factors and k is the number of factors. Interaction among factors was not described in this model and the important factors that influence cell growth were screened by using this model [21,22]. The factors with p-values <0.05 are considered the most significant factors that affect cell growth.

2-5. Cultivation for screening of medium components using Plackett-Burman design and analytical methods

Cultivations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of modified NMS medium in a shaking incubator at 30 °C and 200 rpm. The medium was inoculated with 1% inoculum. Cell growth was monitored by measuring the optical density (OD) at 600 nm (7300 Spectrophotometer, Jenway) after appropriate dilution of culture broth with distilled water. Plackett-Burman design was generated by Minitab 17 Software. In addition, the experimental results of designs were analyzed by Minitab 17 Software.

3. Results and Discussion

3-1. Identification of the isolate by 16S rRNA gene sequence and phylogenetic analysis

The microorganism isolated from the soil sample was related to the genera, *Methylobacterium* based on 16S rRNA gene sequences

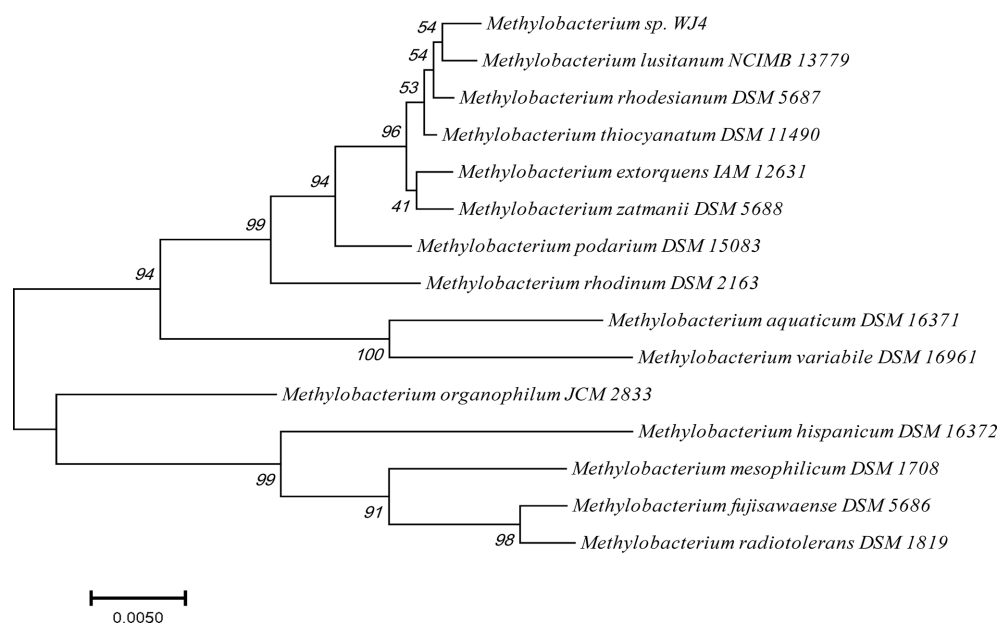


Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between isolate and other methylotrophs. The scale bar represents 0.005 substitutions per nucleotide sequence position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 1000 bootstrap resamplings.

and phylogenetic analysis (Fig. 1). The isolate, identified as *Methylobacterium* sp. WJ4, showed 99.6%, 99.5%, 99.2%, 97.7%, 96.7% and 96.6% similarity to *Methylobacterium rhodesianum*, *M. lusitanim*, *M. thiocyanatum*, *M. rhodinum*, *M. organophilum* and *M. extorquens*, respectively. Levels of 16S rRNA gene sequence similarity were high to assign the isolate to a member of the genus *Methylobacterium*.

3-2. Determination of a suitable nitrogen source for *Methylobacterium* sp. WJ4

The optimal nitrogen source was determined by culturing *Methylobacterium* sp. WJ4 in 250 mL Erlenmeyer flasks containing 50 mL of modified NMS medium with each different nitrogen source in a shaking incubator (200 rpm at 30 °C for 60 h). As shown in Table 1, the highest OD₆₀₀ value of 2.948 and cell dry weight of 0.7 g/L was obtained in medium containing KNO₃ as a nitrogen source. The same cell dry weight and similar OD₆₀₀ values of 1.881 and 1.941 were obtained in medium containing NH₄Cl and (NH₄)₂SO₄ as a nitrogen source, respectively. Thus, KNO₃ was deemed to be the most suitable nitrogen source for *Methylobacterium* sp. WJ4.

Table 1. Effect of nitrogen sources on cell growth

Nitrogen source	Optical density (600 nm)	Cell dry weight (g/L)
KNO ₃	2.948	0.7
NH ₄ Cl	1.881	0.4
(NH ₄) ₂ SO ₄	1.947	0.4

Table 2. Medium components and their levels used in Plackett-Burman design

Symbol	Variable	Experimental value		
		-1	0	+1
X ₁	MgSO ₄ · 7H ₂ O (g/L)	0.2	1.0	1.8
X ₂	CaCl ₂ · H ₂ O (g/L)	0.1	0.2	0.3
X ₃	NaMo · 4H ₂ O (μg/L)	0.5	2.75	5.0
X ₄	Fe-EDTA (μg/L)	0.76	3.80	6.84
X ₅	Trace element solution (mL/L)	0.2	1.0	1.8
X ₆	Vitamin stock (mL/L)	2	6	10
X ₇	Phosphate buffer (mL/L)	1.0	5.5	10
X ₈	CuCl ₂ · 2H ₂ O (mg/L)	0.28	1.4	2.52

Table 3. Plackett-Burman design of variables with corresponding results

Run order	Coded factors								Cell growth (OD ₆₀₀)
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	
1	0	0	0	0	0	0	0	0	2.805
2	-1	-1	-1	-1	-1	-1	-1	-1	3.102
3	-1	-1	1	1	1	-1	1	1	3.388
4	1	1	-1	1	-1	-1	-1	1	2.079
5	-1	1	1	1	-1	1	1	-1	2.233
6	-1	1	-1	-1	-1	1	1	1	0.215
7	1	-1	-1	-1	1	1	1	-1	2.541
8	-1	-1	-1	1	1	1	-1	1	2.827
9	1	1	-1	1	1	-1	1	-1	4.114
10	-1	1	1	-1	1	-1	-1	-1	3.762
11	1	1	1	-1	1	1	-1	1	2.035
12	1	-1	1	1	-1	1	-1	-1	2.607
13	1	-1	1	-1	-1	-1	1	1	2.838

3-3. Determination of the most significant medium components using Plackett-Burman design

Carbon and nitrogen source (methanol and KNO₃) were essentially required for cell growth in microbial cultivation. A total of eight components (X₁-X₈) of NMS medium except for carbon and nitrogen source were analyzed regarding their effects on cell growth using a Plackett-Burman design. Table 2 shows the Plackett-Burman design used in this study. The list of various components and their amounts in the NMS medium is given in Table 2. The Plackett-Burman experiment was performed in 250 mL Erlenmeyer flasks containing 50 mL of growth medium in a shaking incubator (200 rpm at 30 °C for 48 h). A 13-run Plackett-Burman design generated for eight factors and the corresponding results are shown in Table 3. The high OD₆₀₀ values were observed in combinations 9 and 10. Analysis of variance (ANOVA) was performed to estimate the effect of each factor regarding our system (Table 4). Table 4 illustrates the regression coefficient, *t*-value and *p*-value of the eight experimental variables. The equation obtained for Plackett-Burman design was as follows:

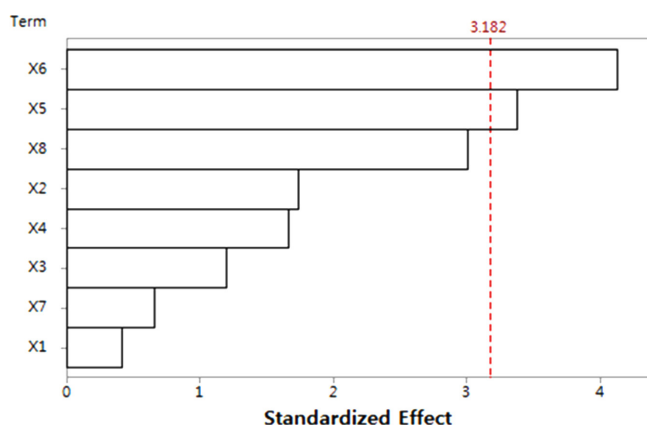
$$Y = 2.645 + 0.057 X_1 - 0.239 X_2 + 0.165 X_3 + 0.230 X_4 + 0.466 X_5 - 0.569 X_6 - 0.090 X_7 - 0.415 X_8 \quad (2)$$

where *Y* = response (cell growth), X₁ = coded value of MgSO₄ · 7H₂O, X₂ = coded value of CaCl₂ · H₂O, X₃ = coded value of NaMo · 4H₂O, X₄ = coded value of Fe-EDTA, X₅ = coded value of trace element solution, X₆ = coded value of vitamin stock, X₇ = coded value of phosphate buffer, X₈ = coded value of CuCl₂ · 2H₂O.

It is clear from Table 4 that MgSO₄ · 7H₂O (X₁), NaMo · 4H₂O (X₃), Fe-EDTA (X₄), and trace element solution (X₅) had positive effects on cell growth, whereas other factors had negative effects. Factors evidencing *p*-values of less than 0.05 were considered to have significant effects on the cell growth. The *p*-values of vitamin stock and trace element solution were 0.043 and 0.026, respectively. Thus, trace element solution (X₅) and vitamin stock (X₆) were determined to be significant components for cell growth. In contrast, the other six variables, MgSO₄ · 7H₂O, CaCl₂ · H₂O, NaMo · 4H₂O, Fe-EDTA, phosphate buffer, CuCl₂ · 2H₂O, were found to be insignificant. MgSO₄ · 7H₂O and

Table 4. Regression coefficients and corresponding *t*-value and *p*-value for cell growth in Plackett-Burman design

Term	Regression coefficient	<i>t</i> -value	<i>p</i> -value
Constant	2.645	19.22	0.000
X ₁	0.057	0.42	0.705
X ₂	-0.239	-1.74	0.181
X ₃	0.165	1.20	0.316
X ₄	0.230	1.67	0.194
X ₅	0.466	3.39	0.043
X ₆	-0.569	-4.13	0.026
X ₇	-0.090	-0.66	0.559
X ₈	-0.415	-3.01	0.057

**Fig. 2. Pareto chart of eight-factor standardized effects on cell growth. X₁: MgSO₄·7H₂O, X₂: CaCl₂·H₂O, X₃: NaMo·4H₂O, X₄: Fe-EDTA, X₅: Trace element solution, X₆: Vitamin stock, X₇: phosphate buffer, X₈: CuCl₂·2H₂O.**

Phosphate buffer showed lower coefficients, 0.057, and -0.090, respectively compared to that of other variables. It indicates that these variables can be deleted in experiments for optimizing the amounts. As the NaMo·4H₂O, Fe-EDTA showed positive coefficients, the higher levels of these components are suggested for further experiments. In contrast, CaCl₂·H₂O and CuCl₂·2H₂O showed negative coefficients. The lower levels of these components are suggested for future experiments.

The Pareto chart of main effects as shown in Fig. 2 displays the order of significance of the factors affecting the cell growth and is a convenient way to view the results of a Plackett-Burman design. The maximal effect was presented in the upper portion of the chart and then progressed down to the minimal effect. The order of significance as indicated by the chart is vitamin stock, trace element solution, CuCl₂·2H₂O, CaCl₂·H₂O, Fe-EDTA, NaMo·4H₂O, phosphate buffer and MgSO₄·7H₂O. The vertical line on the chart judges the effects which are statistically significant at the 95% confidence level. According to the Pareto chart, vitamin stock was the most significant factor, subsequently trace element solution was the next most significant factor.

4. Conclusions

We isolated a novel methylotrophic bacterium, identified as *Meth-*

ylobacterium sp. WJ4, from soil collected in Dongducheon Environment Affairs Agency. Plackett-Burman design was used to screen the most important growth factors among eight medium components of NMS medium for the cell growth. The effects of eight medium components were evaluated by Plackett-Burman design. Among them, trace element solution and vitamin stock were found to be significant components for the cell growth. These results based on ANOVA indicate that trace element solution and vitamin stock play a key role in the cell growth. The effects of trace element solution and vitamin stock can be further investigated to find the optimal concentration.

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