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Statistical Optimization of Medium Composition for Growth of *Leuconostoc citreum*

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Abstract *Leuconostoc citreum* is one of the representative strains of *Leuconostoc* spp. that show fast growth rates in fermented vegetables. Sequential experimental designs including the Plackett-Burman design, fractional factorial design, steepest ascent analysis, central composite design and response surface methodology were introduced to optimize and improve the medium for *L. citreum*. Fifteen medium ingredients were examined and glucose (20 g/L), yeast extract (12.5 g/L), sodium acetate trihydrate (6.12 g/L), potassium phosphate (42.55 g/L) and dibasic ammonium citrate (4.12 g/L) were chosen as the best components to give a critical and positive effect for cell-growth. The biomass was increased to 2.79 g/L (169%), compared to the 1.65 g/L in MRS medium.

Keywords: *Leuconostoc* spp., *Leuconostoc citreum*, Plackett-Burman, fractional factorial, steepest ascent, central composite design, response surface methodology

INTRODUCTION

Escherichia coli is the most widely used prokaryotic system for the synthesis of heterologous proteins, due to the fast growth-rate, well known genetic information and availability of high cell density culture (HCDC) in fed-batch fermentation [1-4]. However, many of these proteins are produced, as their insoluble forms in the intracellularly, from which biologically active proteins can only be recovered by complicated and costly denaturation and refolding processes [5]. Some of these microorganisms are pathogenic. *Leuconostoc* spp. are thought to be an alternative host system to solve the demerits of *E. coli*, as their fast growth rates are similar to the *E. coli* system both in aerobic and anaerobic conditions, the external expression of proteins which enables an efficient purification, desirable recognition which can be used in industrial spheres as food-grade, or GRAS (Generally Recognized As Safe). Dextrans produced from *Leuconostoc* spp. have been approved as food additives [6] and *Leuconostoc* spp. are the major lactic acid bacteria growing in the initial phase of kimchi fermentation. These merits of *Leuconostoc* spp. could lead to various uses in the food and biotechnology industries in the future.

L. citreum HJ-P4, the major lactic bacteria growing in the initial phase of kimchi fermentation, was isolated from lactate-fermented vegetables as a psychrotrophic starter. It is a possible candidate not only for kimchi, sauerkraut, and kefir but also for protein expression [7]. Even with the availability of *Leuconostoc* spp., MRS (De Man, Rogosa, Sharpe) medium is widely used as an optimized medium for the growth of *Lactobacillus* spp., which is generally also used for that of *Leuconostoc* spp. as well [8]. *Leuconostoc* spp., hetero-lactic acid fermenting bacteria, show quite different physicochemical properties to those of *Lactobacilli* spp. and a tailor-made medium remains to be introduced as a compromise.

Statistical experimental designs can be used to optimize both fermentation processes and media. First, categorical factors are studied to determine which nutrients and physical conditions hold the most promise for optimizing the fermentation. Then, a large number of continuous factors are screened and insignificant factors are eliminated in order to obtain a smaller set of factors. The remaining factors are optimized by response surface methodologies. Finally, after model building and optimization, the predicted optimum is verified [9,10]. Maddox and Richert [11] used successive response surface designs for the optimization of media components. Goulas *et al.* [12] used a total of eighty experiments in three sequential experimental designs (a factorial and two response surface designs) to optimize five growth components. Both Alpbaz *et al.* [13] and Bloor *et al.* [14] used steepest ascent subsequent to either factorial or response surface designs to optimize media.

In this research, a statistical medium-optimization study was performed to develop a Leuconostoc-specific medium using sequential experimental designs.

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Table 1. Biomass results of Plackett-Burman experiments with factor levels in g/L

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Biomass (g/L)
1	25	5	5	13.125	13.125	2.5	2.5	2.5	1	1	0.05	0.05	0.5	2.5	2.5	1.84
2	30	0	0	15	11.25	0	5	5	2	2	0	0.1	0	5	0	1.87
3	20	0	10	15	11.25	0	5	0	0	2	0.1	0.1	1	0	5	1.58
4	30	10	10	15	11.25	5	0	5	0	0	0	0	1	0	0	1.76
5	20	10	0	11.25	15	5	5	5	0	2	0	0.1	0	0	0	1.41
6	20	10	0	11.25	11.25	0	5	5	0	0	0.1	0	0	5	5	1.85
7	20	0	0	11.25	15	5	0	0	2	0	0	0.1	1	5	5	2.03
8	30	0	0	15	15	5	5	0	2	0	0.1	0	0	0	0	1.86
9	30	10	0	15	11.25	5	0	0	0	0	0.1	0.1	0	5	5	1.82
10	25	5	5	13.125	13.125	2.5	2.5	5	1	1	0.05	0.05	0.5	2.5	2.5	2.09
11	20	10	10	15	15	0	5	0	2	0	0	0	0	5	5	2.2
12	30	0	0	11.25	1.25	5	5	0	0	2	0	0	1	5	5	1.67
13	20	10	10	11.25	11.25	5	0	0	2	2	0.1	0.1	0	0	0	2.05
14	20	10	0	15	11.25	0	0	0	2	2	0	0	1	0	0	1.79
15	20	0	0	15	15	0	0	5	0	0	0.1	0.1	1	0	0	1.66
16	20	0	10	15	15	5	0	5	0	2	0	0	0	5	5	1.61
17	30	10	0	11.25	15	0	0	5	2	2	0.1	0	1	5	5	1.86
18	30	0	10	11.25	11.25	0	0	5	2	0	0	0.1	0	5	5	1.85
19	25	5	5	13.125	13.125	2.5	2.5	2.5	1	1	0.05	0.05	0.5	2.5	2.5	1.95
20	30	10	10	15	15	5	5	5	2	2	0.1	0.1	1	5	5	2.19
21	20	0	10	11.25	1.25	5	5	5	2	0	0.1	0	1	0	0	1.71
22	30	10	10	11.25	15	0	5	0	0	0	0	0.1	1	0	0	1.47
23	30	0	10	11.25	15	0	0	0	0	2	0.1	0	0	0	0	1.65

MATERIALS AND METHODS

Microorganism and Medium

L. citreum HJ-P4 was grown at 26°C, 100 rpm in Lactobacilli MRS medium and was the strain used in this study, which was stored in 50% (v/v) glycerol stock at - 70°C until required. MRS medium was used as a comparatively standard medium. All medium components used in the experimental designs were prepared separately and sterilized for 15 min at 121°C. After sterilization, all components were mixed according to a predetermined ratio for each experiment. The glucose and dibasic ammonium citrate [$(NH_4)_2HC_6H_5O_7$] were from Duksan (Korea), and the fructose, peptone, and beef extract were from DIFCO (USA). The maltose was from Yakuri (Japan). The tryptone and yeast extract were from Gellix Ventech Bio (Korea), and the $(NH_4)_2SO_4$ was from Tedia (USA). The $MnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, K_2HPO_4, KH_2PO_4, and sodium acetate trihydrate ($CH\ NH_4)_2HC_6H_5$) were from Shinyo (Japan).

Fermentation

Test tubes (working volume: 10 mL) were prepared with the required composition, inoculated with 0.5 mL precultured medium and incubated in a rotary shaker at 26°C and 100 rpm for 12 h and statistically analyzed to determine which factors had a positive effect. The batch medium was composed of 20 g/L glucose, 12.5 g/L yeast extract, 42.55 g/L K_2HPO_4, 6.12 g/L sodium acetate trihydrate and 4.12 g/L dibasic ammonium citrate. To prepare 1 L of batch medium, the stated amounts of yeast extract, K_2HPO_4, sodium acetate and ammonium citrate were dissolved in 900 mL distilled water, the pH was adjusted to 6.5 with 2 M NaOH and the solution was sterilized for 15 min at 121°C. The glucose was prepared separately and added afterwards. The main cultivation

Table 2. Results of Plackett-Burman experiments

Term	Estimate	Standard Error	Prob > ltl
Intercept	1.3200	0.21483	0.0005
Glucose	0.00100	0.00403	0.7479
Fructose	0.00920	0.00403	0.0327
Maltose	0.00240	0.00403	0.4766
Yeast extract	0.02133	0.01075	0.0534
Peptone	-0.00053	0.00806	0.9768
Beef extract	0.00680	0.00806	0.3550
Tryptone	-0.00520	0.00806	0.4437
$(NH_4)_2SO_4$	-0.00680	0.00806	0.3288
K_2HPO_4	0.14700	0.02016	0.0001
$MgSO_4$	-0.02700	0.40324	0.1587
$MnSO_4 \cdot 7H_2O$	0.56000	0.40324	0.1342
$CaCl_2 \cdot 2H_2O$	-0.0200	0.40324	0.9304
Tween 80	-0.0400	0.40324	0.2208
Sodium acetate trihydrate	0.03920	0.00806	0.0010
Ammonium citrate	0.02880	0.00806	0.0049
Curvature	-0.16600	0.05074	0.0110

$R^2 = 0.9662$ Adj $R^2 = 0.8759$ F = 0.85 Pr > F = 0.0129
Root MSE = 0.007364 CV = 4.05505

was carried out at 26°C in 2.5 L a stirred-tank bioreactor (Korea Fermentor Co., Ltd.). The initial batch culture conditions were as follows: working volume = 1 L, air flow rate = 0.2 v.v.m, and agitation = 100 rpm. The pH and the dissolved oxygen concentrations were uncontrolled. The dry cell weight was measured after drying of the cells gathered by filtration through a 0.2 μm filter (ADVANTEC MFS, Inc.). An absorbance of 1.0 was equivalent to 0.55 g dry cells L^{-1}.

Plackett-Burman Design

An initial set of fifteen media components were identified as potentially affecting biomass. The carbon and nitrogen sources included both complex and defined ingredients. A twenty run Plackett-Burman design [15] with three center points was selected as the screening design for the initial set of experiments. The analysis of the data from the Plackett-Burman experiments involved fitting a first order (main effects) model along with a term for the curvature. The low and high factor levels were coded as -1 and +1, respectively, for data analyses. This standardization produced factor main effects (or slopes), which were directly comparable to one another. Similarly, the mid levels (for the center points) were coded as 0. This coding convention was used for all subsequent analyses in this study. The theoretical model accompanying the analysis of the Plackett-Burman results is given by:

$$Biomass = I_0 + b_1X_1 + b_2X_2 + \ldots + b_{15}X_{15} + c_0CP + error$$

where I_0 represents the intercept, b_i is the main effect associated with ith medium component and X_i is the abbreviation for the medium components (X_1: glucose, X_2: fructose, X_3: maltose, X_4: yeast extract, X_5: peptone, X_6: meat extract, X_7: tryptone, X_8: corn steep liquor, X_9: K_2HPO_4, X_{10}: $MgSO_4 \cdot 7H_2O$, X_{11}: $MnSO_4 \cdot 7H_2O$, X_{12}: $CaCl_2 \cdot 2H_2O$, X_{13}: Tween 80, X_{14}: sodium acetate, and X_{15}: ammonium citrate) accompanying each medium effect, while the CP indicates whether the experiment was center point (0) or non-center point (+1). The c_0 term is the gross curvature effect, and indicates the degree to which the center points differ from the non-center points.

Table 3. Range of values of fractional factorial design

Variable name	Level (g/L)		
	-1	0	1
Fructose	0	5	10
Yeast extract	10	12.5	15
H_2O (dummy value)	10	12.5	15
K_2HPO_4	0	1	2
Sodium acetate	0	2.5	5
Ammonium citrate	0	2.5	5

Fractional Factorial Design

The Plackett-Burman experiment was followed by a fractional factorial design (FFD) for five factors. Fractional factorial designs are used in place of Plackett-Burman designs when estimates of two-factor interactions are desired [16-18]. The media components included in the fractional factorial study were the most significant factors. Glucose was fixed at a low level (20 g/L) as it was a major carbon source, even though the glucose was insignificant according to the Plackett-Burman design. All other components were eliminated from the media formulations. A 2^{6-2} fractional factorial design, leading to 16 sets of experiments, was used to verify the most significant factors affecting the biomass. The variables were coded according to the following equation:

$$X_i = (X_i - X_0)\Delta X_i$$

Where X_i is the coded value of an independent variable, X_i the real value of an independent variable, X_0 the real value of an independent variable at the center point, and ΔX_i the step change value. The range and levels of the variable coded values investigated in this study are given in Table 3. Based on the first-order model equation obtained by the FFD, a series of trials were performed in the direction of the steepest ascent.

Central Composite Design

A response surface methodology was applied to optimize the process, using 3×3 factorial central composite design (CCD), according to the method of Box and Behnken [19]. A three-dimensional graph was obtained from the calculated response surface:

$$Y = \beta_0 X_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \text{ (intercept and main effect)} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \text{ (interactions)} + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \text{ (quadratic effects)}$$

where Y is the response and X_1, X_2, and X_3 the coded settings for the three factors. The β terms are the calculated parameter estimates for each main effect, interaction, and quadratic effect. Once the appropriate data were collected, this model could be estimated and the optimal process conditions can be determined.

The statistical experimental designs used in this work were generated using the JMP statistical software (SAS Institute Inc., USA) and the responses from each set of experiments (or design) were collectively analyzed using this software. The analysis of the results from each design produced a predictive model of the response as a function of the process factors. The fitted model differed depending on the experimental stage.

RESULTS AND DISCUSSION

Plackett-Burman Design

The media compositions and the resulting biomass are provided in Table 1. The linear model with the curvature term was statistically significant with a *P*-value of 0.0013 with an R^2 value of 0.97 indicating the model explained over 97% of the total variability in the data. The parameter estimates of the media components are given in Table 2, which were interpreted as the slopes, *i.e.* the change in the response for every unit change in the coded factor level. The media components having the most significance (*P*-value < 0.05) at this stage included fructose, yeast extract, K_2HPO_4, sodium acetate and ammonium citrate. The curvature term was significant, indicating the optimal conditions were likely to be inside the current experimental region. The coefficient of variation (CV) was an indication of the degree of precision with which the treatments could be compared. Usually, the higher the CV value, the lower is the reliability of the experiment. Here, a low value of CV (4.05505) indicated the greater reliability of the experiments performed.

Fractional Factorial Design

A two-level fractional factorial design was employed, and the results are shown in Table 4. The results of the analysis show that fructose, K_2HPO_4, sodium acetate, and ammonium citrate had significant effects on the bacterial growth. Anyway the results in the Table 4 clearly showed that the optimal region was outside of the current design space, as the significant level of curvature was 0.0694. No interactions were observed among these factors. The value of the regression coefficient was calculated, and a first order equation was obtained with a coefficient, R^2, of 0.8960. This result indicates that the optimum point was not in the domain of our experiment. Thus, additional analysis, using steepest ascent path, will be required to reach the optimum domain.

Table 4. Results of FFD regression analysis

Term	Parameter estimate	Regression analysis		
		Standard error	T for H0	Significant level
Intercept	0.83750	0.18391	4.55	0.0007
Curvature	-0.10625	0.05328	-1.99	0.0694
Fructose	0.09188	0.02466	3.73	0.0029
Yeast extract	0.10625	0.11914	0.89	0.3900
H_2O	-0.04375	0.11914	-0.37	0.7199
K_2HPO_4	0.30625	0.04933	6.21	<.0001
Sodium acetate trihydrate	0.32500	0.0595	6.38	<.0001
Ammonium citrate	0.26500	0.04766	5.56	0.0001

R^2 = 0.8960 Adj R^2 = 0.8354 F = 14.78 Pr > F = <.0001 Root MSE = 0.09531 CV = 7.03410

* KH_2PO_4 showed insignificant effect on cell growth. (P-value = 0.1492)

Steepest Ascent Experiment

Steepest ascent method uses the magnitude and sign of the linear effects to determine the direction toward a predictive higher response. The path begins at the center of the current design space and stretches well outside the design space [20]. The other factors were fixed at zero. The highest biomass was achieved with 6 g/L sodium acetate, 4 g/L ammonium acetate and 45 g/L K_2HPO_4 (data not shown). To determine the difference of biomass as increasing glucose content, the same composition of newly optimized medium except for glucose was used. Glucose contents were 0, 10, 15, 20, and 30 g/L and the maximum biomass was obtained with 20 and 30 g/L glucose, thus the glucose concentration was fixed at 20 g/L (low level). The yeast extract was at 12.5 g/L (zero level) as this was significant in the Plackett-Burman design, but not in the fractional factorial design because it was the major nitrogen source. Even though fructose was significant in all designs, it was eliminated from this experiment as it is much more expensive than glucose, and an enhancement of the biomass should still be expected without fructose.

Central Composite Design (CCD) and Response Surface Analysis

The central composite design, with three settings for

Table 5. Variables, levels and results of 3-factor central composite design

Run	Sodium acetate (g/L)		Ammonium citrate (g/L)		K_2HPO_4 (g/L)		Biomass (g/L)
1	-1	5.5	-1	3.5	0	42.5	2.60
2	-1	5.5	0	4	-1	40	2.55
3	-1	5.5	0	4	+1	45	2.59
4	-1	5.5	+1	4.5	0	42.5	2.61
5	0	6	-1	3.5	-1	40	2.58
6	0	6	-1	3.5	+1	45	2.65
7	0	6	+1	4.5	-1	40	2.70
8	0	6	+1	4.5	+1	45	2.61
9	+1	6.5	-1	3.5	0	42.5	2.60
10	+1	6.5	0	4	-1	40	2.62
11	+1	6.5	0	4	+1	45	2.69
12	+1	6.5	+1	4.5	0	42.5	2.72
13	0	6	0	4	0	42.5	2.79
14	0	6	0	4	0	42.5	2.76
15	0	6	0	4	0	42.5	2.81

The settings for each factors are 5.5 (-1), 6 (0), 6.5(+1) for sodium acetate trihydrate, 3.5(-1), 4(0), 4.5 (+1) for ammonium citrate, and 40(-1), 42.5(0), 45(+1) for K_2HPO_4.

each of 3 factors, was run to optimize the process. The settings for each factors are 5.5(-1), 6(0), 6.5(+1) for sodium acetate trihydrate, 3.5(-1), 4(0), 4.5(+1) for ammonium citrate, and 40(-1), 42.5(0), 45(+1) for K_2HPO_4. The results of the experiment are shown in Table 5. An insignificant value of Lack of Fit ($Pr>F$ = 0.4216) means that the range of variables and levels used in the CCD were compatible. The R^2 was 96%. This means that 96% of the variability can be accounted for by the second-order polynomial prediction equation given below:

$$Y = -41.043750 + 3.615000 \times SA + 2.859167 \times AC + 1.263833 \times DPP - 0.353333 \times SA \times SA + 0.110000 \times AC \times SA - 0.263333 \times AC \times AC + 0.00600 \times DPP \times SA - 0.032000 \times DPP \times AC - 0.013733 \times DPP \times DPP$$

where Y is the biomass, and SA, AC, and DPP the coded settings for sodium acetate, ammonium citrate and potassium phosphate, respectively. The three-dimensional graph obtained from the calculated response surface is shown in Fig. 1. The fitted surface had a maximum point with 6.12 g/L sodium acetate, 4.12 g/L ammonium citrate and 42.55 g/L K_2HPO_4, for which the model predicted a maximum response of 2.79 g/L at this point.

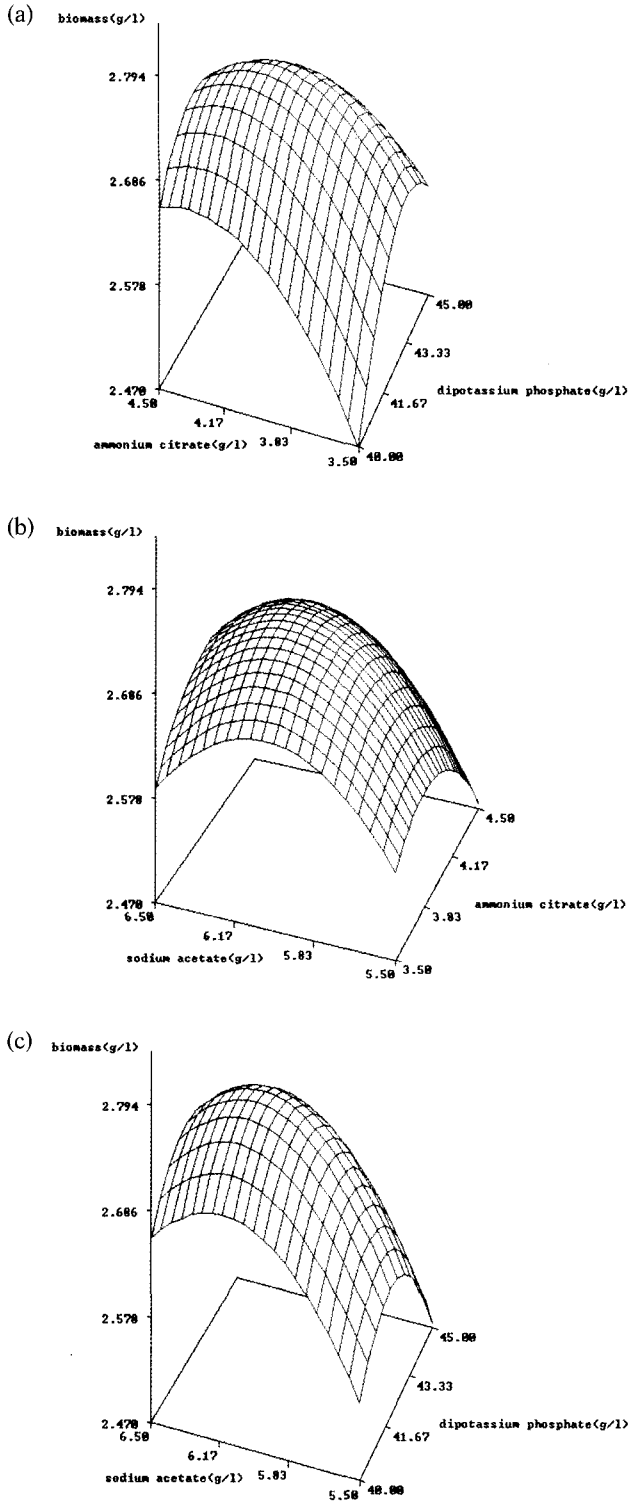

Fig. 1. The response surface plots of biomass on dipotassium phosphate, sodium acetate and ammonium citrate. (a): ammonium citrate and dipotassium phosphate, (b): sodium acetate and ammonium citrate, (c): sodium acetate and dipotassium phosphate.

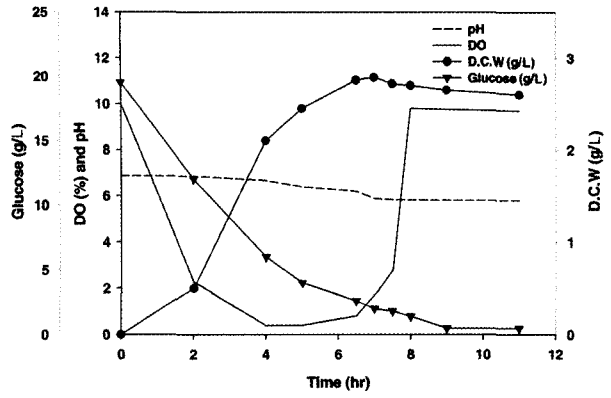

Fig. 2. Batch culture of *L. citreum* in a newly optimized medium. *L. citreum* HJ-P4 was cultivated at 26°C and pH 6.5 in medium containing glucose (20 g/L), yeast extract (12.5 g/L), K_2HPO_4 (42.55 g/L), sodium acetate (6.12 g/L), and ammonium citrate (4.12 g/L).

Table 6. Regression coefficient of a full second-order polynomial model

Parameter	Standard estimate	t Value	Pr > ltl
Intercept	-41.043750	-6.41	0.0014
SA	3.615000	4.02	0.0101
AC	2.859167	3.73	0.0136
PP	1.263833	5.74	0.0022
SA*SA	-0.353333	-5.90	0.0020
AC*SA	0.110000	1.91	0.1142
AC*AC	-0.263333	-4.40	0.0071
PP*SA	0.006000	0.52	0.6245
PP*AC	-0.032000	-2.78	0.0389
PP*PP	-0.013733	-5.73	0.0023

R^2 = 0.9554 Pr>F = 0.0347 Lack of Fit = 0.4216

Verification

To confirm this result, experimental rechecking was performed using a medium representing this maximum point, and a mean value of 2.79 g/L was obtained. The biomass increased by 69% compared with the 1.65 g/L in MRS medium. The final composition of the optimal medium was as follows: 6.12 g/L sodium acetate trihydrate, 4.12 g/L dibasic ammonium citrate, 20.0 g/L glucose, 12.5 g/L yeast extract and 42.55 g/L K_2HPO_4. Batch fermentation was performed in the newly optimized medium, the results of which are shown in Fig. 2. In the newly optimized medium, after a short lag state the cells grew rapidly, with a maximum specific growth rate of 0.5 h^{-1}, and a dry cell mass of 2.79 g/L was obtained after 7 h, after which the glucose had been completely depleted. The pH did not decrease after the glucose had been consumed. The DO dropped during the exponential growth, but increased sharply once the glucose had been completely consumed.

From the results of the Plackett-Burman design, the glucose was fixed at a low level (20 g/L) throughout all the experiments as a carbon source in the optimized medium. To determine the difference in the biomass as a function of the increasing glucose content, the same composition of newly optimized medium, but with the use of glucose at 0, 10, 15, 20, and 30 g/L. Even though the maximal biomass was obtained with 30 g/L glucose, the biomass variation between the media containing 20, or 30 g/L glucose was not significant. Therefore, this result gives credence to the decision to fix the glucose at a low level (20 g/L) due to the insignificance of glucose during the analysis of the Plackett-Burman design.

CONCLUSION

The final composition of the newly optimized medium was: 20 g/L glucose, 12.5 g/L yeast extract, 42.55 g/L K_2HPO_4, 6.12 g/L sodium acetate trihydrate and 4.12 g/L dibasic ammonium citrate. The biomass production was increased to 2.79 g/L compared with the 1.65 g/L in MRS medium, showing a 169% relative productivity.

This microorganism seems to be sensitive to the pH, so the high amount of K_2HPO_4 may be considered to be related to any buffer effect in concomitant with other physiological requirements. Additional studies on the oxygen effect including the variable pH conditions will be essential to accomplish high cell-density cultures.

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