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Production of Ethanol From Cellulosic Biomass by *Clostridium thermocellum* SS19 in Submerged Fermentation

Screening of Nutrients Using Plackett-Burman Design

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Abstract

Plackett-Burman design, a statistical methodology, was used to screen 23 nutrients belonging to three categories—carbon, nitrogen, and salt/mineral sources—for the production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in anaerobic submerged fermentation. In this design, just *n* number of experiments is required for screening *n*–1 variables. The experimental data were subjected to statistical analysis for calculating the regression coefficients and *t*-values. Filter paper, Solka Floc, corn steep liquor (CSL), cysteine HCl, magnesium chloride, and ferrous sulfate showed relatively higher regression coefficients on ethanol production and growth. Among the 23 nutrients screened, based on their performance in terms of product-promoting ability, availability, and cost, filter paper, CSL, cysteine HCl, magnesium chloride, and ferrous sulfate were identified as the most effective and, therefore, selected for inclusion in further optimization studies.

Index Entries: Cellulosic biomass; Plackett-Burman design; ethanol; anaerobic fermentation; *Clostridium thermocellum*.

Introduction

Over the last century, energy consumption has increased progressively as the result of the growing world population and industrialization *(1)*. Ethanol is a renewable energy source produced through fermentation

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of sugars unlike the fossil fuels. Interest in the bioconversion of abundant and renewable cellulosic biomass into fuel ethanol as an alternative to petroleum is rising around the world owing to the realization of diminishing natural oil and gas resources *(2–5)*. Conventional techniques to achieve this bioconversion include the acid or enzymatic hydrolysis of cellulose followed by fermentation of the resulting soluble sugars into ethanol *(6)*. A better alternative to this multiple-step process, from an economical and technical point of view, is the direct bioconversion of cellulose into ethanol by a bacterium such as *Clostridium thermocellum (7,8)*. *C. thermocellum* is a cellulolytic, ethanologenic, thermophilic, and anaerobic bacterium and produces ethanol, acetic acid, small amounts of lactic acid, CO_2, and H_2 as the fermentation end products *(7–9)*. Wild strains of *C. thermocellum* so far reported produced 0.08–0.37 g of ethanol/g of glucose equivalents fermented *(10–16)*. With this in mind, we have isolated a wild strain of *C. thermocellum* SS19 *(16)* and used it in further studies for increased ethanol production.

Selection of appropriate carbon, nitrogen, and other nutrients is one of the most critical stages in the development of an efficient and economic bioprocess *(17)*. Classic and statistical methodologies are available for screening the nutrients in bioprocess optimization studies. There are various advantages in using the statistical methodologies in terms of rapid and reliable short listing of nutrients, understanding the interactions among the nutrients at varying concentrations, and a tremendous reduction in total number of experiments, resulting in savings of time, glassware, chemicals, and manpower *(18,19)*. Plackett-Burman design *(20)*, a statistical methodology, is used for screening of up to n–1 variables in just n number of experiments *(21)*. In this design, generally a multiple of four, i.e., 4, 8, 12, 16, 20, … 4n, experiments is required to screen 3, 7, 11, 15, 19, … 4n-1 components, respectively, in which n is an integer. Table 1, which shows this design, the columns represent the combinations and the rows represent the variables (nutrients). The ingredients are taken at two levels (lower and higher). The lower level in the design is represented as "–" and the higher level as "+." The contribution of an ingredient toward the growth of the organism or yield of the ethanol and acetic acid is determined based on the t-value (main effect) calculated from the experimental result *(18,22)*. The t-value (main effect) of an ingredient is calculated as follows: t-value or main effect of an ingredient x = (average of sum of the ethanol, acetic acid, or growth in which the ingredient is +) – (average of sum of the ethanol, acetic acid, or growth in which the ingredient is –). The nutrients are ranked based on their t-values. The nutrient with the highest t-value is considered to be the best *(23)*. In spite of various advantages, the statistical designs have been applied to a limited number of aerobic, anaerobic submerged, and solid-state fermentation processes *(17–19,24,25)*, but not in an anaerobic submerged fermentation process. In the present study, we report the screening of 23 nutrients using Plackett-Burman design for the production of ethanol from cellulosic biomass by *C. thermocellum* SS19 in anaerobic submerged fermentation.

Table 1
Plackett-Burman Design for Screening 23 Nutrients Along with Their Lower (–) and Upper (+) Ranges for Production of Ethanol by *C. thermocellum* SS19 in Submerged Fermentation

Serial no.	Nutrient	Concentration of nutrient (g/L) Lower (–)	Upper (+)	Combination no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Filter paper	0.8	8.0	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	–
2	CMC	0.8	8.0	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–
3	Solka Floc	0.8	8.0	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–
4	Avicel	0.8	8.0	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–
5	Native cotton	0.8	8.0	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–
6	$(NH_4)_2SO_4$	0.4	4.0	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	–
7	Urea	0.4	4.0	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–
8	Yeast extract	0.4	4.0	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	–
9	CSL	0.4	4.0	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–
10	Casein hydrolysate	0.4	4.0	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	+	–
11	Trisodium citrate	0.4	4.0	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	+	–
12	KH_2PO_4	0.2	2.0	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–	–
13	K_2HPO_4	0.2	2.0	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–	–
14	KNO_3	0.1	1.0	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+	–
15	NaCl	0.1	1.0	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	+	–
16	Cysteine HCl	0.02	0.20	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–	–
17	$CaCl_2{\cdot}2H_2O$	0.02	0.20	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	+	–
18	Sodium thioglycolate	0.1	1.0	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–	–
19	Na_2HPO_4	0.1	1.0	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	+	–
20	$MgCl_2{\cdot}6H_2O$	0.1	1.0	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	+	–
21	$MgSO_4{\cdot}7H_2O$	0.1	1.0	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–
22	$MnCl_2{\cdot}4H_2O$	0.002	0.02	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	+	–
23	$FeSO_4{\cdot}7H_2O$	0.002	0.02	+	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	+	–	–	–	–	+	–

Materials and Methods

Microorganism and Culture Conditions

The strain *C. thermocellum* SS19 was isolated from goat fecal droppings after enrichment in prereduced CMS medium with cellulose as the sole carbon source *(16)*. Fermentation studies were carried out in 120-mL serum vials containing 20 mL of prereduced CMS medium with appropriate concentrations of nutrients under study. In all experiments, a 5% (v/v) inoculum grown on 0.4% cellulose for 72 h in CMS medium was added and incubated anaerobically at 60°C for 5 d.

Screening of Nutrients Using Plackett-Burman Design

Twenty-three nutrients (Table 1) belonging to three important categories—carbon sources (filter paper [Whatman no. 1]), carboxymethylcellulose [CMC], Solka Floc, Avicel, and native cotton), nitrogen sources (ammonium sulfate, urea, yeast extract, corn steep liquor [CSL], and casein hydrolysate), and salt/mineral sources (trisodium citrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium nitrate, sodium chloride, cysteine HCl, calcium chloride, sodium thioglycolate, disodium hydrogen phosphate, magnesium chloride, magnesium sulfate, manganese chloride, and ferrous sulfate)—were screened using the Plackett-Burman design *(20)*. The concentration of each nutrient was fixed based on the literature and experience gained.

Different carbon sources were weighed and added to serum vials according to their concentrations in each combination. For other nutrients, stock solutions were prepared and added (in such an order that precipitation does not occur) to provide the desired concentration of each nutrient. The total volume of the nutrient solution in each vial was made up to 20 mL with distilled water containing resazurin, a redox potential indicator. The pH of the medium was individually adjusted to 7.2 in each serum vial. All the vials containing medium were kept in a boiling water bath, and then each vial was bubbled with nitrogen gas to make the medium oxygen free. The vials were tightly sealed and autoclaved at 121°C for 30 min and used in the experimental studies.

Estimation of Growth and Fermentation Products

Cell growth was determined by measuring the absorbance of culture broth at 660 nm *(26)*. For estimation of ethanol and acetic acid, 10 mL of fermented broth was centrifuged at 10,000*g* for 30 min at 4°C. The supernatant solution was acidified with 1 mL of 2 *N* phosphoric acid and 2 µL was injected into a Chromosorb 101 column in a CIC gas chromatograph equipped with a flame ionization detector. The following parameters were chosen for analysis: oven temperature, 160°C; injector temperature, 170°C; carrier gas, N_2; flow rate, 20 µL/min *(27)*.

Statistical Analysis

The regression coefficients and *t*-values were calculated by compatible analysis *(20,23)* of the data based on the yields of ethanol and acetic acid obtained in the experiments. The results were analyzed using Indostat, a software developed by Indostat Services, Hyderabad, India, for computing statistics. The ingredients with the highest *t*-values were considered the best nutrients *(23)* and thus chosen for inclusion in further optimization studies.

Results and Discussion

C. thermocellum SS19 optimally grew in CMS medium at 60°C and produced 2.15 and 1.25 g of ethanol and acetic acid, respectively, per liter of culture broth in 5 d *(16)*.

Effect of Nutrients on Growth and Fermentation Products

C. thermocellum SS19 grew optimally and produced highest average yields of ethanol (3.20 g/L), acetic acid (1.56 g/L), and maximum growth (A_{660} = 1.35) in combination 2 of the design followed by combinations 4, 13, and 9 (ethanol); 16, 19, 4, and 6 (acetic acid); and 6, 4, 13, 2, and 3 (growth) in those orders, respectively (Table 2). Table 3 presents the results of the data analysis obtained from growth and ethanol and acetic acid produced by *C. thermocellum* SS19 in screening 23 nutrients through Plackett-Burman design. It is apparent from the data that filter paper, ferrous sulfate, Solka Floc, CSL, trisodium citrate, CMC, K_2HPO_4, and $MgCl_2{\cdot}6H_2O$ showed comparatively higher positive effects on growth. Filter paper, ferrous sulfate, Solka Floc, CSL, Avicel, yeast extract, K_2HPO_4, CMC, KH_2PO_4, cysteine HCl, casein hydrolysate, Na_2HPO_4, and $MgCl_2{\cdot}6H_2O$ contributed a positive effect on ethanol production. Cysteine HCl, Avicel, filter paper, Solka Floc, casein hydrolysate, ferrous sulfate, CSL, $CaCl_2{\cdot}6H_2O$, K_2HPO_4, $MgCl_2{\cdot}6H_2O$, CMC, Na_2HPO_4, and urea showed a comparatively positive effect on acetic acid production. From the results of the data analysis, it is clear that, among the carbon sources screened, filter paper, with the highest positive regression coefficient, was found to be the best substrate for growth and ethanol production by *C. thermocellum* SS19. However, for acetic acid production, filter paper followed Avicel. Among the nitrogen sources, CSL was found to be the best for growth and ethanol production.

Filter paper and Solka Floc were reported as the best cellulosic substrates for ethanol production by strains of *C. thermocellum* SS21 and SS22 *(28)*. In various other studies, cellulosic substrates such as Solka Floc and Avicel were also reported as the carbon source for ethanol production by *C. thermocellum (12,29,30)*. Urea was reported as the best nitrogen source for the growth of certain strains of *C. thermocellum (13–15,31)*, whereas ammonium sulfate served as the best nitrogen source for certain other strains *(11,12,26,32)*. Yeast extract in a concentration range of 0.05–1.4% was found to be optimal for ethanol production by *C. thermocellum (10–15,26,30,31,33)*.

Table 2
Yields of Fermentation Products by *C. thermocellum* SS19
Obtained in Screening of 23 Nutrients Using Plackett-Burman Design

	Growth (A_{660})		Ethanol (g/L)		Acetic acid (g/L)	
Combination no.	Set I	Set II	Set I	Set II	Set I	Set II
1	1.18	1.21	2.16	2.11	0.87	0.84
2	1.31	1.28	3.21	3.19	1.51	1.54
3	1.26	1.29	2.96	2.92	1.39	1.36
4	1.32	1.34	3.18	3.17	1.56	1.52
5	1.26	1.22	2.85	2.89	1.48	1.51
6	1.36	1.34	2.42	2.51	1.58	1.49
7	0.92	0.89	1.86	1.89	0.61	0.66
8	0.99	0.96	1.97	1.88	0.66	0.63
9	1.21	1.25	3.02	3.04	1.38	1.35
10	1.24	1.22	2.63	2.71	1.52	1.49
11	1.08	1.11	2.01	2.06	1.18	1.23
12	1.23	1.21	2.68	2.65	1.38	1.41
13	1.30	1.32	3.17	3.14	1.51	1.50
14	1.14	1.11	2.08	2.12	0.61	0.59
15	1.08	1.12	1.68	1.71	0.57	0.58
16	1.13	1.08	2.71	2.64	1.57	1.55
17	1.19	1.21	2.56	2.59	1.23	1.19
18	1.09	1.06	1.92	1.87	0.81	0.84
19	1.22	1.21	2.26	2.14	1.53	1.58
20	0.98	1.03	1.68	1.73	1.20	1.18
21	1.17	1.15	2.42	2.40	0.88	0.91
22	1.02	0.95	1.58	1.59	1.18	1.19
23	1.06	1.08	1.26	1.19	0.58	0.56
24	1.18	1.15	1.31	1.28	0.62	0.64

Among the salts/minerals, magnesium sulfate was found to be the best nutrient for growth and ethanol production by strains of *C. thermocellum* SS8 and GS1 *(13,14)*. Magnesium chloride and ferrous sulfate were also found to be the best nutrients for ethanol production by *C. thermocellum* *(26,30,31,34)*. Cysteine HCl in a concentration range of 0.015–0.1% was found to be optimal for ethanol production from cellulosic biomass by strains of *C. thermocellum (13,14,30,35)*. To the best of our knowledge, no reports are available on the use of CSL as the nitrogen source for ethanol production by *C. thermocellum* strains. In our studies, CSL showed the highest positive effect on ethanol production. This could be owing to the presence of growth factors *(17,19)*.

From the present study, it is evident that the use of Plackett-Burman design not only helped us in short listing few key nutrients, but also proved to be useful in increasing the yield of ethanol from 2.15 g *(16)* to 3.18 g/L in a limited number of experiments. Overall, based on the product-promoting

Table 3
Regression Coefficients and t-Values Calculated From Ethanol and Acetic Acid Yields Obtained in Screening Experiments

Ingredient	Growth: Regression coefficient	Growth: t-Value	Ethanol: Regression coefficient	Ethanol: t-Value	Acetic acid: Regression coefficient	Acetic acid: t-Value
Intercept	1.16	342.22	2.31	418.76	1.14	309.98
Filter paper	0.05	15.29	0.34	63.00	0.12	33.46
CMC	0.01	4.11	0.07	13.95	0.02	7.41
Solka Floc	0.02	7.31	0.19	34.48	0.12	32.66
Avicel	–0.00	1.78	0.13	23.99	0.14	38.78
Native cotton	–0.01	4.73	–0.01	2.86	–0.10	27.23
$(NH_4)_2SO_4$	–0.02	7.92	–0.12	22.78	–0.08	22.93
Urea	–0.03	9.64	–0.03	5.43	0.01	3.34
Yeast extract	–0.00	2.76	0.09	16.82	0.00	2.54
CSL	0.02	7.43	0.19	35.31	0.08	23.61
Casein hydrolysate	0.00	0.43	0.02	3.99	0.09	24.62
Trisodium citrate	0.02	7.06	–0.05	9.35	0.00	1.07
KH_2PO_4	–0.01	4.73	0.05	9.50	–0.05	14.32
K_2HPO_4	0.01	4.11	0.09	17.73	0.03	10.81
KNO_3	–0.04	12.83	–0.10	19.24	–0.08	23.94
NaCl	–0.00	0.79	–0.02	4.52	–0.03	10.36
Cysteine HCl	0.00	1.53	0.05	9.35	0.15	41.16
$CaCl_2{\cdot}6H_2O$	0.00	1.78	0.00	1.13	0.04	11.94
Sodium thioglycolate	–0.03	9.89	–0.05	10.79	–0.02	6.28
Na_2HPO_4	–0.00	1.90	0.02	5.13	0.02	6.17
$MgCl_2{\cdot}6H_2O$	0.01	5.46	0.01	2.03	0.03	8.21
$MgSO_4{\cdot}7H_2O$	–0.00	2.02	–0.01	3.01	–0.00	1.75
$MnCl_2{\cdot}4H_2O$	–0.03	9.15	–0.08	15.46	–0.08	23.04
$FeSO_4{\cdot}7H_2O$	0.04	12.34	0.22	41.27	0.08	22.81

ability, availability, cost, and need to keep the number of factors as low as possible for optimization studies using response surface methodology, only five nutrients—filter paper, CSL, cysteine HCl, magnesium chloride, and ferrous sulfate—were identified as the most effective. Further, studies are in progress to optimize the concentrations of these selected key nutrients using response surface methodology for the production of ethanol from cellulosic biomass by *C. thermocellum* SS19 in anaerobic submerged fermentation.

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