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Optimization of Culture Medium Composition for Cellulolytic Bacteria by Mathematical Methods

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ABSTRACT. The culture medium composition for cellulolytic bacteria growing on sugar cane wastes was optimized. A modified method of Rosenbrock was employed for shaker culture medium and a factorial plan design for fermentor culture medium optimization. A much more economical and productive medium was obtained for the production of single cell protein (SCP). A biomass concentration of 4.3 g/L was obtained in the optimized medium in batch fermentation, in comparison with 2.8 g/L previously obtained in the traditional medium under similar conditions.

One of the most important aspects of growing cellulolytic bacteria on cellulosic wastes for obtaining SCP is the formulation of the appropriate culture medium to be employed in the industrial process.

The analysis of the medium employed so far in our laboratories for this process shows an excess of phosphorus and potassium and a slight lack of nitrogen (Enriquez 1978). Besides that, other components have been reported to be important for the growth of cellulolytic bacteria, such as $CaCl_2$ and mineral trace elements (Srinivassan 1975; Schmid and Bomar 1975*a*, *b*).

Mathematical methods were recently employed for the optimization of microbiological processes, among them, that of Box and Wilson (1951) and Auden *et al.* (1967) and, especially in the optimization of culture media, the modified method of Rosenbrock (Votruba *et al.* 1975; Pilát *et al.* 1976*a*, *b*). The aim of our present work was to establish whether it was possible to apply modified Rosenbrock's method for the optimization of culture medium and to obtain a new medium composition more convenient for the industrial production of SCP from bagasse.

MATERIALS AND METHODS

Microorganisms. Cellulomonas sp. IIbc and GiIII (Enriquez 1978; Rodriguez *et al.* 1982) were transferred in CMC-agar tubes at 30 °C and maintained at 4 °C.

Medium. We used traditional medium M9 (Miller 1972) with the following composition (g/L) : $Na_2HPO_4.12H_2O$ 15, KH_2PO_4 3, NaCl 0.5, $MgSO_4.7H_2O$

0.25, NH_4Cl 1, thiamine 0.001, distilled water to 1 L. Pretreated bagasse (Dunlap 1969) was used at 1 % concentration.
CMC-agar: 0.5 % carboxymethyl cellulose and 2 % agar were used in medium M9.
Trace mineral solution (Schmid and Bomar 1975) had the following composition (mg/L): $FeCl_3.6H_2O$ 10, $CuSO_4.5H_2O$ 0.5, $MnCl_2.4H_2O$ 0.45, $CoCl_2.6H_2O$ 0.03, $Na_2MoO_4 . 2H_2O$ 0.4, $ZnSO_4.7H_2O$ 0.5.

Cultivation in flasks. The culture was grown on CMC-agar slopes at 30 °C for 2 d. The cells were then suspended in a sterile way and inoculated in 80 mL of medium with 1 % pretreated bagasse in 500-mL cultivation flasks. The cultivation was carried out on a reciprocal shaker at 30 °C.

Cultivation in laboratory fermentor. A Biolafitte glass fermentor was used under optimum cultivation parameters (Enriquez 1981): culture volume of 5 L, temperature 32 °C, pH 6.5, airflow 10 L/min and impeller frequency 10 Hz. The pH level was automatically regulated by adding 10 % NaOH solution. The medium was inoculated with 10 % (*V*/*V*) vegetative inoculum.

Vegetative inoculum. This was prepared in the fermentor. In the exponential growth phase the appropriate part of the culture was centrifuged and the biomass was resuspended in the same part of sterile medium.

Analyses. Growth was followed turbidimetrically at 600 nm after filtration of samples through a sintered glass filter (pore diameter 90—150 μm). This eliminates most of the residual bagasse.

During the cultivation the dry mass of the bacterial biomass was determined gravimetrically after filtering the sintered glass filtrates through a Synpor No. 6 membrane, washing and drying the cells at 105 °C to constant mass. The protein content of bacteria was determined by the biuret method (Herbert *et al.* 1971).

Mathematical methods of optimization. Rosenbrock's method. The modified method of Rosenbrock and Storey (1970) was employed for the optimization of the medium for cultivation in flasks. A detailed explanation of the method is given by Pilát *et al.* (1976*a*). The method is based on the evaluation of an optimization criterion in successive experimental runs, where each variable takes the value

$$X = X_0 + k_1\bar{v}_1 , \qquad (1)$$

where X is experimental value for the variable,

X_0 start point,
k_1 step length and
$\bar{v}_1$ orthogonal unit vector.

In our case the optimization criterion was the final biomass concentration obtained.

Each new experiment is designed according to the results of the previous one and following equation (1). When increasing the nutrient concentration „success" is defined by the condition:

$$F(X) > F(X_0) .$$

When decreasing the concentration "success" is given by

$$F(X) \geq F(X_0)$$

TABLE I. Variables for optimization of shaker medium composition (Rosenbrock method)

Variables		Nutrient	Levels	
			minimum	maximum
X_1	(proportion) g/L	$Na_2HPO_4.12H_2O/KH_2PO_4$	0	15/3
X_2	g/L	NaCl	0	6
X_3	g/L	NH_4Cl	0	6
X_4	g/L	$MgSO_4.7H_2O$	0	1
X_5	(fold concentrated)	trace mineral solution	0	1
X_6	g/L	$CaCl_2$	0	1
X_7	mg/L	thiamine	0	1

In case of "success" we will have: $k = 3k$, $\bar{X}_0 = X_1$ (the new X_0 is equal to X_1 from last step) and $\bar{X}_1 = X_0 + 3k$. (2)
For a failure:

$$k = -1/2k,\ \bar{X}_0 = X_0 \text{ and } \bar{X}_1 = X_0 - 1/2k\ . \quad (3)$$

Our plan included 7 variables (Table I). The nutrients to be optimized and their minimum and maximum values were selected in the basis of economic considerations. For variable No. 1 and 5 the k values are factors multiplying the concentration of a stock solution, where $X_1 = k$.

Concentrated stock solution from each nutrient were employed for all experiments. These were made in triplicate and statistical analysis was carried out by Student's test.

Factorial design plan (Box & Wilson method). For the fermentor cultivation medium a 2^2 factorial plan was employed (Bacon and Henson 1971). The plan is represented by the experimental matrix D:

Variables	Levels		Matrix			Run no.
	low (−1)	high (+1)				
Source of phosphorus (X_1)	KH_2PO_4	$(NH_4)_2HPO_4$				
				$X1$	$X2$	
Addition of trace mineral solution (X_2)	No	Yes	$D =$	−1	−1	1
				1	−1	2
				−1	1	3
				1	1	4

Each experiment was performed according to the signs of the experimental matrix, for a total of 4 experiments. Repetitions were made at one point for an internal estimate of variance.

RESULTS AND DISCUSSION

Media for cultivation in flasks (Rosenbrock's method)

The conditions of the different variables for each experimental run are summarized in Table II, and the corresponding results in Table III.

This result represents the final optimization. Comparison between optimized (MO) and traditional (M9) media is shown in Table IV.

TABLE II. Conditions for each experimental run[a]

Variables	Run No.														
	1			2			3			4			5		
	X_{10}	k_1	X_{11}	X_{10}	k_1	X_{11}	X_{10}	k_1	X_{11}	X_{10}	k_1	X_{11}	X_{10}	k_1	X_{11}
X_1[b]	15/3	1	4/1	15/3	2	8/2	15/3	2.5	10/2.5	10/2.5	—	—	10/2.5	—	—
X_2	0.5	0.5	1	1	1.5	2.5	1	−0.75	0.25	0.25	−2.25	0	0.25	1.13	1.4
X_3	1	1	2	3	3	5	2	−1.5	0.5	2	0.75	2.75	2	—	—
X_4	0.25	0.05	0.3	0.25	−0.025	0.23	0.25	—	—	0.25	—	—	0.25	—	—
X_5	0	1	1	0	−0.5	0	0	0.25	0.25	0	—	—	0	—	—
X_6	0	0.1	0.1	0	−0.5	0	0	0.025	0.025	0	—	—	0	—	—
X_7	0	1	1	0	−0.5	0	0	0.25	0.25	0	—	—	0	—	—

[a] X_{10} is the start point k_1 is the step length and X_{11} the experimental value for the variable.
[b] Proportion of $Na_2HPO_4.12H_2O$ to KH_2PO_4.

TABLE III. Results for the different experimental runs

Run No.	Variant[a]	Biomass g/L	Comparison with variant 0
1	0	3.6	0
	1	1.8	−
	2	3.7	+
	3	4.0	+
	4	2.7	−
	5	3.1	−
	6	3.6	−
	7	3.4	−
2	0	4.2	0
	1	3.5	−
	2	3.8	−
	3	2.5	−
	4	2.9	−
3	0	4.5	0
	1	4.7	+
	2	4.5	+
	3	3.0	−
	5	4.2	−
	6	3.6	−
	7	3.6	−
4	0	4.0	0
	3	3.8	−
5	0	4.4	0
	2	4.3	−

[a] Variant 0 — start point, variants 1 − 7 — conditions of variant 0 except for the corresponding variable.

The results of biomass concentration were recalculated to be comparable with those obtained by centrifugation (dry biomass by centrifugation corresponds to 0.66 dry biomass by filtration).

We decreased the concentration of three of the five salts in the medium, and increased one which was found to be deficient. Beside this, the addition of trace mineral elements, thiamine and $CaCl_2$ proved to have no effect in the assayed conditions.

Medium for cultivation in fermentors (factorial plan design)

The medium composition was based on the optimized medium MO (Table IV). Since no buffering capatity is needed at this level (pH is automatically regulated) we have in this case only one phosphate salt of a lower concentration.

Strain IIbc was employed in this experiment since although it requires thiamine in the culture medium, its growth at the shaker level in optimized medium (MO) was better than that of strain GiIII (Table V).

Then we decided between two different sources of phosphorus, at concentrations assessed from theoretical requirements for bacterial growth (Ribons 1970), as well as the influence of trace mineral elements at fermentor level (Table VI).

TABLE IV. Comparison of traditional with optimized media

Medium	Thiamine mg/L	$Na_2HPO_4.12H_2O$ g/L	KH_2PO_4 g/L	NaCl g/L	NH_4Cl g/L	$MgSO_4.7H_2O$ g/L	Biomass g/L
Shaker culture[a]							
Traditional (M9)	1	15	3	0.5	1	0.25	2.5
Optimized (MO)	—	10	2.5	0.25	2	0.25	2.8
Fermentor culture[b]							
Optimized	1	—	0.6	0.25	2	0.25	4.3

[a] Strain GiIII.
[b] Strain IIbc.

TABLE V. Comparison of the growth of strains GiIII and IIbc in cultivation flasks

Strain	Medium	Biomass, g/L	Yield[a]
IIbc	MO	3.6	0.326
GiIII	MO	2.8	0.263

[a] g biomass per g feed bagasse.

In Table VI are shown the results based on the biomass production for the 4 runs of the plan.

From the 4-run design it is possible to estimate the parameters of the polynomial equation

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_1 .$$

The coefficients can be calculated from matrix X (matrix of results) and they therefore determine the significance of the corresponding variable (Bacon and Henson 1971). The results of the calculation of the coefficients are given in Table VII. The significance was analyzed by Fisher's test. The addition of mineral salts (variable X_1) as well as the interaction between both variables has no significant effect on biomass production. On the contrary, coefficient b_2 is significant and negative, indicating that variable X_2 (KH_2PO_4) must be properly chosen and, moreover at low level.

TABLE VI. Results from the 2^2 factorial plan optimization

Run no.	Variable	Biomass, g/L
1	KH_2PO_4	4.2
2	$(NH_4)_2HPO_4$	0.1
3	KH_2PO_4 + mineral salts	4.2
4	$(NH_4)_2HPO_4$ + mineral salts	0.4

Poor growth was observed when $(NH_4)_2HPO_4$ was used as the source of phosphorus. In one of these runs, and after growth had become stationary, sterile KH_2PO_4 (0.6 g/L) was added to to the medium (Fig. 1). After several hours the strain started to grow again to high absorbance and biomass production values. The most logical explanation is that potassium is required for the growth of the bacteria, knowing the effect of this element in the growth of many microorganisms and especially in the cellulolytic ones (Siu 1951).

A new set of cultivations were carried out with double the assayed concentration of KH_2PO_4 (1.2 g/L). An average result of 4.2 g/L of biomass was

TABLE VII. Values of the calculated coefficients from the polynomial equation

Coefficient	Value	Significant
b^0	2.2193	—
b_1	−2.0018	yes
b_2	0.3410	no
b_{12}	0.0873	no

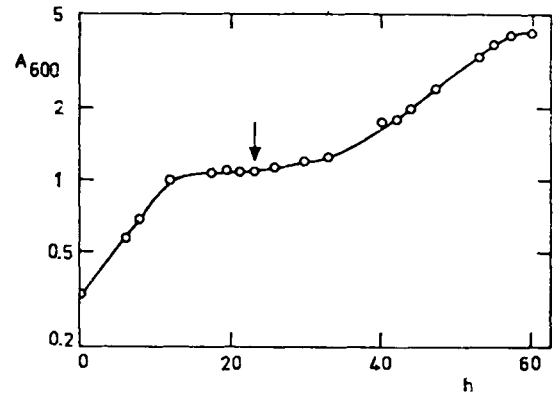

FIG. 1. Growth of *Cellulomonas* sp. IIbc (absorbance, A_{600}) in medium with $(NH_4)_2HPO_4$ as the source of phosphorus with a further addition of KH_2PO_4 (0.6 g/L; *arrow*).

obtained, not being significantly different from the 4.3 g/L value at 0.6 g/L concentration. We may then consider this as the approximately optimum concentration for this nutrient.

We should point out that the results of biomass production in these cultures are partially influenced by the remaining small cellulosic particles in the biomass, so "pure" biomass production should be determined by other means. Therefore, the final concentration of protein in the medium is also of important practical value, since it is in fact the production of protein which is the objective of this work.

The results of cultivation of the strain in the optimized medium are compared with previous results in the traditional M9 medium in Table V. The final protein content of biomass was 49 $\pm$ 1 % in all cases.

As can be seen the new results compare favourably with previous ones, probably due to the higher NH_4Cl concentration.

Another reason could be the lower concentrations of K^+ and NaCl, which are reported to be, in high concentrations, inhibitory to cellulolytic activity (Siu 1951).

It thus appears that an application of mathematical methods for the optimization of the cultivation medium is also suitable for cellulolytic bacteria and in fact a new medium has been obtained. By its application the saving of mineral salts in the production of 1 Mg (1 ton) of bacterial biomass from bagasse amounts to 5.36 Mg of $Na_2HPO_4.12H_2O$ and 0.93 Mg of KH_2PO_4.

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