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Application of Mathematical Optimization Methods in Microbiology

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ABSTRACT. Mathematical methods of experiment design have so far found little use in the optimization of microbiological processes. The conventional optimization procedure is still the transformation of functional relationship of n variables into n unidimensional optimizations; furthermore, the Box-Wilson gradient method is often used. This paper presents a review of methods used in other fields, and their application in microbiological practice. The methods are classified according to whether they require, besides the simple determination of the objective function (direct search methods), also the finding of its first (gradient methods) or second derivative (Newton-Raphson method). A modified Rosenbrock's method of direct optimum search and the gradient Box-Wilson method were used in parallel for the optimization of yeast growth on methanol. Their comparison showed that Rosenbrock's method is more suitable for multiparameter systems.

OPTIMIZATION METHODS

The economical utilization of every microbiological process requires the optimization of cultivation conditions. These include the selection and dosage of energy and carbon source, nitrogen, phosphorus, potassium and magnesium sources, growth substances, *etc.*, both at the qualitative and quantitative level. Other equally important cultivation parameters are *e.g.* level of dissolved oxygen in the medium, pH of cultivation medium, and cultivation temperature. In the way of optimization criterion, the highest cell yield, growth rate, or the amount of product can be chosen. The optimization of cultivation conditions is still done mostly using empirical methods which either fail to project adequately the mutual relationships of individual optimized parameters or are excessively laborious and time-consuming, especially when a higher number of variable parameters is involved. The optimization should generally take into account, and analyse systematically, the influence of all factors under study on optimization criterion, *e.g.* the growth rate. A number of optimum search methods based on mathematical experiment design have been evolved in the chemical industry to bear on various technological regimes (Nalimov and Chernova, 1965; Biryukov, 1969; Himmelblau, 1970; Adler *et al.*, 1975). These methods are designed for multidimensional systems and make it possible to seek the free extreme or the extreme with limitations. Each of the methods has its specific properties that restrict the extent of its use. The selection of the method itself represents thus the keynote step of every optimization treatment. The factors to be taken into account include *e.g.* the number of variables defining the objective func-

tion under study, the accuracy of their measurement, required accuracy of optimum attainment, computation facilities, and, above all, the type of the process examined and the way of obtaining experimental data (the values of variables and the objective function). One method is suitable for a set of parallel experiments, another for a successive series of experiments. This last criterion is of special importance in biological processes where single successive experiments are sometimes hard to reproduce. The extreme search methods can, in general, be divided into three groups: direct search methods requiring no derivative of the objective function according to parameters, gradient methods requiring the first derivative, and the Newton-Ralphson method requiring both first and second derivative. The methods applicable in microbiology are surveyed in the paper by Maksimov and Fedorov (1969).

DIRECT SEARCH METHODS

Alteration of single variable. This method reduces the multidimensional optimum search procedure to a one-dimensional one ("one-factor-at-a-time method"). One parameter is variable while all the others are kept constant. This process results in determining a value of the variable which corresponds to an optimum value of the criterion under study. The procedure is carried out with all optimized factors and is repeated until the required accuracy of objective function determination is reached. The large number of experiments needed to find individual local optima and the overall optimum of the process makes the method very slow and unwieldy; it also fails to project adequately the mutual interactions of individual optimized parameters. Despite these shortcomings, the method is still currently used in microbiological practice.

Optimization by multidimensional factor plan. The method is based on a factor plan of the experiment (Nalimov and Chernova, 1965; Nalimov, 1975) set up by dividing the searched parameter region into uniform segments, performing experiments in the points so obtained, and determining the magnitude of the objective function. The network is then condensed in the places of the optimum values found. The method thus represents a mapping of the whole parameter area. Its application to biological problems was described *e.g.* by Lewis and Ijichi (1957), Auden *et al.* (1967), Fedorov *et al.* (1968), McDaniel *et al.* (1970), Grachev *et al.* (1970), Krayushkina *et al.* (1973), and Dumenil *et al.* (1975*a*). Details of the multiplimensional factorial plan are given below in the paragraph on the Box-Wilson method.

Rosenbrock's method. The original method (Rosenbrock and Storey, 1970) had to be modified for the work with biological material (Votruba *et al.*, 1975). The method makes use of an information on the objective function (optimization criterion) determined by measurement. At the beginning of optimization for n variables $x_1, x_2, \ldots$ $\ldots x_n$, one has to chose n orthogonal unit vectors $\bar{v}_1, \bar{v}_2, \ldots \bar{v}_n$ and a set of n lengths of unit steps $k_1, k_2, \ldots k_n$. The step lengths may be both positive and negative. Segments of $k_1\bar{v}_1, k_2\bar{v}_2, \ldots k_n\bar{v}_n$ are gradually added to the selected starting point $\bar{x}_o = (x_1, x_2, \ldots x_n)$, yielding new points $\bar{x}_1, \bar{x}_2, \ldots \bar{x}_n$. A further step is the comparison of the values of objective function at points $\bar{x}_o$ and $\bar{x}_1, \bar{x}_2, \ldots \bar{x}_n$, namely $F(\bar{x}_o)$ and $F(\bar{x}_1), F(\bar{x}_2), \ldots F(\bar{x}_n)$. If the objective function is to be minimized, such as is the case *e.g.* with cultivation period, then if $F(\bar{x}_1) \leqq F(\bar{x}_o)$, the $\bar{x}_o$ is in the next step replaced by $\bar{x}_1$ and, consequently, $\bar{x}_o = \bar{x}_1$ and $F(\bar{x}_o) = F(\bar{x}_1)$. Provided that $F(\bar{x}_1) > F(\bar{x}_o)$, the shift of the coordinate in direction v_1 has been unsuccessful, and $\bar{x}_o$ and $F(\bar{x}_o)$ retain their original values during the following measurement. A similar procedure is adopted during the search of objective function changes in

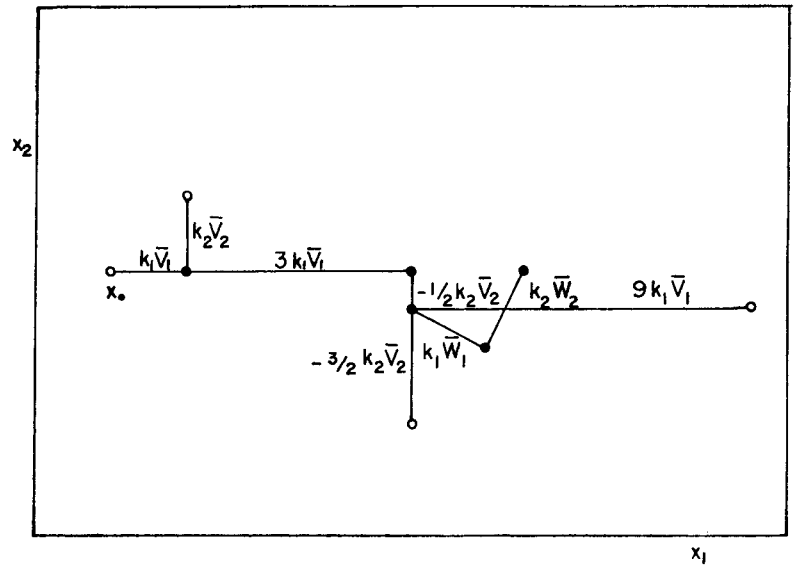

FIG. 1. Schematic outline of Rosenbrock's optimization method for $n = 2$.

directions $\bar{v}_2, \ldots \bar{v}_n$ and thus at points $\bar{x}_2, \ldots \bar{x}_n$. The maximization of the objective function, *e.g.* with specific growth rate, is done likewise but the expected outcome is that $F(\bar{x}_1) \geqq F(\bar{x}_o)$, *etc.* Changes in all optimized directions are measured at the same time. The procedure remains the same in the following experiments, the only change being in the lengths $k_j (j = 1, \ldots n)$. After each comparison of objective functions $F(\bar{x}_o + k_j \bar{v}_j)$ and $F(\bar{x}_o)$ the length of step k_j is multiplied by factor 3 (in the case of success) or $-1/2$ (after a failure). The values of these factors have been recommended by Rosenbrock and Storey (1970) on the basis of a numerical experiment. A sequence of unsuccessful experiments results thus in the shortening of step length and an oscillation in the sign, a series of successful experiments leads to a rapid advance towards the optimum. If at least one unsuccessful step has been done during the evaluation in each of the directions $\bar{v}_j$, the original set of vectors $\bar{v}_j$ $(j = 1, \ldots n)$ is superseded by a new system of orthogonal unit vectors w_j $(j = = 1, \ldots n)$ using Gram-Schmidt orthogonalization (Rosenbrock and Storey, 1970; Votruba *et al.*, 1975). After the rotation of the original vectors, the new vectors are used to carry out a new computation cycle with the original step lengths k_j $(j = = 1, \ldots n)$ or with lengths used for the last unsuccessful step in the given direction. The original starting point $\bar{x}_o$ is replaced by point $\bar{x}_j$ obtained from the previous measurements. For the sake of clarity, the procedure is illustrated graphically in Fig. 1 (for 2 factors). The method has not yet been used in microbiology, but it has served for a successful optimization of chemical processes (Hoffmann and Hoffmann, 1971).

Hook-Jeeves method. The basic tenets of the procedure are the same as with Rosenbrock's method but it differs from the former technique in that the search vectors are not rotated after an unsuccessful step but the measurement is carried on with original vectors until the selected accuracy limit is exceeded (Hooke and Jeeves, 1961). Historically, the Hooke-Jeeves method is a predecessor of the Rosenbrock's

method and served as a basis which was further developed and modified by Rosenbrock and Storey (1970).

Simplex method. This method requires a selection, in an n-dimensional space, of a regular polyhedron determined by $n + 1$ points, called simplex (Hoffmann and Hoffmann, 1971; Kubíček a Hlaváček, 1972). In a two-dimensional space, three points are thus selected, forming an equilateral triangle, and the values of objective function at these points are determined. In the next step the following point is defined so that it forms a new regular triangle with two vertexes of the original triangle; three possible selections offer themselves in a two-dimensional space. The comparison of objective function values of the eliminated and the newly chosen point in the triangle gives the direction of approach to optimum. The length of the triangle side can be adjusted so that the functional value (optimization criterion) increases or decreases depending on the aim of the procedure. The method is extremely suitable for multidimensional systems. Its practical use is mostly with asymmetrical polyhedron based on a modified empirically derived computation procedure described by Nelder and Mead (1965). Each step permits the performance of only one experiment, which is unsuitable for microbiological processes. Like Rosenbrock's method, this procedure can also be modified. Simplex method was practically applied in the optimization of intermediary product in successive reactions (Morgan and Deming, 1973, 1974; Dean *et al.*, 1975). The application of the method in biology was described by Fedorov *et al.* (1968), Pisarenko *et al.* (1971), Tsaplina *et al.* (1973) and Dumenil *et al.* (1975*b*). Older literature was reviewed by Nalimov (1975).

Other procedures for direct optimum search are *e.g.* the techniques described by Powell (1962; 1964). Combination of Powell's and the Hook-Jeeves technique was used by Undén and Hedén (1973) for optimal fermentor control.

GRADIENT METHODS

In these methods, the search for optimum proceeds in the direction of the steepest slope, *i.e.* in the direction of objective function gradient

$$\nabla F(x) = \text{grad } F(x) = e_1 \frac{\partial F}{\partial x_1} + e_2 \frac{\partial F}{\partial x_2} + \dots e_n \frac{\partial F}{\partial x_n},$$

where $x_1, x_2, \dots x_n$ are individual optimized variables and $e_1, e_2, \dots e_n$ are base unit vectors.

Box-Wilson method. The principle of the method is the interpolation of regression function through experimentally obtained objective function values in points defined according to a predetermined plan. The optimum is then approached in the direction of the gradient of regression function since in this direction the function has its steepest ascent (Box and Wilson, 1951). $F = F(x) = F(x_1, x_2, \dots x_n)$ is the optimized objective function (*e.g.* specific growth rate) that depends on n variables $(x_1, x_2, \dots x_n)$. The point $x^o = (x_{10}, x_{20}, \dots x_{no})$ is an arbitrary starting point and values $d_1, d_2, \dots d_n$ are differences corresponding to coordinates $x_{10}, x_{20}, \dots x_{no}$. The values $x_{jo} \pm d_j$, where $j = 1, 2, \dots n$, define generally two levels for each coordinates. All combinations of these values, whose total number is 2^n, define then the coordinates of points in the vicinity of the starting point x^o. A 2^n factorial plan can then be set up for n coordinates as follows:

Coordinate of vicinity point	x_1	x_2	x_3	...
x^1	+	+	+	...
x^2	−	+	+	...
x^3	+	−	+	...
x^4	−	−	+	...
x^5	+	+	−	...
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If $F_1, F_2, \ldots F_{2n}$ are values of function F in points $x_1, x_2, \ldots x_{2n}$, the n-dimensional linear regression function interpolated through these points by the least squares method has the equation $y = a_o + a_1x_1 + a_2x_2 + \ldots a_nx_n$. This equation is the first approximation of function F at point x^o. The regression function gradient determines the direction of steepest ascent of function F at point x^o

$$\text{grad } y = \nabla y = e_1 \frac{\partial y}{\partial x_1} + e_2 \frac{\partial y}{\partial x_2} + \ldots e_n \frac{\partial y}{\partial x_n} .$$

The comparison implies that

$$\frac{\partial y}{\partial x_1} = a_1 , \quad \frac{\partial y}{\partial x_2} = a_2, \quad \ldots \quad \frac{\partial y}{\partial x_n} = a_n ,$$

where $a_1, a_2, \ldots a_n$ are regression function coefficients. The next step is the selection of the optimum approach step length (H) and an advance from the starting point x^o in the direction of function y gradient by whole multiples of step length H. The coordinates of the next points are thus defined by:

$$x^H = \left(x_{10} + H\frac{\partial y}{\partial x_1} ; x_{20} + H\frac{\partial y}{\partial x_2} ; \ldots x_{no} + H\frac{\partial y}{\partial x_n}\right)$$

$$x^{2H} = \left(x_{10} + 2H\frac{\partial y}{\partial x_1} ; x_{20} + 2H\frac{\partial y}{\partial x_2} ; \ldots x_{no} + 2H\frac{\partial y}{\partial x_n}\right)$$

$$x^{mH} = \left(x_{10} + mH\frac{\partial y}{\partial x_1} ; x_{20} + mH\frac{\partial y}{\partial x_2} ; \ldots x_{no} + mH\,\partial y\,\partial x_n\right) ,$$

i.e.

$$x^H = (x_{10} + Ha_1; x_{20} + Ha_2; \ldots; x_{no} + Ha_n)$$

$$x^{2H} = (x_{10} + 2Ha_1; x_{20} + 2Ha_2; \ldots; x_{no} + 2Ha_n)$$

$$x^{mH} = (x_{10} + mHa_1; x_{20} + mHa_2; \ldots; x_{no} + mHa_n) ,$$

where $m = 1, 2, \ldots, M$; M is the total number of approach steps. These relationships hold for maximization of objective function, in the case of minimization the

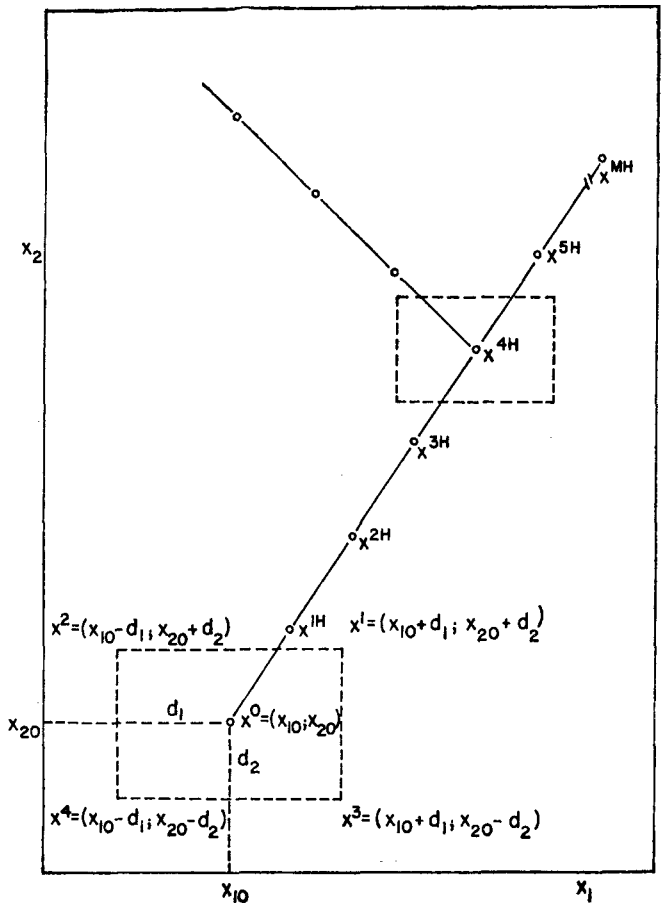

FIG. 2. Schematic outline of Box-Wilson optimization method for $n = 2$.

signs of the inequalities change. The coordinates x^{mH} correspond to functional values of $F^{mH} = F(x^{mH})$. The approach towards the optimum ends at step m in point x^{mH} which satisfies the formula:

$$F^{(m-1)H} \leqq F^{mH} \geqq {}^{(m+1)H} .$$

The value $F^{mH} = F(x^{mH})$ is assumed to be the optimum of function F attained in point x^{mH}. The criterion for the optimum is based on testing if the functional value in the assumed optimum point is higher or equal to functional values of the points of the 2^n-factorial plan with the optimum as the central point.

If the attained accuracy of approach is considered insufficient, the point x^{mH} is taken as the new starting point and the whole procedure is repeated. Fig. 2 demonstrates the whole procedure for $n = 2$. The above equations are valid when the magnitudes of individual coordinates and differences are mutually compatible, *i.e.* of the same order. In the opposite case coordinate transformation is to be carried out (Schröder, 1972).

The Box-Wilson method is used in chemistry, predominantly for the optimization of catalysed reactions. It has also been used in microbiology to a considerable extent (Bogorov *et al.*, 1965; Auden *et al.*, 1967; Maksimova *et al.*, 1968; Veksler *et al.*, 1970;

Loginova *et al.*, 1970*a*,*b*; Zeltin', 1970; Voino *et al.*, 1970; Iegorov *et al.*, 1971; Pisarenko *et al.*, 1971; Balitskaya *et al.*, 1973; Ustinnikov *et al.*, 1973; Murgov and Zaitseva, 1973; Schröder and Weide, 1974; Palagina *et al.*, 1975).

Box-Wilson method with half factorial plan. The satisfactory number of linear independent equations necessary to calculate the coefficients of n-dimensional regression function is $n + 1$. Since the number of experiments defined by the 2^n-factorial plan is excessive for $n > 1$, it can be reduced (most often by half, *i.e.* to $2^n/2$) while preserving the validity of the condition $2^n/2 \geqq n + 1$. This condition is satisfied for $n \geqq 3$. The criterion for the selection of $2^n/2$ measured points is such that for the selected point the number of levels of individual parameters $(x_{j0} + d_j)$ is even. This simplification of the above full-factorial-plan Box-Wilson method may bring about an increased effect of a random error on the level of optimization direction. This shortened Box-Wilson method has also been used for microbiological applications (Bogorov *et al.*, 1965; Zeltin', 1970; Maksimov, 1971; Schröder, 1972).

NEWTON-RAPHSON METHOD

The search by this method is commenced by assuming that grad $F(x) = O$. On expanding this function into the Taylor series (up to the second order) for several variables, a simple iteration formula ensues

$$x^{(i+1)} - x^{(i)} = -[F_{xx}(x^i)]^{-1} \cdot F_x(x^i) ,$$

where one terms denotes the Hessian and the other the Jacobian (Hoffmann and Hoffmann, 1970; Kubíček and Hlaváček, 1972). The method is applicable when second derivatives of objective function according to parameters are known. However, the determination of second derivatives may pose considerable difficulties and the number of experiments necessary to search the vicinity rises undesirably.

The optimization of vitamin requirements and the composition of medium used for cultivating yeast on methanol served to compare the modified Rosenbrock method with the Box-Wilson method with both complete and half factorial plan, with respect to the speed of optimum attainment and laboriousness.

MATERIALS AND METHODS

Cultivation methods. The culture used was *Candida boidinii* 11 Bh (Volfová and Pilát, 1974) transferred in shaking flasks with methanol. The basic cultivation medium contained: 1.65 g NH_4Cl, 6.3 g KNO_3, 7 g KH_2PO_4, 0.5 g $MgSO_4 . 7 H_2O$, 0.1 g NaCl, 0.1 g yeast extract in 1000 ml tap water. Methanol, as the carbon and energy source, was added after medium sterilization to a concentration of 1% v/v. Cultivation temperature was 30 °C, pH was 4.9—5.0. Experiments were carried out on a reciprocal shaker at a frequency of 1.67 s^{-1}.

Analytical methods. The concentration of yeast cells was determined spectrophotometrically (spectrophotocolorimeter SPEKOL) at 540 nm. Calibration curve was obtained gravimetrically (Volfová and Pilát, 1974).

RESULTS

The character of work with microbiological material makes it difficult to carry out a series of successive experiments, maintaining at the same time the same level

of accuracy and reproducibility. It was therefore necessary to find such methods which would make it possible to perform a series of experiments simultaneously, *i.e.* in parallel. This condition is satisfied by the Box-Wilson method which employs an alternate measurement of the vicinity of the starting point according to the 2^n-factorial plan with the measurement of approach steps, and is thus well feasible for a parallel set of experiments. This gradient method has its merits and shortcomings; the main shortcoming is the possibility of a distortion of optimization direction near optimum, especially when the optimized objective function assumes there a flat course. Another drawback is the appreciable laboriousness when measuring the vicinity of the starting point in $2^n + 1$ experiments for n variables. For this reason Rosenbrock's direct search method was also employed and compared with the Box-Wilson procedure. The method was modified so as to suit a parallel, simultaneous, measurement of $(n + 1)$ experiments (Votruba *et al.*, 1975). To evaluate the optimization procedure, the corresponding objective function (optimization criterion) had to be defined. Our studies were concerned with the growth of a culture hence, after preliminary experiments, the most suitable criterion appeared to be the cultivation time required to attain a certain culture growth, or the culture growth within a selected time interval, both at a constant initial yeast concentration. The criterion is very simple and encompasses both the magnitude of specific growth rate and the length of the lag-phase of growth. The concentration of yeast cells was determined by absorbence measurement. The initial absorbence value was 0.02 and the final 0.2, corresponding to 0.55 g/litre yeast dry weight; this final concentration (and absorbence level) represented an upper limit since at higher cell concentrations in the flasks the growth was retarded due to oxygen limitation.

Both methods were compared using a two-parameter (vitamin requirement) and five-parameter (composition of cultivation medium) optimization.

Two-parameter optimization (requirement for vitamins)

The yeast *Candida boidinii* 11 Bh is capable of growing on a mineral medium without the addition of growth factors but the growth rate is strongly stimulated by the addition of biotin or thiamine. The experiments were conducted with the medium described above, in which yeast extract was replaced by biotin and thiamine. The culture, grown on a medium containing yeast extract (2% v/v methanol, 48 h) brings with it a certain level of vitamins; prior to the experiment, the yeast was therefore centrifuged, washed, and kept for 24 h in a medium without yeast extract (1% v/v methanol) on a shaker. The resulting culture was used in the experiments, adjusted to starting absorbence 0.02, 1% v/v methanol. All calculated variants were cultivated in 2—4 parallel flasks to exclude the possibility of an effect

TABLE I. 2^n-Factorial plan for optimization by the Box-Wilson method

Measured variant	Levels of factors	
	x_1, μg/litre	x_2, mg/litre
1	$x_{10} + d_1 = 0.04$	$x_{20} + d_2 = 0.4$
2	$x_{10} - d_1 = 0.02$	$x_{20} + d_2 = 0.4$
3	$x_{10} + d_1 = 0.04$	$x_{20} - d_2 = 0.2$
4	$x_{10} - d_1 = 0.02$	$x_{20} - d_2 = 0.2$
0	$x_{10} = 0.03$	$x_{20} = 0.3$

TABLE II. Initial conditions for optimization by the Rosenbrock method

Measured variant		Levels of factors	
		x_1, μg/litre	x_2, mg/litre
0	$\bar{x}_o$	0.02	0.2
1	$\bar{x}_o + k_1\bar{v}_1$	0.04	0.2
2	$\bar{x}_o + k_2\bar{v}_2$	0.02	0.4

of a random error. The starting point and individual differences were chosen so as to ensure identical conditions of the initial experiment for both methods.

Box-Wilson method: $x_{10} = 0.03$ μg/litre (biotin); $x_{20} = 0.3$ mg/litre (thiamine); $d_1 = 0.01$ μg/litre; $d_2 = 0.1$ mg/litre. Table I shows the 2^n-factorial plan for initial experiment.

Rosenbrock method: $x_1 = 0.02$ μg/litre (biotin); $x_2 = 0.2$ mg/litre (thiamine); $k_1 = = 0.02$ μg/litre; $k_2 = 0.2$ μg/litre; $\bar{v}_1 = (1.0)$; $\bar{v}_2 = (0.1)$. Initial experimental conditions are given in Table II.

Initial experiments, representing the measurement of the starting point vicinity, are followed in the Box-Wilson method by the selection and measurement of approach steps towards the optimum (Fig. 2). The results of determination of the starting point vicinity were evaluated using a Hewlett-Packard 9810 A calculator. The difference in the concentration of the two vitamins between two successive steps was chosen so as to ensure a sufficient difference in the value of the optimization criterion. It was also necessary to find a suitable relationship between the length and the number of steps. In our experiments, the number was always 5.

The Rosenbrock procedure is much simpler up to the stage of unit vector rotation. If the value of the optimization criterion in the i-th direction improves, the length of initial approach step is multiplied by 3, in the opposite case it is multiplied by $-1/2$. The survey of optimization results with the two methods is given in Table III.

Five-parameter optimization (cultivation medium composition)

This represents a more complex system which permitted a better comparison of the two methods both with respect to the speed of optimum attainment and the amount of labour required. The optimization was done with 5 components of the medium: x_1 — yeast extract (source of growth factors), x_2 — nitrogen source, x_3 — KH_2PO_4 (phosphorus and potassium source), x_4 — $MgSO_4 . 7 H_2O$ (magnesium source), and x_5 — $ZnSO_4 . 7 H_2O$ (source of zinc). The influence of zinc was proved

TABLE III. Optimization of biotin and thiamine requirement

Method	Biotin concentration μg/litre	Thiamine concentration mg/litre	Number of experiments	Number of variants
Box-Wilson	7.5	2.6	6	33
Rosenbrock	7.3	2.8	7	21

TABLE IV. Starting point and differences (Box-Wilson)

	x_1, mg/litre	x_2, g/litre	x_3, g/litre	x_4, g/litre	x_5, mg/litre
x_{i0}	3	0.10	0.10	0.05	1.0
d_i	2	0.05	0.05	0.03	1.0

in a preceding study (Pilát, 1975). Zinc ions stimulated the growth rate somewhat and enhanced the content of proteins in yeast biomass. The maximum concentration of yeast extract in the medium was set at 100 mg/litre (optimization by seeking an optimum with constraints or restrictions). Culture grown in basic medium with 2% (v/v) methanol for 48 h was transferred into 10-fold diluted medium with 1% (v/v) methanol without yeast extract and cultivated for 8 h. The ensuing inoculum was used in further experiments. The cultivation was carried out in 500-ml cultivation flasks filled each with 50 ml medium of a calculated composition, prepared by dis-

TABLE V. Schematic 2^n-factorial plan for 5 variables in transformed coordinates

Variant	Levels of factors				
	1	2	3	4	5
1	+1	+1	+1	+1	+1
2	−1	+1	+1	+1	+1
3	+1	−1	+1	+1	+1
4	−1	−1	+1	+1	+1
5	+1	+1	−1	+1	+1
6	−1	+1	−1	+1	+1
7	+1	−1	−1	+1	+1
8	−1	−1	−1	+1	+1
9	+1	+1	+1	−1	+1
10	−1	+1	+1	−1	+1
11	+1	−1	+1	−1	+1
12	−1	−1	+1	−1	+1
13	+1	+1	−1	−1	+1
14	−1	+1	−1	−1	+1
15	+1	−1	−1	−1	+1
16	−1	−1	−1	−1	+1
17	+1	+1	+1	+1	−1
18	−1	+1	+1	+1	−1
19	+1	−1	+1	+1	−1
20	−1	−1	+1	+1	−1
21	+1	+1	−1	+1	−1
22	−1	+1	−1	+1	−1
23	+1	−1	−1	+1	−1
24	−1	−1	−1	+1	−1
25	+1	+1	+1	−1	−1
26	−1	+1	+1	−1	−1
27	+1	−1	+1	−1	−1
28	−1	−1	+1	−1	−1
29	+1	+1	−1	−1	−1
30	−1	+1	−1	−1	−1
31	+1	−1	−1	−1	−1
32	−1	−1	−1	−1	−1

TABLE VI. Starting point and steps (Rosenbrock)

	x_1, mg/litre	x_2, g/litre	x_3, g/litre	x_4, g/litre	x_5, mg/litre
x_{10}	1	0.05	0.05	0.02	0
k_i	4	0.10	0.10	0.05	2

solving separate components in distilled water. Additional components included NaCl (0.1 g/litre), 1% (v/v) methanol, and solution of trace elements. The measurement was done in two parallel flasks. The optimization criterion was an absorbence of the culture attained after a selected cultivation interval. Initial absorbence was 0.02 and individual cultivation intervals were chosen so that the growth of the culture corresponded to an absorbance of 0.2. The selection of the starting point was based on the elementary composition of cells. The starting point and the individual differences were chosen so that the conditions of the initial experiments were the same for both methods. The initial conditions for the Box-Wilson method are presented in Table IV; Table V gives the plan of individual measurement variants. In the Table, +1 stands for $(x_{io} + d_i)$, level −1 denotes $(x_{io} - d_i)$. Box-Wilson method with half factorial plan was done with variants having an even number of positive φ_i, *i.e.* variants 2, 3, 5, 8, 9, 12, ... (a total of 16 variants). The initial conditions used for the Rosenbrock method are shown in Table VI. The selected unit vectors were $\bar{v}_1 = (1, 0, 0, 0, 0)$, $\bar{v}_2 = (0, 1, 0, 0, 0)$, $\bar{v}_3 = (0, 0, 1, 0, 0)$, $\bar{v}_4 = (0, 0, 0, 1, 0)$, $\bar{v}_5 = (0, 0, 0, 0, 1)$ and all corresponding variants were measured (Table VII). Variants given in Tables V and VII represent the plan of the first optimization experiment. The subsequent procedure used with the two methods has been described above. A Hewlett-Packard 9810 A calculator was used for a simultaneous evaluation of the direction of optimization approach steps for both modifications of the Box-Wilson method. The differences in the determination of this direction between the two modifications were practically nonexistent, although the shortened Box-Wilson method employs the appraisal of only half the measured points as compared to the original version. It was not necessary to perform the vector orthogonalization in Rosenbrock's method.

The results of the optimization with the two basic methods are summarized in Table VIII. The scanning of individual values of the optimization criterion (absorbence after 24 h of growth) shows them to be practically identical (the error of the measurement was 0.002—0.004) and it can be concluded that no increase in

TABLE VII. Measured variants in initial experiment optimized by Rosenbrock's method

Variant	Coordinates
0	x_o (starting point) $= (x_{10}, x_{20}, x_{30}, x_{40}, x_{50})$
1	$x_o + k_1\bar{v}_1 = (x_{10} + k_1, x_{20}, x_{30}, x_{40}, x_{50})$
2	$\bar{x}_o + k_2\bar{v}_2 = (x_{10}, x_{20} + k_2, x_{30}, x_{40}, x_{50})$
3	$\bar{x}_o + k_3\bar{v}_3 = (x_{10}, x_{20}, x_{30} + k_3, x_{40}, x_{50})$
4	$\bar{x}_o + k_4\bar{v}_4 = (x_{10}, x_{20}, x_{30}, x_{40} + k_4, x_{50})$
5	$\bar{x}_o + k_5\bar{v}_5 = (x_{10}, x_{20}, x_{30}, x_{40}, x_{50} + k_5)$

TABLE VIII. Five-parameter optimization of cultivation medium composition and its comparison with the composition of original medium

Method	Yeast extract mg/litre	N g/litre	KH_2PO_4 g/litre	$MgSO_4 . 7 H_2O$ g/litre	$ZnSO_4 . 7 H_2O$ mg/litre	Optimization criterion
Box-Wilson	100	0.73	0.86	0.20	14.4	0.198
Rosenbrock	100	0.675	1.35	0.22	8.0	0.199
Original	100	1.32	7.00	0.50	10.0	0.200

TABLE IX. Laboriousness of individual optimization methods

Method	Number of experiments	Number of variants
Box-Wilson	6	101
1/2 Box-Wilson	6	61
Rosenbrock	5	30

the growth rate has been achieved; however, an equally important positive result of the series of optimization steps is the definition of a medium which ensures equal growth of the culture as the original medium but which contains much lower quantities of individual components, in particular the phosphorus source. This represents a considerable achievement from the viewpoint of economy of the process. The methods were also compared with respect to their laboriousness (Table IX). As seen from the Table, the number of variants to be measured is considerable and it increases, with both the Box-Wilson and Rosenbrock methods, with rising number of optimized parameters (*cf.* Table III). The number of variants can be reduced by using the Box-Wilson method with half factorial plan; the accuracy of optimum determination is not significantly lowered.

DISCUSSION

Out of the above array of mathematical methods of experiment design, the Box-Wilson method (in two modifications) and Rosenbrock's method were chosen for optimization of cultivation conditions. Both procedures require the solution of several methodical problems prior to the experiment. The first of these is the selection of optimization criterion. This problem is particularly significant in the work with biological material. Decisive role is played by the aim of the experiment; it can be the achievement of a maximum growth of the culture tested, accumulation of a certain metabolite (antibiotic, amino acid, *etc.*), or other requirements. The corresponding criterion may be simple (in our case the growth of culture within a certain time interval or an interval needed to attain a preset growth at constant inoculum size). A more complex criterion may include *e.g.* economical fermentation aspects such as the price of raw material, product price, *etc.* (Votruba *et al.*, 1975). The accuracy of attainment of optimum conditions is affected by the relative or absolute experimental error. Although in our case this error was small (about 2%),

the optimization had to be eventually discontinued because the error exceeded the differences between the values of optimization criterion in individual measured variants. This fact has also a bearing on the shape of the objective function. Another problem is the selection of the starting point for optimization and of the magnitudes of appropriate steps (or differences) during the search for optimum. The selected initial conditions are to be chosen so as to ensure a sufficiently sizable optimization effect and thus a sufficiently accurate determination of optimization direction. This holds especially for the Box-Wilson gradient method. With this method, the determination of the vicinity of the starting point is followed by the measurement of approach steps in the direction of the gradient of the determined regression function. It is imperative that a suitable relation be found between the size and number of these steps. In our case the number of approach steps was always 5; experience showed that this number is suitable especially in the initial phases of the procedure while near the optimum a higher number of relatively fine steps is better suited for increasing the accuracy of the method and reducing the number of experiments. This fact is of particular importance with higher numbers of optimization parameters. The Rosenbrock method, as a direct search procedure, leads to a rapid advance to the optimum but a significant factor is even here the measure of missing the optimum between the last successful and the first unsuccessful step. On registering a failure in all directions, initial unit vectors are swivelled (rotated) and the optimization proceeds anew in the resulting new directions. A selection of excessively large initial step may cause a complication in the procedure following the axis rotation, which may necessitate a repeated rotation of the vectors to achieve the optimum.

A separate problem is the selection of the optimized cultivation parameters; certain knowledge of the optimized process is required to avoid excessive complexity of the model to be set up.

The comparison of the two methods showed clearly the advantages of the Rosenbrock method, above all concerning laboriousness. Its superiority over the alternative procedure would be even more pronounced on using a larger number of parameters measured. Another advantage of Rosenbrock's method is the very simple mathematical apparatus used up to the stage of vector rotation. On the other hand, the results indicate that these advantages of the method would be greatly reduced if the optimized process had an objective function of a non-Monod character with an unambiguous extreme. This would certainly require the rotation of the initial vectors and the whole series of experiments would be appreciably extended as compared to the Box-Wilson method. With a more numerous set of optimized parameters the method of choice would nevertheless be the Rosenbrock procedure since in it each experiment includes the measurement of $n + 1$ variants while with Box-Wilson method this number increases in an exponential manner, being equal to $2^n + 1$ for the measured vicinity of the initial point ($2^n/2 + 1$ for the half-factorial modification), n being the number of optimized parameters. Due to this fact the Box-Wilson method would be unfeasible with a simultaneous optimization of 8 or more cultivation parameters whereas Rosenbrock method permits the simultaneous optimization of 10—15 parameters.

REFERENCES

Adler Yu. P., Markova E. V., Granovskii Yu. V.: The design of experiments to find optimal conditions. Mir Publs., Moscow. 1975.

Auden J., Gruner J., Nüesh J., Knüsel F.: Some statistical methods in nutrient medium optimalisation. *Pathol. Microbiol.* **30**, 858 (1967).

BALITSKAYA P. M., KAZARINOVA L. A., BALITSKII V. A., MUZYCHENKO L. A.: Application of mathematical experiment design methods in the selection of an optimal medium for the synthesis of inosine by the culture of *Bacillus subtilis* Gen-265. (In Russian) *Prikl. Biokhim. Mikrobiol.* **9**, 694 (1973).

BIRYUKOV V. V.: Practical instructions for the use of mathematical methods of experiment design in the search for optimum conditions in multifactorial processes. (In Russian) Zinatne, Riga, 1969.

BOGOROV V. G., MAKSIMOV V. N., FEDOROV V. D.: Selection of optimum medium composition for photosynthesizing serobacteria with the aid of mathematical statistical methods. (In Russian) *Dokl. Akad. Nauk SSSR* **165**, 686 (1965).

BOX G. E. P., WILSON K. B.: On the experimental attainment of optimum conditions. *J. Roy Stat. Soc.* **B 13**, 1 (1951).

DEAN W. K., HEALD K. J., DEMING S. N.: Simplex optimization of reaction yields. *Science* **189**, 805 (1975).

DEMING S. N., MORGAN S. L.: Simplex optimization of variables in analytical chemistry. *Analyt. Chem.* **45**, 278 A (1973).

DUMENIL G., CREMIEUX A., PHAN-TAN-LUU R., COMBET M.: Bioconversion from DL-homoserine to L-threonine. I. Application of factorial design. *Europ. J. Appl. Microbiol.* **1**, 213 (1975*a*).

DUMENIL G., CREMIEUX A., PHAN-TAN-LUU R., AUNE J. P.: Bioconversion from DL-homoserine to L-threonine. II. Application of the simplex method of optimization. *Europ. J. Appl. Microbiol.* **1**, 221 (1975*b*).

FEDOROV V. D., MAKSIMOV V. N., BOGOROV V. G.: Experimental development of nutritive media for micro-organisms. *Biometrika* **55**, 43 (1968).

GRACHEV Yu. P., TUBOL'TSEV A. K., LUSHCHIK T. A.: Optimization of cultivation conditions for *Bacillus mesentericus* strain 11-11 with the use of a mathematical process model in the form of power function. (In Russian) *Mikrobiologiya* **39**, 945 (1970).

HIMMELBLAU D. M.: Process analysis by statistical methods. Wiley, New York, 1970.

HOFFMANN V., HOFFMANN H.: Einführung in die Optimierung mit Anwendingskeispielen aus Chemie Ingeneur-Wesen. Verlag Chemie, Weinheim, 1971.

HOOKE R., JEEVES T. A.: Direct rearch solution on numerical and statistical problems. *J. Assoc. Comput. Mach.* **8**, 212 (1961).

IEGOROV N. S., BARANOVA I. P., KOZLOVA Yu. I.: Optimization of the composition of nutrient medium for *Streptococcus lactis*, the producer of the antibiotic nisine. (In Russian) *Mikrobiologiya* **40**, 993 (1971).

KRAYUSHKINA E. A., ZHEREBITSOV N. A., ZVYAGINTSEV V. I.: Optimization of the composition of nutrient medium for the cultivation of the mould fungus *Mucor pusillus* 917. (In Russian) *Biokhim. Mikrobiol.* **9**, 883 (1973).

KUBÍČEK M., HLAVÁČEK V.: Numerical Methods and Optimization in Chemical Engineering. (In Czech) SNTL, Prague, 1972.

LEWIS J. C., IJICHI K.: Multiple factor experimentation with reference to biosynthetic yield of aterrimin. *Appl. Microbiol.* **5**, 124 (1957).

LOGINOVA L. G., IEGOROVA L. A., TSAPLINA I. A., MAKSIMOV V. N.: Optimization of synthetic medium composition by the steep ascent method serving for the activation of neutral protease synthesis by the thermophilic bacterium *Bacillus brevis* 224. (In Russian) *Mikrobiol. Promyshl.* 1970 (4), 11 (1970*a*).

LOGINOVA L. G., IEGOROVA L. A., TSAPLINA I. A., MAKSIMOV V. N.: Activation of growth and protease synthesis in the thermophilic bacterium *Bacillus brevis*. (In Russian) *Mikrobiologiya* **39**, 588 (1970*b*).

MCDANIEL L. E., BAILEY E. G., ETHIRAJ S., ANDREWS H. P.: Factorial and central composite experiments with computer analysis for optimizing fermentation processes. Bacteriol. Proc. 1970, 70th Ann. Meet., Boston, Mass. 26 April—1 May, 1970. Abstract A 42, p. 7.

MAKSIMOV V. N., FEDOROV V. D.: Application of mathematical methods of experiment design in the search for optimum conditions in microorganisms cultivation. (In Russian) MGU, Moscow, 1969.

MAKSIMOV V. N.: Search for optimum medium composition for the cultivation of green serobacteria (*Chl. thiosulphatophilum*). (In Russian) *Mikrobiologiya* **40**, 258 (1971).

MAKSIMOVA E. P., USTINNIKOV B. A., MAKSIMOV V. N., FEDOROV V. D.: Determination of optimum conditions for the cultivation of *Bac. diastaticus* with the use of the steep ascent method. (In Russian) *Prikl. Biokhim. Mikrobiol.* **3**, 373 (1968).

MORGAN S. L., DEMING S. N.: Simplex optimization of analytical chemical methods. *Analyt. Chem.* **46**, 1170 (1974).

MURGOV I. D., ZAITSEVA Z. M.: Optimization of the composition of nutrient medium for L-lysine biosynthesis by *Brevibacterium flavum* 178 by the method of mathematical experiment design. (In Russian) *Prikl. Biokhim. Mikrobiol.* **9**, 845 (1973).

NALIMOV V. V., CHERNOVA N. A.: Statistical Methods of Extreme-search Experiment Design. (In Russian) Nauka, Moscow, 1965.

NALIMOV V. V.: Theorie des Experiments. VEB Deutsche Landwirtschaftverlag, Berlin, 1975.

NELDER J. A., MEAD R.: A simplex method for function minimization, *Comput. J.* **7**, 308 (1965).

PALAGINA N. A., ROZMANOVA N. V., VITRINSKAYA A. M., KHRYCHEVA A. I.: Optimization of cultivation conditions for baker's yeast. (In Russian) *Prikl. Biokhim. Mikrobiol.* **11**, 500 (1975).

PILÁT P.: Study of growth kinetics of *Candida boidinii* 11 Bh on methanol. (In Czech) Ph.D. thesis, Prague, 1975.

PISARENKO T. N., YAROVENKO V. L., DOBROLINSKAYA G. M.: Effect of medium salt composition on glucoamylase biosynthesis by the fungus *Aspergillus awamori* 224-21 as determined by the random equilibrium method. (In Russian) *Mikrobiol. Promyshl.* 1970(3), 9 (1971).

POWELL M. J. D.: An iterative method for finding stationary values of a function of several variables. *Comput. J.* **5**, 147 (1962).

POWELL M. J. D.: An efficient method for finding the minimum of a function of several variables without calculating derivatives. *Comput. J.* **5**, 155 (1964).

ROSENBROCK H. H., STOREY C.: Computational techniques for chemical engineers. Pergamon Press, Oxford, 1970.

SCHRÖDER K. D.: Optimierung der Kulturbedingungen für der Hefe-Stamm *Candida guilliermondii* H 17 beim Abbau von Kohlenwasserstoffen. Ph. D. thesis, Greifswald, 1972.

SCHRÖDER K. D., WEIDE H.: Optimization of growth conditions for yeasts. *Biotechnol. Bioeng. Symp.* No. 4 (Pt II), 713 (1974).

TSAPLINA I. V., MAKSIMOV V. N., VOROVYEVA G. I.: *Mikrobiol. Promyshl.* **12**, 19 (1973).

UNDÉN G. Å., HEDÉN C.-G.: An approach to on-line continuous culture optimization. In: Proc. 1st Europ. Conf. on Computer process control in fermentation. Sept. 3.–5., 1973, Dijon, INRA, Dijon. Session 2, 1973.

USTINNIKOV B. A., TUSEVA V. M., MAKSIMOV V. N., MOSKVICHEVA E. P.: Optimization of cultivation medium and conditions for ASP Batatal 61 by the method of mathematical design. (In Russian) *Prikl. Biokhim. Mikrobiol.* **9**, 32 (1973).

VEKSLER M. A., ZAVADU V. A., MARKOVA E. V., KHEFETS S. E.: Optimierung der Processe zur Drogengewinnung mit Hilfe mathematisch-statischer Methoden III. Optimierung des Process der Nicodingewinnung. *Chim. Pharmacol.* **4**, 31 (1970).

VOINO L. I., GRACHEV Yu. P., SIVACHEVA T. A.: Determination of optimum cultivation conditions for *Aspergillus oryzae* with the use of mathematical methods of experiment design. (In Russian) *Mikrobiologiya* **39**, 827 (1970).

VOLFOVÁ O., Pilát P.: Studies on methanol-oxidizing yeast I. Isolation and growth studies. *Folia Microbiol.* **19**, 249 (1974).

VOTRUBA J., PILÁT P., PROKOP A.: Optimization of nutrient medium for culturing microorganisms by means of modified Rosenbrock's method. *Biotechnol. Bioeng.* **17**, 1833 (1975).

ZELTIN' R. P.: Selection of optimum medium for the biosynthesis of cellulolytic enzymes by *Aspergillus terreus* 17 P by the steep ascent method. (In Russian) *Mikrobiologiya* **39**, 832 (1970).