

## Experimental Design in Microbiology

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### Summary

The field of predictive microbiology is rapidly widening and ranges from bioreactor engineering to the modeling of foodstuff degradation and contamination. In this chapter, we point out how important the choice of experimental conditions is. We also briefly describe some tools (methodology of experimental research) that can be used to design optimal experimental strategies with respect to the study's aim. Emphasis is given on the optimization of microbial metabolite production, with an example showing the screening of factors and another illustrating the use of the response surface methodology.

**Key Words:** Predictive microbiology; methodology; experimental research; optimal design; response surface methodology; screening of factors, experimentation plan.

### 1. Introduction

Experiments are essential to scientific exploration and are still highly favored by researchers. Any work relying on a large number of them is normally considered of good standard. The methods of study and analysis of the physical, chemical, and biological phenomena have recently been rapidly improving, thanks to increasingly refined techniques and equipment (often linked to a computer in charge of part of the data analysis) and the development of mathematical and statistical methods for the analysis and processing of numerical data (factorial analysis, pattern recognition, classification, etc.).

By increasing the number of sensors, recorders, analyzers, and so forth, it is now possible to have an almost unlimited quantity of information concerning the study of a phenomenon. It is understandable that the researcher wants to gather as much “material” as possible during his experimentation even if this means delaying (sometimes forever) the analysis of these data. However, for some time, the tendency has been to reduce the number of experiments mainly because of their cost. Nevertheless, a few researchers point out that a plethora of results

does not guarantee valuable scientific information. Significant improvement has taken place in the field of methodology of experimental research, but, to date, it has not succeeded in convincing a wide audience of experimental scientists. Two important facts are often overlooked. First, as powerful as the hardware and software might be, one cannot extract more information than contained in the experimental data. Second, in spite of the increase in the number of measurements, it is not rare that essential information is lacking.

It is difficult to persuade researchers that the result of an experiment contains no information and that all information is contained in the chosen experimental conditions. For example, with an infinite number of experiments carried out at two precise points, it is only possible to study a linear first-order model. The information quality does not depend on the number of experiments. The planning of the experiments is of the highest importance, although often neglected. In most cases, a classical approach is needed: The researcher makes assumptions from which he then deduces consequences. If the necessary information required to check these assumptions is not available, he must undertake the necessary experiments. The aim of the “methodology of experimental research” (see **Note 1**) is to control, describe, foresee, or explain the phenomenon under study.

In **Subheading 3.**, we give a brief overview of the ERM (Experimental Research Methodology) and illustrate how it can be used with the example of microbial metabolite production optimization.

## 2. Materials

NEMRODW software (Mathieu D., Nony J. and Phan Tan Luu R., New Efficient Methodology for Research Using Optimal Design, Windows version; LPRAI, Marseille, France) has been used to study the given examples and create the corresponding input tables and output figures.

## 3. Methods

### 3.1. Methodology of Experimental Research

We propose a methodological approach that emphasizes the importance of planning the experiments rather than running the experiments. Planning is necessary not only during the initial stages but also throughout all the research process. New information obtained from optimally designed experiments must be used to redefine the experimental strategy if required. The different steps of this procedure are as follows:

1. Clear definition of the problem being studied: proposed targets, consequences of a wrong decision, budget (in time, cost, means, etc.).
2. Compilation of the current local and bibliographical knowledge. If some necessary information is not available, experiments must be undertaken. Therefore, a complete and precise list of the factors likely to be influential, the responses, and the con-

straints must be established. The area of the experimental domain in which the missing information is to be sought has to be defined. It is referred to as the experimental domain of interest.

3. Setting up an experimental strategy (or experimental design) (i.e., to choose the experiments to be carried out according to the defined targets, the means available, and the desired information). The researcher seeks a relationship of cause and effect between some parameters of the phenomenon (called factors) (*see Note 2*), which are supposed to influence the behavior of the phenomenon and other parameters (called responses) (*see Note 3*) that define the result of the phenomenon. The planning of experiments consists in forcing the factors (input) to vary in a precise way, measuring the induced variations of the answers (output) and then deducing the relationships between causes and consequences.
4. Carrying out the experiments that will give us the values of the studied responses.
5. Deduction of the answers to the questions either directly or with the help of a mathematical model.

There are many different types of study, depending on the proposed targets. This implies different experimental strategies. We will give an example of two of them with the adequate experimental design.

### **3.2. Exploratory Research**

At the beginning of a new study, the researcher usually does not know much about the phenomenon. He sometimes even ignores if he will be able to reproduce it. Therefore, he undertakes preliminary experiments to ensure that he has control over the phenomenon, to choose the favorable experimental fields and responses, and to check the reproducibility. These experiments are usually done without planning. However, there are simple methods to perform the exploratory research in a more organized way with grids, saturated designs of experiments, random research, and so forth.

### **3.3. Screening of Factors**

This step is often done after the exploratory research. The researcher quickly picks the factors potentially influential in the chosen experimental field. Because of the belief that an increasing number of factors increases the number of experiments exponentially, a high number of factors is often reduced to a number that seldom exceeds three or four. This reduction is artificial and relies only on laboratory practices and the researcher's feeling (i.e., they retain the factors they like and reject those they do not). For a more scientific screening, there are methodological techniques with a low number of experiments. If the number of factors is very high (50–10,000), group screening or sequential bifurcation or supersaturated designs can be used. If it is lower, symmetrical and asymmetrical fractional factorial designs can be used. In any case, each factor is weighed, which allows choosing the most important ones (and not the preferred ones) for a later

**Table 1**  
**Factors and Experimental Domain**

	Factors	Unit	Level (–)	Level (+)
$X_1$	Glucose concentration	g/L	1	5
$X_2$	Initial pH	—	5	7
$X_3$	Inoculum size	Spores/mL	$10^6$	$10^8$
$X_4$	Agitation rate	rpm	100	200
$X_5$	Inducer added	—	Yes	No
$X_6$	Nitrogen source	—	Corn steep	Peptone
$X_7$	Temperature	°C	20	30

**Table 2**  
**Hadamard Matrix**

$X_1$	$X_2$	$X_3$	$X_4$	$X_5$	$X_6$	$X_7$
+	+	+	–	+	–	–
–	+	+	+	–	+	–
–	–	+	+	+	–	+
+	–	–	+	+	+	–
–	+	–	–	+	+	+
+	–	+	–	–	+	+
+	+	–	+	–	–	+
–	–	–	–	–	–	–

and more precise analysis. This approach is very effective but not often used mainly because researchers are afraid of working with more than three factors.

Screening designs can be written  $s_1^{k_1}s_2^{k_2} \dots s_l^{k_l}/N$ , where  $s_i$  is the number of levels (i.e., values) of  $k_i$  factors and  $N$  is the minimal number of experiments required (see **Note 4**). If several factors have a different number of levels, the design is asymmetrical, whereas if  $k$  factors have the same number  $s$  of levels (i.e.,  $s^k/N$ ), it is symmetrical. Optimal experimental designs such as Addelman’s (1) or Hadamard’s also known as Plackett and Bluman (2) can be used. A Hadamard design is a symmetrical design for two-level factors, and for the design to be optimal, it requires that  $N$  be a multiple of 4.

This design was used in a build-up example to screen seven factors supposed to have an influence on the lipase production of a *Penicillium* sp. (see **Table 1**). The response studied is the enzymatic activity of the culture broth after 150 h (in U/mL). The factors are transposed in the Hadamard matrix (see **Table 2**), giving the datasheet of the experiments to run (see **Table 3**). In a screening

**Table 3**  
**Experimental Datasheet**

Run	Glucose conc.	Initial pH	Inoculum size	Agitation rate	Inducer	Nitrogen source	Temp.	Enzymatic activity
1	5	7.0	10 <sup>8</sup>	100	No	Corn steep	20	61
2	1	7.0	10 <sup>8</sup>	200	Yes	Peptone	20	284
3	1	5.0	10 <sup>8</sup>	200	No	Corn steep	30	23
4	5	5.0	10 <sup>6</sup>	200	No	Peptone	20	69
5	1	7.0	10 <sup>6</sup>	100	No	Peptone	30	238
6	5	5.0	10 <sup>8</sup>	100	Yes	Peptone	30	236
7	5	7.0	10 <sup>6</sup>	200	Yes	Corn steep	30	105
8	1	5.0	10 <sup>6</sup>	100	Yes	Corn steep	20	167

**Table 4**  
**Calculation of the Estimation of  $b_i$  Effects**

$b_i$	Value
$b_0$	147.87
$b_1$	-30.12
$b_2$	24.12
$b_3$	3.12
$b_4$	-27.62
$b_5$	-50.12
$b_6$	58.87
$b_7$	2.62

study, the effects are supposed to be additive; this implies that the relationship between the experimental responses and the studied variables is a first-order polynomial model with values of  $X_i = \pm 1$ :

$$\eta \text{ (response)} = \beta + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7$$

From the experimental data, the estimations of the  $\beta_i$  effects can be calculated (see **Table 4**). These values can then be represented with various diagrams such as a bar chart (see **Fig. 1**). This study clearly highlights two very influent key factors: the nature of the nitrogen source and the presence of the inducer. Glucose concentration, agitation rate, and initial pH are less influent. Inoculum size and temperature are without any significant effect on the response in the chosen experimental field. Other graphical tools can be used to analyze the screening results, such as the Pareto technique or normal and half-normal plot charts.

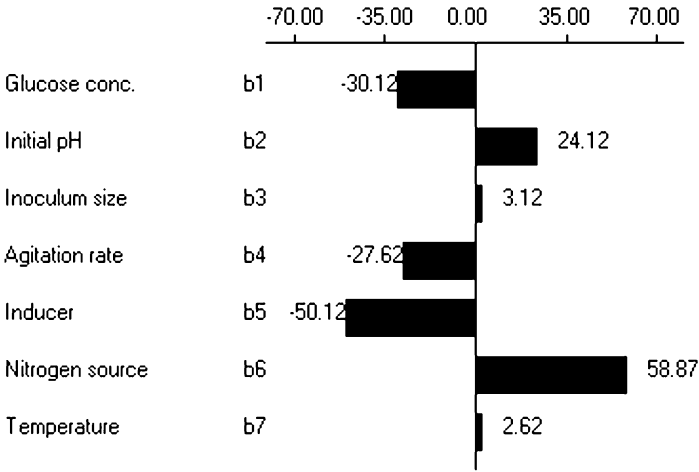


Fig. 1. Bar chart of the factor screening results.

3.4. Quantitative Studies of Factors

Once the influent factors are known, they can be studied more precisely. The hypothesis of additive effects used in the screening of factors is abandoned. The old strategy, which consists in studying a factor at a time while the others are maintained at a fixed value, requires many experiments and does not take into account the possible interactions (*see Note 5*). Information obtained is incomplete and might not allow the solving of the studied problem.

There are two kinds of interaction: those that we can postulate (of which we want to know their possible existence importance) and those of which we are unaware and wish to discover. To study them, the desired information is well defined and factorial experiment designs, whether symmetrical or asymmetrical, complete, or fractional, are used (3). These experiment designs have all the desired qualities and especially “sequentiality.” A complete factorial design is, by definition, composed of all the possible combinations of the levels of each factor. The number  $N$  of different combinations is equal to the product of the number of levels (i.e.,  $N = s_1 s_2 s_3 \dots s_k$ ). These designs, especially the two-level factorial ones, are very often used and they have many applications.

3.5. Quantitative Studies of Responses

In many applications, the interest does not lie in studying the effects of the factors or the importance of the interactions but in knowing how one or several measured characteristics (responses) behave in a well-defined experi-

mental domain. It is then possible to seek the optimum of one or more experimental responses without having to undertake a great number of experiments. Whatever the domain of application, the objective is to find a region of the experimental domain in which all of the studied properties meet the desired constraints as closely as possible. This region is called the area of acceptable compromise. To find the relationship between the factors and the responses, the phenomenon studied is simplified by mathematical modeling. Depending on the problem studied, the model could be linear or nonlinear, a differential equation, and so forth. This part of the methodological tool is called “response surface methodology” (4–6). Experimentation will determine the values of the mathematical model’s coefficients. However, prediction is of little value if one does not know how precise it is. The precision of the model depends on the precision of its coefficients. However, the quality (variance) of these depends only on the experimental measuring accuracy (experimental or residual variance), the structure of the experimental design, and the postulated mathematical model; it is completely independent of the experimental results. Therefore, the quality of an experimental design can be either the precision of the prediction in a given experimental field or the precision with which the coefficients are known. These models must be good representations of the experimental response within the domain of interest, and if this condition is fulfilled, they must give an estimation of the response that is acceptable, qualitywise. It is considered acceptable if it can be compared (for a given experimental point) to the quality obtained by running the experiment. Any model type can be chosen, provided it possesses the two aforementioned properties. Polynomial models are very often used because of their simplicity and their sequential approach: Once the model and a corresponding optimal design are chosen, it is tested for validity. If it is valid (i.e., it is a good representation of the phenomenon), the response can be calculated for every point of the experimental domain.

The most commonly used models are first- and second-order polynomial models and the corresponding classical optimal designs are as follows:

For first-order models:

1. Hadamard or Plackett and Burman experimental designs
2. Full ( $2^k$ ) or fractional ( $2^{k-r}$ ) factorial experimental designs
3. Equiradial experimental designs
4. Simplex experimental designs

For second-order models:

1. Composite experimental designs
2. Doehlert uniform shell designs
3. Equiradial experimental designs

**Table 5**  
**Factors and Experimental Domain**

Factor	Unit	Center	Variation step
Nitrogen conc.	%	3.0	2.0
Inducer conc.	%	0.50	0.46
pH	U	6.0	2.0

**Table 6**  
**Doehlert Matrix**

Run	$X_1$	$X_2$	$X_3$	$Y_1$
1	1.0000	0.0000	0.0000	298.00
2	−1.0000	0.0000	0.0000	260.00
3	0.5000	0.8660	0.0000	290.00
4	−0.5000	−0.8660	0.0000	248.00
5	0.5000	−0.8660	0.0000	269.00
6	−0.5000	0.8660	0.0000	278.00
7	0.5000	0.2887	0.8165	274.00
8	−0.5000	−0.2887	−0.8165	267.00
9	0.5000	−0.2887	−0.8165	290.00
10	0.0000	0.5774	−0.8165	284.00
11	−0.5000	0.2887	0.8165	268.00
12	0.0000	−0.5774	0.8165	264.00
13	0.0000	0.0000	0.0000	285.00
14	0.0000	0.0000	0.0000	288.00
15	0.0000	0.0000	0.0000	284.00

- 4. Box and Behnken experimental designs
- 5. Hybrid experimental designs
- 6. Hoke experimental designs

Here, we give an example of a Doehlert (7) uniform shell design. The settings are the same as in the screening example. The three most influent factors (see **Table 5**) were retained in order to maximize lipase production (experimental response). The Doehlert matrix (see **Table 6**) was used to create the experimental datasheet (see **Table 7**). Experiment 13 is done in triplicate to check reproducibility. The resulting coefficients are shown in (**Table 8**). With the complete equation, response surfaces can be drawn (see **Figs. 2–4**). From **Fig. 2**, it can be seen that the response is minimal when both concentrations are low. When the inducer concentration rises from 0.04 to 0.50, the response rap-



**Table 7**  
**Experimental Datasheet**

Run	Nitrogen concentration (%)	Inducer concentration (%)	pH	Enzymatic activity (UI/mL)
1	5.0	0.50	6.0	298.00
2	1.0	0.50	6.0	260.00
3	4.0	0.90	6.0	290.00
4	2.0	0.10	6.0	248.00
5	4.0	0.10	6.0	269.00
6	2.0	0.90	6.0	278.00
7	4.0	0.63	7.6	274.00
8	2.0	0.37	4.4	267.00
9	4.0	0.37	4.4	290.00
10	3.0	0.77	4.4	284.00
11	2.0	0.63	7.6	268.00
12	3.0	0.23	7.6	264.00
13	3.0	0.50	6.0	285.00
14	3.0	0.50	6.0	288.00
15	3.0	0.50	6.0	284.00

**Table 8**  
**Model Coefficients**

Coefficient	Value
$b_0$	285.667
$b_1$	17.250
$b_2$	12.846
$b_3$	-7.144
$b_{11}$	-6.667
$b_{22}$	-17.001
$b_{33}$	-10.832
$b_{12}$	-5.196
$b_{13}$	-8.573
$b_{23}$	-2.594

idly increases; whereas from 0.50 upward, it does not change significantly. When the nitrogen concentration increases, so does the response. From **Fig. 3**, we can see a response enhance with the increase of nitrogen concentration if the initial pH is under 6.0. If the pH is higher than this limit, the response decreases.

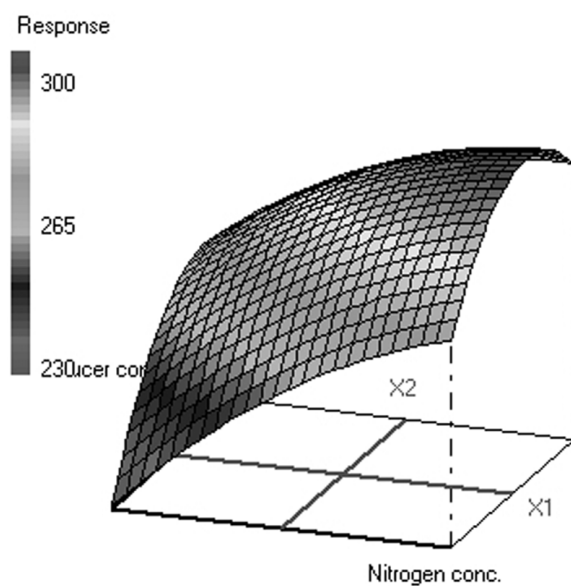
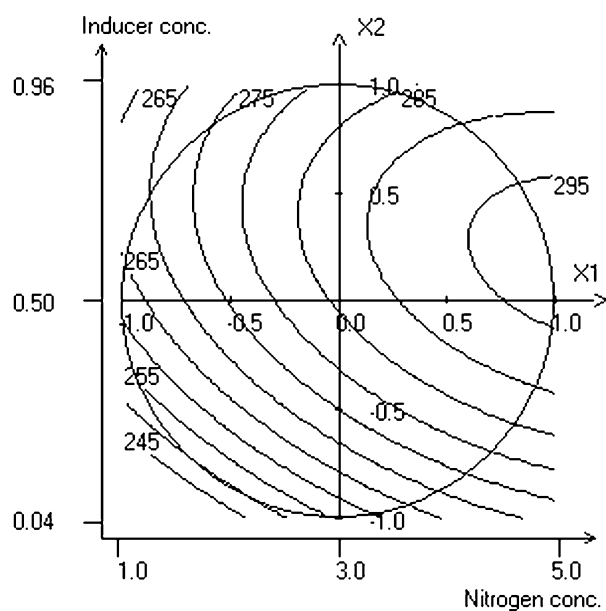


Fig. 2. A two-dimensional (2D) and three-dimensional (3D) graphical study of the response variation in the plane: nitrogen concentration and inducer concentration. Fixed factor: pH = 6.0.

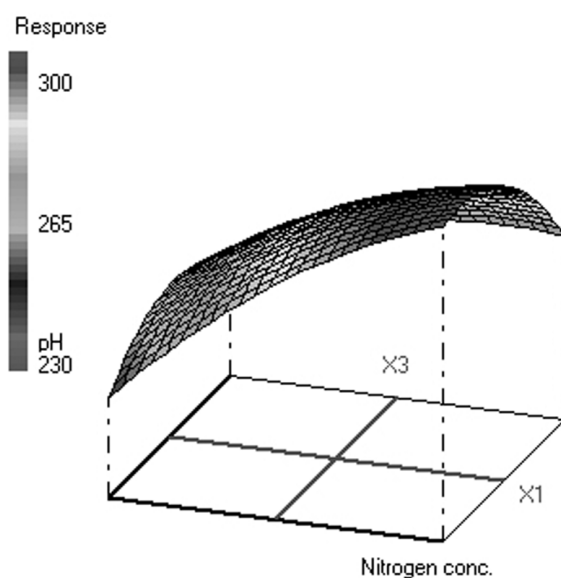
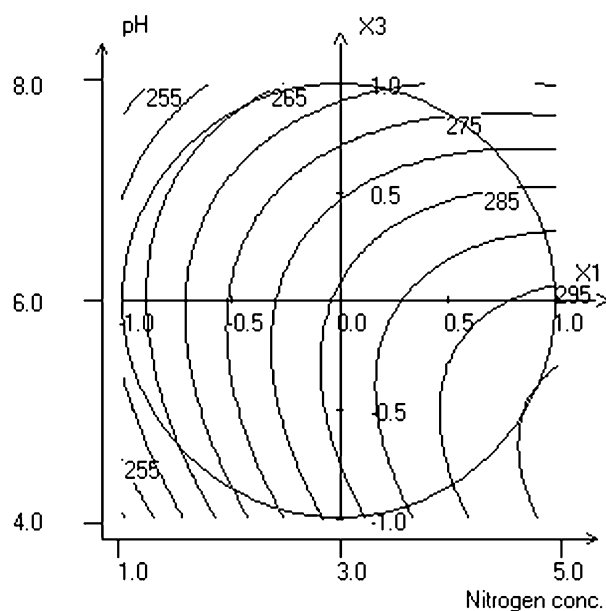


Fig. 3. A 2D and 3D graphical study of the response variation in the plane: nitrogen concentration and pH. Fixed factor: inducer concentration = 0.50 %.

Finally, **Fig. 4** shows that the response is high for inducer concentrations above 0.04 but no longer rises when it equals 0.50. The maximum response is obtained for inducer concentration above 0.50 and pH under 6.0.

### 3.6. Mixtures

In many industries, a great number of products are obtained by mixing two or more components or ingredients. The properties of the final product depend on the proportion of each component in the studied mixture. In the case of mixtures, the factors are the proportions of each component. They have two significant characteristics: (1) Their total amount is equal to one and they are, thus, not independent and (2) their values are dimensionless numbers, perfectly comparable.

These constraints on the values that components can take account for the fact that the mixtures cannot be treated as usual. Considering these constraints, any variation of the proportion of a component causes a variation of the proportions of the other components. The problems involving mixtures have two main differences: the experimental domain (a regular polyhedron of dimension  $[q-1]$  for a mixture of  $q$  components) and the form of the mathematical model (8). For this type of study, specific experimental designs are available, such as Scheffe's (9) simplex lattice designs or particular optimal designs when the components are under constraints.

### 3.7. Particular Experimental Designs

The traditional experimental designs presented cover a significant share of the experimenter needs. There are, however, many circumstances under which these designs are inapplicable:

1. Nonsymmetrical experimental domains. This is a very significant limitation to the use of the traditional experimental designs that are usable only in the case of a symmetrical experimental domain. In certain cases, the experimental domains limited by technological or economic constraints that give it a nonsymmetrical form, some combinations of factors leading to expensive, dangerous, or impossible experiments, and discontinuities can be feared. It can also happen that some experimental fields are discrete and have a reduced number of possible experiments.
2. A fixed number of experiments. It can happen for economic reasons that the number of experiments is limited. This number is seldom in agreement with that of a traditional experiment design.
3. Unspecified linear mathematical model. The traditional experiment matrices are designed to study well-defined linear models and do not allow, economically, the study of a particular linear model postulated according to existing information.
4. Complement of a design. It is extremely rare that previous experiments can be reused. The traditional experimental designs, because they are rigid and pre-

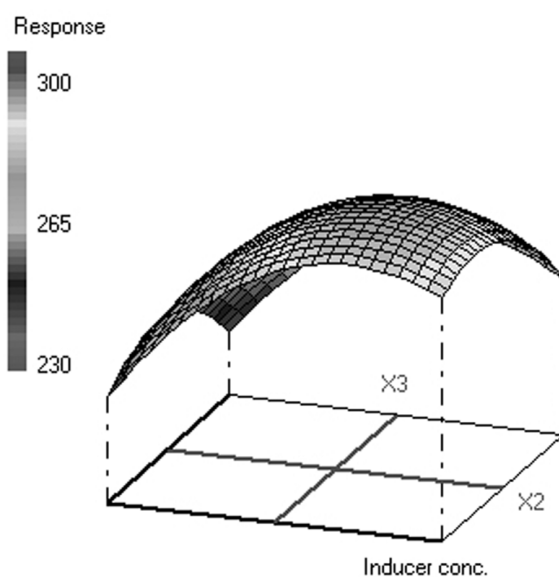
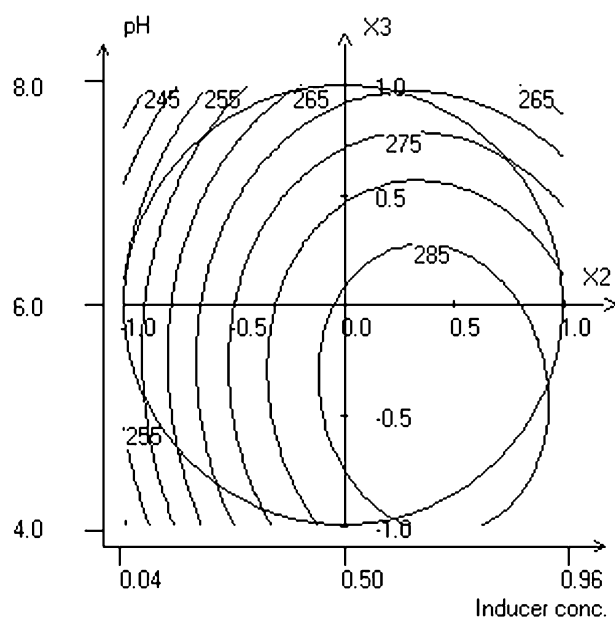


Fig. 4. A 2D and 3D graphical study of the response variation in the plane: inducer concentration and pH. Fixed factor: nitrogen concentration = 3.0 %.

established, usually do not allow modifications (such as adding new factors), especially if they were not planned in advance.

5. Repair of an experimental design. Even if none of the above cases prevents the construction of a traditional experimental design, new difficulties can emerge. We can imagine many situations in which the selected experimental design does not work. For example, if one or more experiments are impossible to perform in the course of experimentation, the experimental results will be incomplete and will not satisfy the studied objectives. The missing information will have to be acquired by adding one or more experiments.

In conclusion, we currently have new, powerful, and very flexible tools that make it possible to build, according to the problem arising, the most economic and most informative experimental strategies by taking account of the reality of the studied problem. The application field of this methodology is very broad because it includes not only the traditional applied sciences (physics, chemistry, biology, etc.), but also, for example, social sciences or economy and various situations of simulation (the experiment being taken in the broad sense).

#### 4. Notes

1. For a long time, the expression “experimental design” has been used to describe a group of hitherto well-known experimental strategies for variance analysis such as factorial designs, Latin squares, Greco-Latin squares, and so forth. In 1970, when we began our research in this field, in order to avoid the ambiguous use of the over-worked expression “experimental design” and to underline the extreme importance of the planning stage, we decided to use the expression “methodology of experimental research.” “Methodology” describes all the methods and tools that can be used to define the studied problem, undertake the necessary experiments, and exploit the results. This also includes the concept of “experimental strategy,” where “experimental” indicates experimentation is the only way of obtaining the as-yet unavailable information.
2. The factors are the causes, either supposed or certain, responsible for the studied phenomenon. They can be controlled or not (noise factors), but only those we can control are taken into account. They can be quantitative or qualitative, continuous or discontinuous.
3. An experimental response is a measurable change observed when the factors vary. A phenomenon can be described by several responses. Problems can arise during interpretation of the results if the response can take only discrete values.
4. With a design including  $k$  factors and  $s$  levels, the number of coefficients to calculate is  $p = \sum_{i=1}^k (s_i - 1)$  and, therefore, the minimal number of experiments  $N$  to undertake is:

$$N \geq 1 + \sum_{i=1}^k (s_i - 1) \quad (1)$$

5. An interaction effect between two factors means that the effect of one of them depends on the value of the other one.

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