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Full Length Research Paper

The utilization of the response surface methodology for the optimization of cultivation medium and growth parameters in the cultivation of the yeast strain *S. cerevisiae* 3.20 on ethanol

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A mutant strain of the yeast *Saccharomyces cerevisiae* growing on ethanol as single source of carbon and energy was used in optimization experiments at laboratory and micropilot scale, following the surface response methodology. The cultivation medium optimization was performed on the basis of maximization of dry cell weight and the process parameters optimization on the basis of substrate yield maximization.

Key words: Ethanol, optimization, response surface methodology, yeast.

INTRODUCTION

The use of yeast as therapeutic agent has been well known since antiquity. But only at the end of nineteenth century, the brewing yeast *Saccharomyces cerevisiae* was first published in a pharmacopeia. In 1899 Brocq was the first scientist who studied the action of the yeast *S. cerevisiae* in the cutaneous diseases, starting from the observation that the workers from the beer plants were not affected by furunculosis.

Later studies made possible the administration of *Saccharomyces* yeast in the treatment of B avitaminosis, colitis, acute diarrhea and diabetes (Hochter, 1990; Offenbacher et al., 1985; Massot et al., 1984) due to its high content in the vitamins B group. The viable yeast biomass is also used as an additional source for proteins and vitamins in malnutrition and denutrition cases (Segal, 1991). For all these reasons, there is a growing interest in the cultivation of yeast of genera *Saccharomyces* and *Candida*, advised by FAO for human use, on substrates also accepted for human use.

The attractiveness of ethanol as a substrate for microbial cultivation comes from its availability as a petrochemical pure feedstock and from the acceptance of the final product grown on this substrate as an edible protein. Furthermore the standard quality of the substrate, in contrast to molasses variability, guarantees for the constant quality of the product.

Several yeast strains are cited by the literature for their ability to grow on ethanol as the single source of carbon and energy: *S. cerevisiae* (Keulers et al., 1996; Wasungu and Simard, 1982; Mor and Fiechter, 1968a), *Candida utilis* (Peskova, 1998; Paca and Votruba, 1994; Votruba and Paca, 1992; Prior et al., 1980; Watteeuw et al., 1979), *Torulopsis ethanolitolerans* (Potucek, 1989), *Pichia pastoris* (Duff et al., 1989) and *Candida krusei* (Kilian et al., 1981). Several papers studying the cultivation conditions for yeast growing on ethanol as well as the influence of different process parameters on yeasts' performance were reported (Kilian et al., 1981; Prokop et al., 1978; Eroshin et al., 1976; Mor and Fiechter, 1968b).

This paper aims to optimize the composition of the growth medium and the cultivation parameters of the bioprocess for obtaining biomass on ethanol with the strain *S. cerevisiae* 3.20, using the response surface methodology. Different studies presented in the literature on the ethanol tolerance of yeast (Norton et al., 1995;

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Table 1. The limits and coding of medium components (independent variables).

Variable	Code	Variable level					Range
		-1.6817	-1	0	1	+1.6817	Δ = 3.3634
KH_2PO_4	x_1	188	800	1700	2600	3213	3025 mg/l
$MgSO_4 \cdot 7H_2O$	x_2	114	270	500	730	886	772 mg/l
Corn steep liquor	x_3	0.3	1	2	3	3.7	3.4 g/l

D'Amore et al., 1990) made us determined to use yeast strain resistant to higher ethanol concentrations.

The response surface methodology is a three-factorial design which provides the relationship between one or more measured dependent responses and a number of input (independent) factors (Kafarov, 1976; Box and Hunter, 1957). The response surface method has some advantages that include a smaller number of experiments, suitability for multiple factor experiments, search for relativity between multiple factor experiments and finding of the most suitable correlation and forecast response (Sayyad et al., 2007; Shieh et al., 2003). This facilitates the determination of the optimum values for the factors under investigation and the prediction of a response under optimized conditions (Smigelschi and Woinarovschy, 1978).

MATERIALS AND METHODS

Materials

Ethanol, kalium dihydrogen phosphate, magnesium sulphate and diammonium sulphate are all purchased from CHIMO-PAR Bucharest. Yeast extract, peptone, glucose and malt extract were obtained from SIGMA.

Strain, media and cultivation conditions

The used yeast strain *S. cerevisiae* 3.20 is a UV mutant from the Collection of Industrial Microorganisms of the National Institute for Chemical Pharmaceutical R and D Bucharest, registered WFCC 232. The selection criterion for mutagenesis was the resistance to acetaldehyde.

The composition (w/v) of the inoculum medium is: yeast extract 1%, peptone 1%, malt extract 1%, and glucose 1%. Equal aliquots of ethanol were added at 0, 12, 24 and 36 h, so the total concentration of the added ethanol was 2% (v/v). pH was adjusted at 4.0 - 4.5 with 12.5% (v/v) NH_4OH solution at every 12 h.

The composition (w/v) of the cultivation medium for the second stage of the bioprocess is: KH_2PO_4 0.10%, $(NH_4)_2SO_4$ 0.20%, corn steep liquor (50% D.C.W.) 0.20% and $MgSO_4 \cdot 7H_2O$ 0.05%. Equal aliquots of ethanol were added at 0, 12, 20 and 28 h, so the total concentration of added ethanol was 2% (v/v). The pH was adjusted at 4.0 - 4.5 with 12.5% (v/v) NH_4OH solution before each addition of the ethanol.

The inoculum phase and the second cultivation one (first lasting 48 h and the second one, 36 h) were performed at laboratory scale, in 500 ml Erlenmeyer flasks, with 100 ml medium, closed with cotton stoppers, on a rotary shaker (240 rpm) at 28 $\pm$ 0.5° C. The inoculum culture was transferred 10% (v/v) to the flasks of the second generation and the bioprocess continued for 36 h in the same conditions described above.

The assays for the determination of the optimum composition of the fermentation medium by the response surface method were performed during the second stage of the cultivation of the yeast *S. cerevisiae* 3.20.

The next stage experiments in order to establish the optimum values for some cultivation parameters (pH, T) were performed in a New Brunswick bench scale bioreactor with 8 l working volume (geometric volume = 12 l), inoculated with 10% (v/v) second generation culture, obtained in the laboratory experiments. The bioreactor was equipped with automatic control of temperature, pH, air admission, agitation speed, the concentrations of dissolved oxygen and carbon dioxide in the exhausted gases.

The medium composition was the same as determined in the laboratory phase experiments. The pH was maintained between 4.0 - 4.2 by automatic correction with a 12.5% (v/v) NH_4OH solution.

The addition of ethanol was automatically performed in order to maintain the dissolved oxygen concentration in the medium during the fermentation greater than 10% of the saturation value.

Analytical methods

The evolution of yeast cell development during the cultivation was evaluated by determining the optical density (λ = 570 nm) correlated to the dry cell weight, each value being the mean value of two determinations.

Ethanol was determined by gas chromatography, using the external standard method, from the supernatant samples resulted after the centrifugation of measured volumes of fermentation broth. Analyses were performed on a FID Carlo-Erba FRACTOVAP 4200 gas chromatograph, using a glass column (1,500 x 24 mm), packed with 80 - 100 mesh Porapack Q. The nitrogen flow was 50 ml/min, temperature 200° C.

RESULTS AND DISCUSSION

In order to quantify the influence of the cultivation medium components on the biomass concentration, a central composite design was used, by which we studied the influence of three factors in 17 runs. The design is to be run in a single block. To provide protection against the effects of lurking variables, the order of experiments has been fully randomized.

As independent variables, the concentrations of potassium dihydrogen phosphate, magnesium sulphate and corn steep liquor were selected. The range of variation and the codification of the variables are presented in Table 1, and the experimental matrix is given in Table 2.

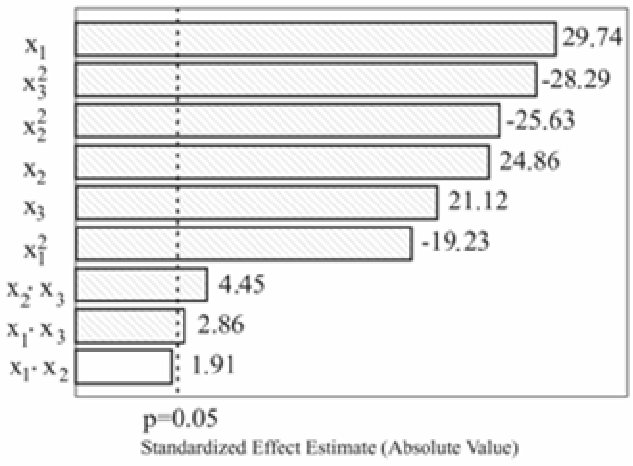

Figure 1. Pareto chart of standardized effects.

In order to analyze the experimental data, a second-order regression equation was proposed:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

Where Y - Normalized dry cell weight; x_1, x_2, x_3 - the independent variables; b_0, b_1, …b_{33} – the coefficients of the regression equation.

The Pareto chart (Figure 1) shows each of the estimated effects, interactions and the standard error of each of the effects, which measures their sampling error. In the experimental design the Pareto chart is a Frequency Histogram that shows the amount of influence each factor has on the response in decreasing order. Because the interaction between x_1 and x_2 has no statistical significance, the term $x_1 \cdot x_2$ will be cancelled from the final equation:

$$Y = 95.9538 + 17.9097 \cdot x_1 + 14.9741 \cdot x_2 + 12.7175 \cdot x_3 + 2.2500 \cdot x_{13} + 3.5000 \cdot x_{23} - 12.7455x_1^2 - 16.9881 \cdot x_2^2 - 18.7559 \cdot x_3^2$$

Figure 1 shows that the variable x_1 has the greatest influence on Y, followed by x_3^2 and x_2^2. The great numerical values for the quadratic terms justify the selection of the response surface method in the experimental design. The results presented in Table 3 showed an excellent correlation between the calculated and the experimental values.

Plot of the response surfaces for $Y = f(x_1, x_2, 0.5)$, $f(x_1, 0.5, x_3)$, $f(0.5, x_2, x_3)$ showed a global maximum situated in the area $x_1, x_2, x_3 \in (0, 1)$, as it can be observed in Figures 2, 3 and 4. The maximum value for Y was obtained using the gradient method with the start point (1, 1, and 1). The optimal values of parameters for Y_{max} =108.94 are: $x_1 = 0.74$, $x_2 = 0.48$, $x_3 = 0.43$. These normalized values correspond to the real optimal values of the medium's components presented in Table 4.

Table 2. The experimental matrix for rotatable central composite design: (2^3 + star).

Block	x_1	x_2	x_3	Y
1	0	-1.68179	0	20
1	1	1	-1	62
1	0	0	1.68179	65
1	-1	-1	1	25
1	0	0	-1.68179	20
1	0	0	0	95
1	1	-1	-1	40
1	-1	1	1	55
1	0	0	0	97
1	1.68179	0	0	90
1	-1	1	-1	30
1	-1	-1	-1	10
1	1	-1	1	60
1	0	1.68179	0	75
1	-1.68179	0	0	29
1	1	1	1	100
1	0	0	0	96

The next step in our experiments was the determination of the optimum values for pH and temperature. The experiments were performed in a New Brunswick bio-reactor, using as inoculum (10% v/v) the culture obtained in the second laboratory phase, having the parameters described in Table 5.

Several cultivations were run in the bioreactor in the conditions described above. All were stopped at 24 h, at a final DCW around 35 g/l, when a severe decrease in the ethanol consumption rate was observed. The specific growth rate for the exponential phase (8 - 20 h) was 0.157 h^{-1}, with a maximum of 0.165 h^{-1} between 16 - 20 h. Under these conditions the total substrate yield was 0.66 [g biomass/ g ethanol].

The experiments for the optimization of the cultivation parameters (pH, T) were performed in the conditions described above. We used a central composite design (2^2 + star) to study the effects of the two factors on the substrate yield, in 10 runs. The order of the experiments was completely randomized. The codification of the variables is presented in Table 6. The experimental matrix is shown in Table 7.

The value 1 corresponds to a substrate yield of 0.66 [g/g]. The dependence Z (pH, T) is described by a second-order regression equation:

$$Z = 0.983 + 0.314\ x_4 - 0.079\ x_5 - 0.212\ x_4^2 - 0.0025\ x_4x_5 - 0.195\ x_5^2$$

Drawing a new Pareto chart for this dependence (not shown) the term $x_4 \cdot x_5$ was removed from the final equation as it had no statistical significance and the

Table 3. Comparison between experimental and calculated data for Y.

Row	Experimental value	Fitted value	Lower 95.0% CL for mean	Upper 95.0% CL for mean
1	10	7.62	3.25	11.98
2	20	21.52	16.17.90	26.13
3	100	98.82	94.95	103.18
4	55	58.50	54.13	62.86
5	29	29.78	25.17	34.40
6	95	95.95	92.54	99.36
7	65	64.29	59.68	68.90
8	30	30.56	26.19	34.91
9	97	95.95	92.54	99.36
10	62	61.88	57.51	66.25
11	20	22.72	18.11	27.33
12	40	38.93	34.56	43.30
13	25	21.55	17.18	25.92
14	60	61.87	57.50	66.24
15	75	73.09	68.47	77.70
16	90	90.02	85.41	94.64
17	96	95.95	92.54	99.36

Table 4. The values of the independent variables leading to the maximum biomass concentration.

Parameter	Component	Normalized values	True values
x_1	KH_2PO_4	0.74	2366 [mg/l]
x_2	$MgSO_4 \cdot 7\ H_2O$	0.48	610 [mg/l]
x_3	Corn steep liquor	0.43	2.43 [g/ l]
Y	Dry cell weight	109	10.7 [g/ l]

Table 5. The characteristics of the standardized inoculum used for the bioreactor cultivation.

Parameter	Value
Strain	*S. cerevisiae* 3.20
Time of cultivation [h]	36
Temperature [^{o} C]	28 ± 0.5
pH	4.0 ± 0.2
Culture medium	Previously determined
Working Volume [ml]	Vu/Vg = 100/500
Ethanol	2 %
Dry cell weight [g/l]	10.7
M [h^{-1}]	0.135

equation became:

$$Z = 0.983 + 0.314\ x_4 - 0.079\ x_5 - 0.212\ x_4^2 - 0.195\ x_5^2$$

The response surface and contour plots for Z = f (pH, T) are presented in Figure 5. The coordinates of maxim were (+ 0.76, -0.203). The maximum values obtained for x_4 and x_5 correspond to the real values: pH = 4.76, T= 27.5^{o}C. The theoretical Z_{max} obtained by applying the statistical method was 1.11.

In order to verify the accuracy of the theoretical predictions, three other cultivations were performed in the bioreactor, keeping all the parameters described above at the mentioned values, except for the pH and the temperature. The temperature was kept between 27.4 - 27.6^{o}C, and the pH between 4.65 and 4.8. In these conditions the mean value obtained in the three runs for the substrate yield was 0.61 g ethanol/ g biomass, corresponding to an experimental value for Z_{max} of 1.08.

Conclusions

The mutant strain *S. cerevisiae* 3.20 was used both in laboratory and micropilot experiments to determine the optimal composition of the growth medium and also the optimal values for the process parameters pH and temperature. In both sets of experiments, the response

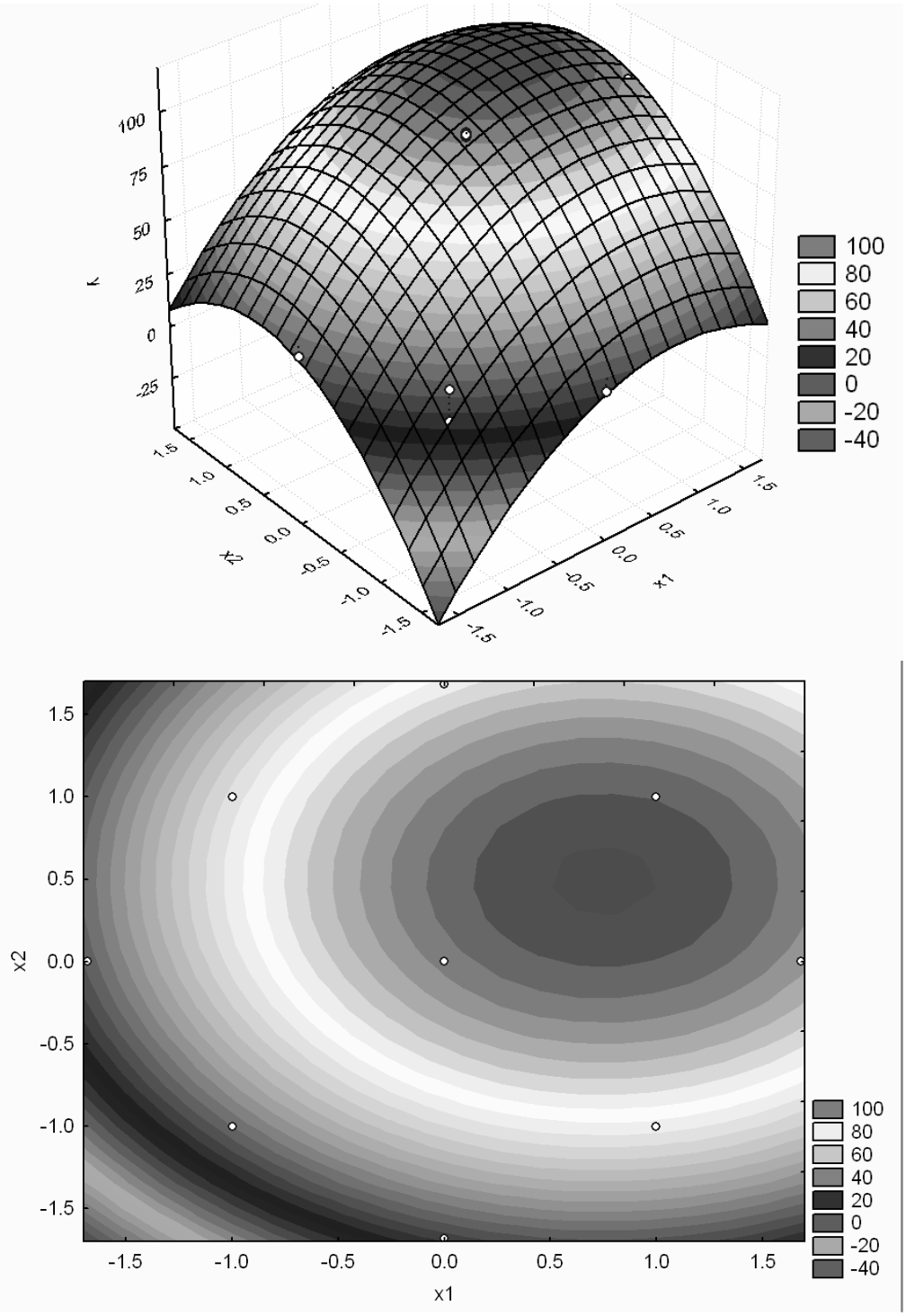

Figure 2. Response surface and contour plots f(x_1,x_2,0.5).

Table 6. The codification of the model's variables.

Variable	Code	Variable levels					Range
		-1.414	-1	0	1	1.414	2.828
pH	x_4	2.6	3.0	4.0	5.0	5.4	2.8
Temperature	x_5	24.5	25.5	28	30.5	31.5	7

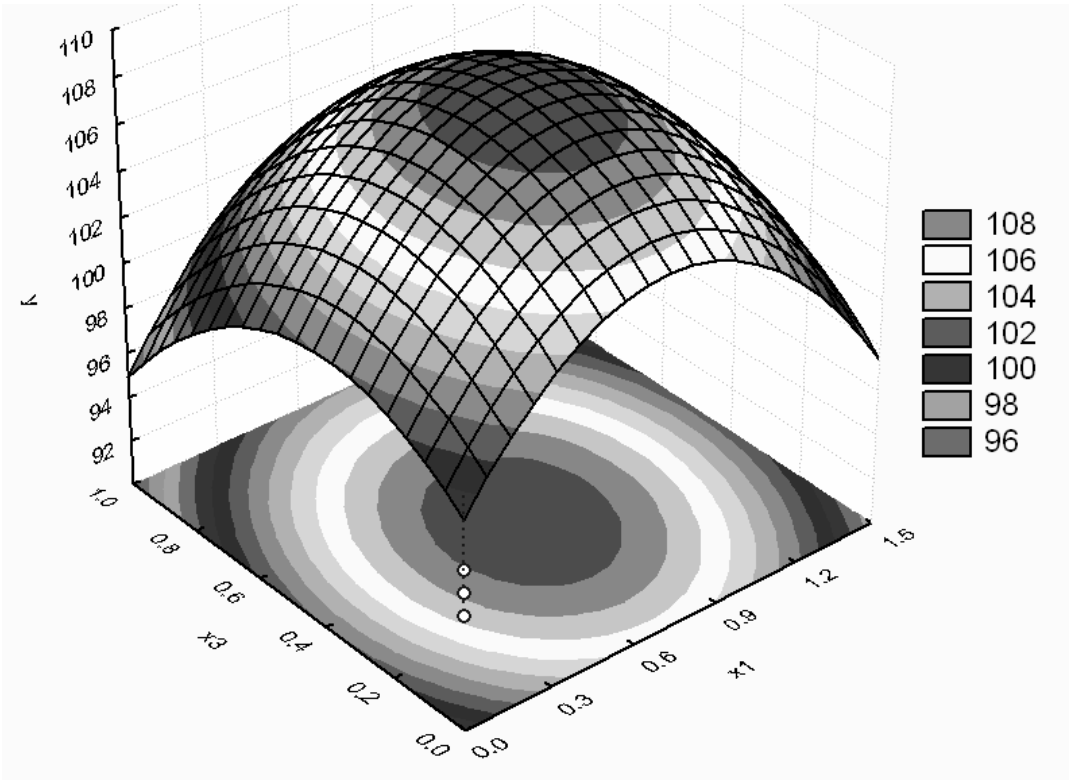

Figure 3. Response surface and contour plots for f(x_1,0.5,x_3).

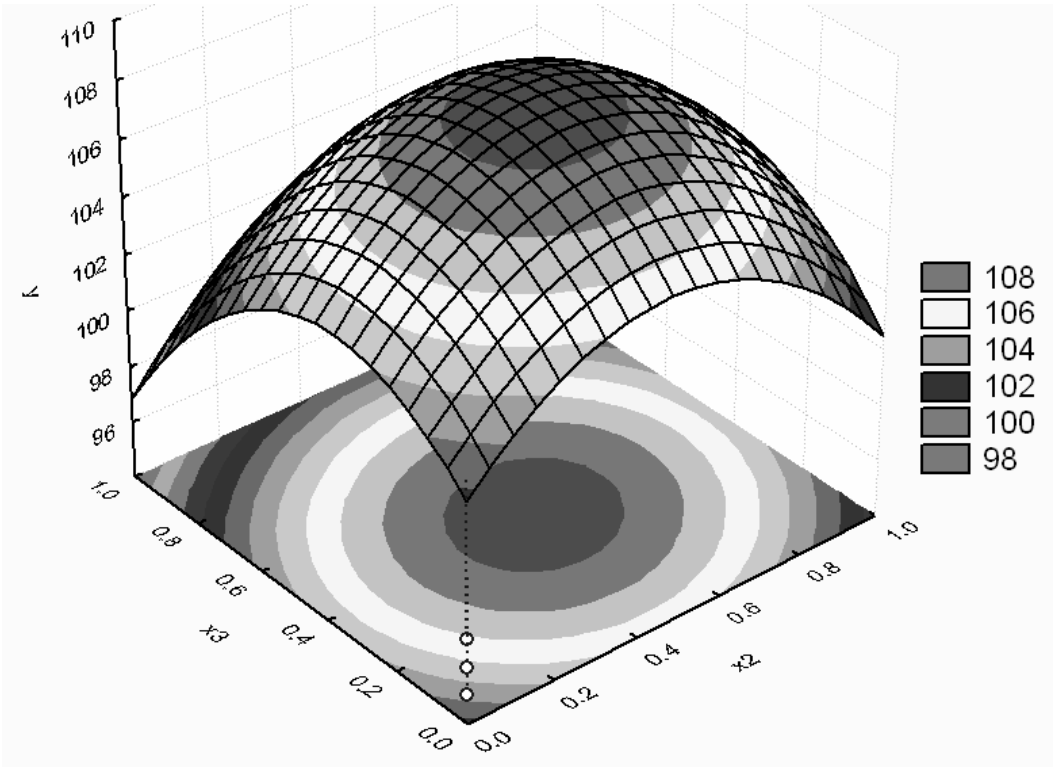

Figure 4. Response surface and contour plots for f (0.5, x_2,x_3).

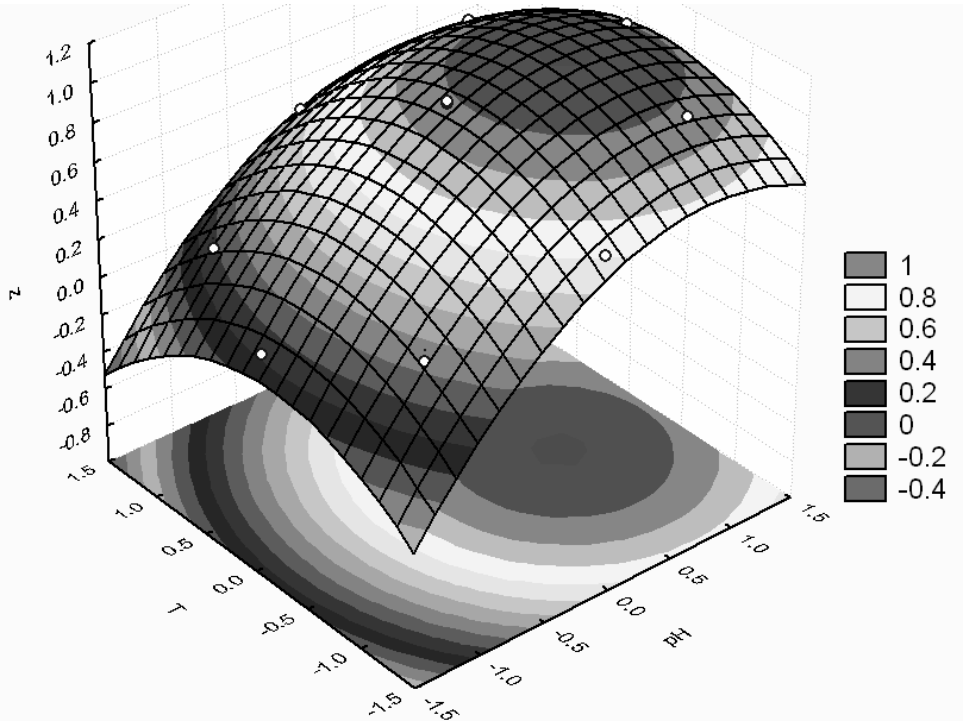

Figure 5. Response surface and contour plots for $Z_2 = f$ (pH, T).

Table 7. The experimental matrix for the central composite design (2^2 + star).

Block	pH	T	Z
1	1.414	0	1
1	1	-1	0.97
1	0	-1.414	0.71
1	-1	1	0.18
1	0	1.414	0.48
1	-1	-1	0.33
1	0	0	0.98
1	1	1	0.81
1	-1.414	0	0.12
1	0	0	0.98
1	0	0	0.99

surface methodology was used. The optimal composition of the growth medium, established by a central composite design (2^3 + star) was: KH_2PO_4- 2366 mg/l, $MgSO_4 \cdot 7H_2O$- 610 mg/l, corn steep liquor (50% dry substance) 2.43 g/l which yielded to a final dry cell weight of 10.7 g/l. The optimal values for the cultivation parameters pH and temperature, determined by a similar experiment were pH = 4.76, and T = 27.5°C, for which a substrate yield of about 0.6 g ethanol/g biomass was obtained.

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