Optimisation of inulinase production by *Kluyveromyces bulgaricus*

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Summary

Present work is based on observation of effects of pH and temperature of fermentation on the production of microbial enzyme inulinase by *Kluyveromyces bulgaricus* (former *Kluyveromyces marxianus*). Inulinase hydrolyses inulin, an oligosaccharide which can be isolated from plants such as Jerusalem artichoke, chicory or dahlia, into pure fructose (1). Fructooligosaccharides have great potential in food industry because they can be used as calorie-reduced and noncariogenic sweeteners. Fructose formation from inulin is a single step enzymatic reaction and yields are up to 95 % fructose. On contrary, conventional fructose production from starch needs at least three enzymatic steps, yielding only 45 % fructose (2).

Process of inulinase production was optimised by using experimental design method. pH value of the cultivation medium showed to be the most significant variable and it should be maintained at optimum value, 3.6. The effect of temperature was slightly lower and optimal values are between 30 and 33 ºC. At a low pH value of the cultivation medium, the microorganism was not able to produce enough enzyme and enzyme activities were low. Similar effect was caused by high temperature. Highest values of enzyme activities were achieved at optimal fermentation conditions and the values were: 100.16-124.36 IU/ml (with sucrose as substrate for determination of enzyme activity) or 8.6 -11.6 IU/ml (with inulin as substrate), respectively.

The method of factorial design and response surface analysis makes it possible to study several factors simultaneously, to quantify the individual effect of each factor and to investigate their possible interactions (3).

The model based on physiological assumptions is also applied. Assumed is a single enzyme rate determing growth (Monod kinetics) with proportional inulinase production rate. Applied are the models of reversible temperature and acidity inhibition based on thermodynamic equilibrium between active and inhibited enzyme states.

Predictions by the two models are compared by ANOVA.

Key words: Kluyveromyces bulgaricus, inulin, inulinase, optimisation, response surface method

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Introduction

Microbial enzymes can be roughly classified into three major fields of application: 1) those which can be used to synthesise useful compounds; 2) which can stereospecifically carry out important bioconversion reactions; 3) and which are able to hydrolyse polymers into interesting monomers (1).

Microbial enzyme inulinase hydrolyses plant polymer inulin into pure fructose with some glucose. Inulin is the storage carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory or dahlia. Inulin and inulin analogs are polyfructans, consisting of linear ß-2,1-linked polyfructose chains displaying a terminal glucose unit. The average length of an inulin chain varies as a function of the plant and a season. Theoretically, inulin should contain 30 sugar units at a minimum. Oligosaccharides are compounds with great potential of use in food industry. Particularly, fructooligosaccharides (FOS) are interesting, because of their favourable functional properties such as low calorie and noncariogenic sweeteners, improvement of the intestinal microbial flora, relief of constipation, decrease of total cholesterol and lipid in the serum and promotion of animal growth (1).

Lately in Croatia there is a great interest in adding FOS to dairy products, because prebiotic inulin enhances absorption of calcium (4). Inulin can be considered as dietary fibre, substitute for fat and low calorie sweetener (5). The use of prebiotic ingredients in combination with probiotics (e.g. high quality synbiotic yoghurts) offers an exciting possibility to enhance the health effects (6). Prebiotics exert their beneficial effects through direct and selective stimulation of healthy bacterial species in the colon flora. Ingestion of the prebiotic inulin leads to increased content of *Bifidobacteria* and *Lactobacilli* in the colon, thereby promoting gut health. The fermentation products from these species give rise to local and systemic health effects. Those effects are: lowered colonic pH, increased bioavailability of minerals, lowering of serum lipids levels (relevant for cardiovascular disease) and

stimulation of the immune response (7). Brand names of inulin are raftilin® or raftilose® (ORAFTI Active food ingredients, Belgium) and frutafit® (SENSUS, Netherlands).

Fructose formation from complete hydrolysis of inulin is a single step inulinase reaction and yields up to 95 % of fructose. On contrary, conventional fructose production from starch needs at least three enzymatic steps, yielding only 45 % of fructose. Inulinase can be used for production of pure fructose syrups and "Ultra High Fructose Glucose Syrups" (UHFGS)-not from starch, but from inulin (2). Inulinases can be found in plants and microorganisms. It is difficult to isolate plant inulinases in sufficient quantity. Therefore, microbial inulinases, which can be induced by growing microorganisms have a potential for industrial use in the production of fructose from inulin (8, 9, 10).

In the present paper, the inulinase production by *Kluyveromyces bulgaricus* in a shake flask was optimised using factorial design and response surface analysis.

Materials and Methods

Microorganism and growth conditions

Kluyveromyces bulgaricus was the microorganism used for the production of inulinase. First, microorganism was grown in test tubes filled with medium with agar for 24 hours, and afterwards in medium without agar for the next 24 hours. The inoculum cultures were grown on a medium containing 2 % sucrose and pH adjusted at 6.8. During this phase, 500 ml Erlenmeyer flasks were used, containing 100 ml of culture medium. Temperature was 30 ºC, at 150 rpm for 24 hours. Duration of the fermentation was 48 hours for the first series of experiments and 42 hours for the second series of experiments. Inulinase was produced in a 1000 ml flasks containing 300 ml of culture medium. Fermentation was carried out with 10 % of inoculum on a rotary shaker and different temperatures, depending on experiment. Fermentation medium contained: sucrose, 14 g/L, yeast extract, 10 g/L, peptone, 20 g/L,

K_2HPO_4, 1 g/L (11). Range of pH was from 2.3 to 3.7, and range of temperature was from 24 ºC to 36 ºC in the first series of experiments, and in the second series of experiments pH range was 2.7-4.7, and temperature range was 28-42 ºC.

Determination of inulinase activity

Determination of inulinase activity is based on rate of liberation of free sugar units in controlled conditions. Activity was assayed as follows: 1 ml enzyme solution was mixed with 5 ml sucrose or inulin, 2.5 ml acetate buffer and 1.5 ml distilled water. Usually, enzyme is appropriately diluted. The mixture was maintained at 50 ºC in an incubator and the rate of appearance of fructose by the DNS method was determined. One unit of inulinase activity is defined as the amount of enzyme catalysing the liberation 1μmol of fructose min^{-1} under the specified conditions.

Experimental design

The major difference from a classical methodology (univariable analysis) is that this method enables to vary all of factors. The classical method is laborious and time-consuming, especially for a large number of variables. In this work, the effects of pH and temperature T on inulinase production were studied, using a fractional design of 2^2 trials.

$$?(pH,T) = b_o + b_1\ pH + b_2\ T \qquad /1/$$

A full factorial design (2^3) with four central point replications was used for two variables- pH and T – having invertase and inulinase activities as responses.

$$?(pH,T)=b_0+b_1\,pH+b_2\,T+b_3\,pH^2+b_4\,pH\,T+b_5\,T^2 \qquad /2/$$

Table 1.

Table 2.

Theoretical model of activity

Besides optimisation of the fermentation parameters by the first and second order polynomial approximation /1-2/ an approximate phenomenological model may be applied. Assumed is growth associated production of inulinase with Michaelis-Menten kinetics for specific growth rate dependency on substrate with maximum growth rate dependent on pH and temperature T. The balance for inulinase during batch production is given by /3/

$$\frac{d}{dt}c_P = Y_{p/x}\cdot v_{\max}(pH,T)\cdot\left(\prod_{i=1}^{N}\frac{c_{Si}}{K_{mi}+c_{Si}}\right)\cdot c_x \qquad /\mathbf{3}/$$

Optimal concentrations of substrates have been predetermined in the previous work (11).

Theoretical dependence of maximum growth rate on temperature and pH is based on assumption of reversible temperature and pH inhibition of a single pseudo enzyme E:

$$substrates \xrightarrow{E\,(pH,T)} X + products \qquad /\mathbf{4}/$$

Reversible temperature inhibition of the enzyme is determined by the equilibrium between active E and inactive state E_d:

$$E \underset{}{\overset{K_1\,(T)}{\rightleftharpoons}} E_d \qquad /\mathbf{5}/$$

Reversible inhibition of the enzyme by acidity is modelled as equilibrium between active enzyme E and inhibited protonated enzyme EH^+ and hydroxylated enzyme EOH^-:

$$E + H^+ \underset{}{\overset{K_2(pH)}{\rightleftharpoons}} EH^+$$
$$E + OH^- \underset{}{\overset{K_3(pH)}{\rightleftharpoons}} EOH^- \qquad /\mathbf{6}/$$

Concentration of active state of enzyme E is determined by evaluation of the equilibrium states for reactions /5/ and /6/. The result is the following functional dependence of maximum enzyme activity on temperature T and pH:

$$n_{\max}(pH,T) = b_0 \frac{Exp\left(-\frac{b_1}{R\cdot T}\right)}{1+b_2\cdot Exp\left(-\frac{b_3}{R\cdot T}\right)} \cdot \frac{1}{1+b_4\cdot 10^{-pH}+b_5\cdot 10^{pH}} \qquad /\mathbf{7}/$$

The expression is derived on the assumption of temperature dependence of thermodynamic equilibrium between active and inactive states of enzyme, and pH dependence of equilibrium between ionisation states of enzyme (12, 13). Use of approximate theoretical models provides more accurate interpolation of data, such as Arrhenius type dependence of reaction rates on temperature, yielding more accurate approximation of process optimality. However, theoretical models are nonlinear with respect to parameters and minimisation of variance becomes a difficult numerical problem. The problem can be solved by the robust Levenberg-Marquardt iteration procedure (14) based on analytical evaluation of gradients. The method is based on application of one dimensional optimisation /8/ of parameter change $\boldsymbol{\Delta}$ evaluated by Jacobian and gradient of the sum of squares S^2 of errors between experimental data and model predictions:

$$\underset{l}{opt}\,? = -\left(\mathbf{J}^T\cdot\mathbf{J} + l\cdot\mathbf{I}\right)\cdot\frac{\partial}{\partial\mathbf{b}}S^2(\mathbf{b}) \qquad /\mathbf{8}/$$

The method integrates the best properties of the gradient method, for starting values of parameters ($\lambda\rightarrow 0$), and fast convergence in vicinity of a minimum, provided by approximated

Hessian evaluation ($\lambda \to \infty$). Used is high precision numerical evaluation provided with software *Mathematica* (15).

Results and Discussion

Results of the first series of experiments

Enzyme activity was measured at 24, 30, 36 and 48 hours of fermentation and the data are depicted on Fig. 1. and the values are given in Table 3. Analysis by response surface method was applied for the results obtained at 48 hour of fermentation. Activity varied according to the fermentation conditions. After 48 hours of fermentation the highest achieved value of enzyme activity with sucrose as substrate was 100.16 IU/ml at pH 3.7 and temperature 30 ºC, while with inulin as a substrate the highest value of enzyme activity was 8.6 IU/ml.

Fig. 1

Table 3.

Results of the second series of experiments

Enzyme activity was measured at 24, and 42 hours of fermentation with sucrose and at 42 hours with inulin and values are given in Table 4. Analysis (response surface method) was applied using results at 42 hours of fermentation. The highest values; 124.36 IU/ml with sucrose and 11.62 IU/ml with inulin were achieved after 42 hours of fermentation at pH 3.0 and temperature 30 ºC.

Table 4.

Fig. 2.

Fig. 3.

Comparisons between experimental data and predictions by surface response method ,given by /2/, for the first and second series of experiments with sucrose and inulin as

substrates are depicted in Fig. 2-3. Coefficients of correlation, R^2, were: 0.81 (for the first series of experiments) and 0,88 (second series of experiments) for substrate sucrose, and 0.67 (I)and 0.80(II) for substrate inulin. For the both series of experiments, the errors of model predictions are randomly distributed around the line of symmetry, which indicates random character of experimental error.

Fig. 4.

Fig. 5.

The response surfaces and contour diagrams for enzyme activities with the substrates are shown in Fig. 4-5. The surfaces are computed and graphically presented by use of *Mathematica* software (15). From the surfaces can be deduced higher sensitivity of enzyme activity on pH compared to temperature for the selected range of experimentation. Optimal conditions can be deduced from the corresponding contour diagrams.

Fig. 6.

Fig. 7.

Optimal conditions of enzyme activity can be recalculated by use of theoretical model of maximum rate dependence on pH and temperature /3/. Determination of activity surfaces requires nonlinear iterative procedure, and results obtained by Levenberg-Marquardt algorithm with *Mathematica* software are depicted in Fig. 6-7. The method is numerically more complex compared to the surface response method, but provides better interpolation and more accurate prediction of optimal conditions. Parameters in the theoretical model are energies of activation and equilibrium constants, which can be interpreted by physical and chemical mechanisms.

Conclusions

The main achievment of this work is in optimisation of the inulinase production by *Kluyveromyces bulgaricus* by use of sucrose as a carbon source, instead of inulin which is available in limited quantities and at a high price.

pH value of medium showed to be the most important parameter in the experiments when sucrose is used for determination of enzyme activity, while temperature has a slightly weaker influence. The optimal region for pH is 3.4 - 3.6, and for temperature 29 - 31 ^{0}C.

Similar conclusions are obtained with inulin as a substrate. The optimum values of pH were between 3.3 and 3.5 and optimum values of temperature are between 31 and 33 ºC.

Application of theoretical model for optimisation provides better interpolation of data which results in more accurate regions of optimality and model parameters. Energies of activation and equilibrium constants can be interpreted based on mechanism of enzyme activity. However, model is nonlinear in parameters and requires Levenberg-Marquardt numerical iterative algorithm.

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Symbols

b_i	model parameters
c_p	inulinase concentration
c_{si}	concentration of i-th substrate
c_x	biomass concentration
E	enzyme
E_d	deactivated enzyme
$\boldsymbol{J}$	Jacobian matrix
K_{mi}	Monod constant with respect to i-th substrate
K_i	thermodynamic equilibrium constant
pH	acidity
S^2	sum of squares of errors
T	temperature ^{0}C
V	volume L
X	biomass
$Y_{p/x}$	yield factor of inulinase production rate per biomass growth rate
$\boldsymbol{\Delta}$	vector of parameter changes
λ	gain factor
μ_{max}	maximum specific growth rate
ν	enzyme activity IU ml^{-1}

Table 1. Values of coded levels used in the fractional factorial design – first (I) and second (II) series of experiments

Coded variable levels	pH		$\frac{T}{^0C}$	
	I	II	I	II
-1	2.5	3.0	26	30
0	3.0	3.7	30	35
+1	3.5	4.4	34	40

Table 2. Values of coded levels used in the full factorial design – first (I) and second (II) series of experiments

Coded variable levels	pH		$\frac{T}{^0C}$	
	I	II	I	II
-1,41	2,3	2,7	24	28
-1	2.5	3.0	26	30
0	3.0	3.7	30	35
+1	3.5	4.4	34	40
+1,41	3,7	4,7	36	42

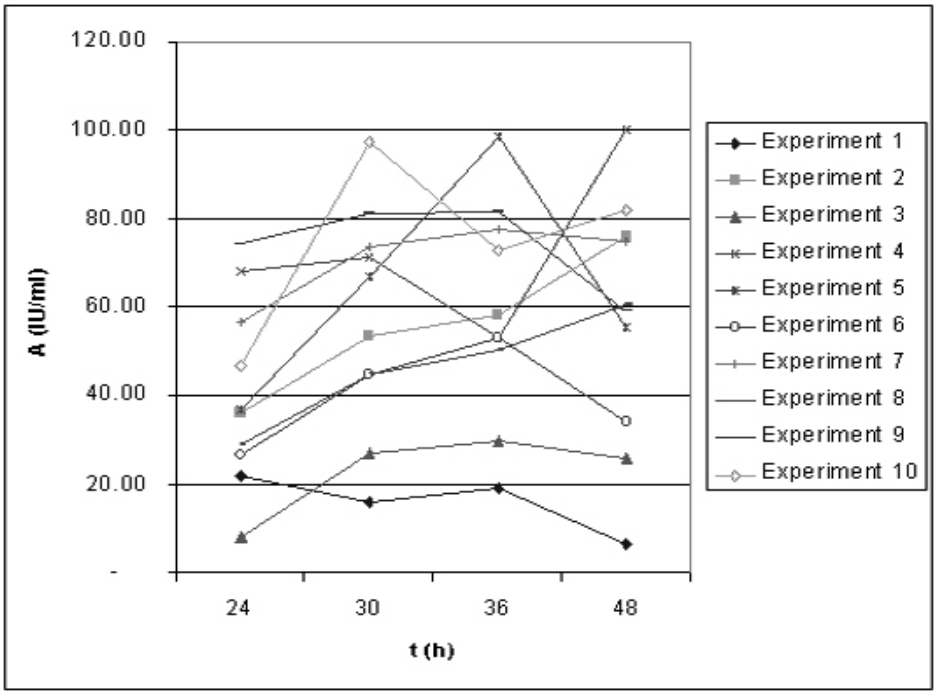

Fig.1. Enzyme activity A (sucrose) measured during fermentation of 48 h at the following pH and temperature : exp.1: 2.5, 26; exp. 2: 3.5, 26; exp. 3:3.5,34; exp. 4: 3.7, 30; exp. 5: 3.0, 24; exp. 6: 3.0, 36; exp. 7: 3.0, 30; exp. 8: 3.0, 30; exp. 9: 3.0, 30; exp. 10: 3.0, 30

Table 3. Results of full factorial design for the first series of experiments

Number of trial	Level		Value		$\frac{Enzyme\,activity}{IU\ ml^{-1}}$				
					Succrose				Inulin
	pH	$\frac{T}{^0C}$	*pH*	$\frac{T}{^0C}$	*t*=24 *h*	*t*=30 *h*	*t*=36 *h*	t=48 *h*	t=48 *h*
1	-1	-1	2.5	26	22.07	15.65	19.00	6.20	1.12
2	+1	-1	3.5	26	36.08	53.46	58.34	76.02	1.82
3	-1	+1	2.5	34	0.56	0.09	0.00	0.00	1.20
4	+1	+1	3.5	34	7.95	26.81	29.75	25.95	8.60
5	-1.41	0	2.3	30	0.13	0.00	0.32	0.17	0.00
6	+1.41	0	3.7	30	68.16	71.29	53.16	100.16	5.84
7	0	-1.41	3.0	24	36.81	66.98	98.29	55.33	5.36
8	0	+1.41	3.0	36	26.47	44.76	53.29	34.20	3.28
9	0	0	3.0	30	56.58	73.52	77.41	74.84	2.68
10	0	0	3.0	30	28.75	45.00	50.13	60.63	2.82
11	0	0	3.0	30	74.23	81.12	81.35	59.24	4.96
12	0	0	3.0	30	46.61	97.22	72.71	81.66	6.86

Table 4. Results of full factorial design for the second series of experiments

Number of trial	Level		Value		$\frac{Enzyme\,activity}{IU\ ml^{-1}}$		
					Succrose		Inulin
	pH	$\frac{T}{^0C}$	*pH*	$\frac{T}{^0C}$	*t*=24 *h*	*t*=42 *h*	*t*=42 *h*
1	-1	-1	3.0	30	155.64	124.36	11.62
2	+1	-1	4.4	30	31.61	20.59	2.77
3	-1	+1	3.0	40	34.55	27.71	1.75
4	+1	+1	4.4	40	20.05	30.71	2.80
5	-1.41	0	2.7	35	9.48	18.93	1.06
6	+1.41	0	4.7	35	38.31	50.92	2.29
7	0	-1.41	3.7	28	88.18	112.96	6.90
8	0	+1.41	3.7	42	7.52	9.28	1.67
9	0	0	3.7	35	96.36	104.53	4.84
10	0	0	3.7	35	70.24	90.10	4.67

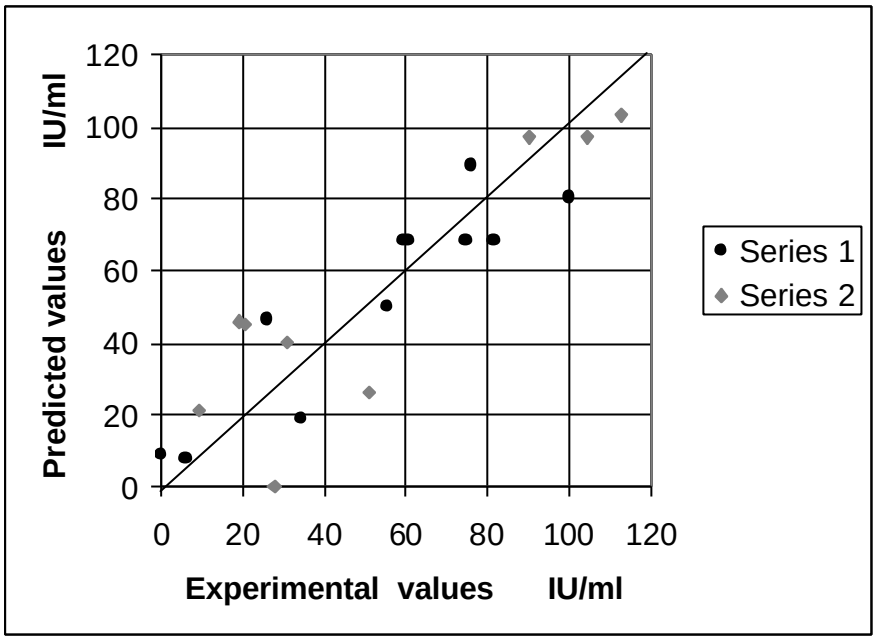

Fig.2. Values of enzyme activities predicted by surface response method versus experimental data achieved with substrate sucrose.

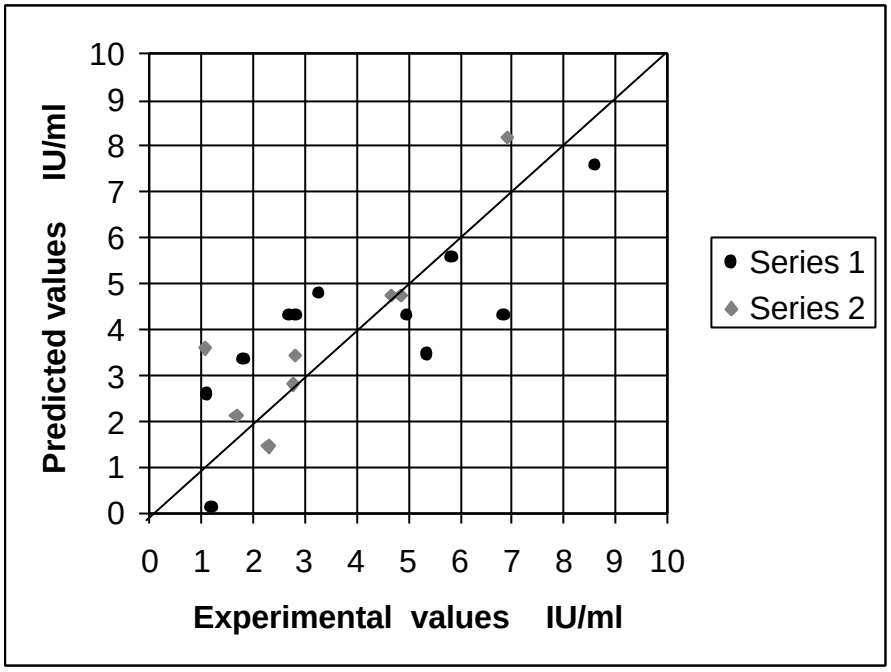

Fig. 3. Values of enzyme activities predicted by surface response method versus experimental data achieved with substrate inulin.

1-st experiment, substrate is sucrose

Effect estimates. Dependent variable: enzyme activity, substrate sucrose
R^2=0.87897, pure error=119,8872

Factor	Effect	Standard error	t(3)	p	Confiance lim. –90%	Confiance lim. +90%	Coefficient	Standard error coefficient
Mean	69,09	5,47	12,62	0,001	56,21	81,97	69,09	5,47
1. pH (L)	59,35	7,74	7,66	0,004	41,13	77,57	29,67	3,87
pH (F)	-29,20	8,65	-3,37	0,043	-49,57	-8,83	-14,60	4,33
2. T (L)	-21,53	7,74	-2,78	0,068	-39,75	-3,31	-10,76	3,87
T (F)	-34,52	8,65	-3,98	0,028	-54,89	-14,15	-17,26	4,33
1 x 2	-21,93	10,94	-2,00	0,138	-47,69	3,84	-10,96	5,47

Analysis of variance

Source of variation	Sum of squaresSQ	Degrees of freedom	Mean squares-MS	F-test
Regression	11189,08	5	2237,81	8,71
Residual	1540,73	6	256,78	
Lack of fit	1181,07	3	393,69	
Pure error	359,66	3	119,88	
Total	12729,81	11	1157,25	

$F_{0,1}(5/6)=3,11$
$F_{rac}/F_{tab}=2,8$

2-nd experiment, substrate is sucrose

Effect estimates. Dependent variable: enzyme activity, substrate sucrose
R^2=0,81491, pure error =104,1125

Factor	Effect	Standard error	t(3)	p	Confiance lim. –90%	Confiance lim. +90%	Coefficient	Standard error coefficient
Mean	97,31	7,21	13,49	0,05	51,76	142,87	97,31	7,21
1. pH (L)	-13,62	7,17	-1,89	0,31	-58,95	31,69	-6,81	3,59
pH (F)	-59,91	9,37	-6,39	0,09	-119,132	-0,69	-29,95	4,69
2. T (L)	-58,50	7,25	-8,06	0,08	-104,29	-12,72	-29,25	3,62
T (F)	-35,59	9,71	-3,66	0,17	-96,91	25,71	-17,79	4,85
1 x 2	53,38	10,20	5,23	0,12	-11,04	117,81	26,69	5,10

Analysis of variance

Source of variation	Sum of squaresSQ	Degrees of freedom	Mean squares-MS	F-test
Regression	14359,86	5	2871,97	3,522
Residual	3261,63	4	815,41	
Lack of fit	3157,52	3	1052,51	
Pure error	104,11	1	104,11	
Total	17621,49	9	1957,94	

$F_{0,1}(5/6)=3,11$
$F_{rac}/F_{tab}=2,8$

1-st experiment, substrate is inulin

Effect estimates. Dependent variable: enzyme activity, substrate inulin R^2=0.67762, pure error=3,9334								
Factor	Effect	Standard error	t(3)	p	Confiance lim. –90%	Confiance lim. +90%	Coefficient	Standard error coefficient
Mean	4,33	0,99	4,36	0,02	1,99	6,66	4,33	0,99
1. pH (L)	4,08	1,40	2,91	0,06	0,78	7,39	2,04	0,70
pH (F)	-1,62	1,56	-1,03	0,37	-5,32	2,06	-0,81	0,78
2. T (L)	0,98	1,40	0,69	0,53	-2,32	4,28	0,49	0,70
T (F)	-0,22	1,56	-0,14	0,89	-3,92	3,46	-0,11	0,78
1 x 2	3,35	1,98	1,69	0,19	-1,32	8,02	1,67	0,99

Analysis of variance				
Source of variation	Sum of squaresSQ	Degrees of freedom	Mean squares-MS	F-test
Regression	51,02	5	10,20	2,55
Residual	24,02	6	4,00	
Lack of fit	12,22	3	4,07	
Pure error	11,80	3	3,93	
Total	75,04	11	6,82	

$F_{0,1}(5/6)=3,11$
$F_{rac}/F_{tab}=2,8$

2-nd experiment, substrate is inulin

Effect estimates. Dependent variable: enzyme activity, substrate inulin R^2=0.80424, pure error=0,01445								
Factor	Effect	Standard error	t(3)	p	Confiance lim. –90%	Confiance lim. +90%	Coefficient	Standard error coefficient
Mean	4,75	0,08	55,94	0,011	4,22	5,29	4,75	0,08
1. pH (L)	-1,49	0,08	-17,68	0,035	-2,03	-0,96	-0,75	0,04
pH (F)	-2,18	0,11	-19,80	0,032	-2,88	-1,49	-1,09	0,05
2. T (L)	-4,33	0,08	-50,73	0,012	-4,87	-3,79	-2,16	0,04
T (F)	0,42	0,11	3,67	0,169	-0,30	1,14	0,21	0,06
1 x 2	4,95	0,12	41,18	0,015	4,19	5,71	2,47	0,06

Analysis of variance				
Source of variation	Sum of squaresSQ	Degrees of freedom	Mean squares-MS	F-test
Regression	14359,86	5	2871,97	3,522
Residual	3261,63	4	815,41	
Lack of fit	3157,52	3	1052,51	
Pure error	104,11	1	104,11	
Total	17621,49	9	1957,94	

$F_{0,1}(5/6)=3,11$
$F_{rac}/F_{tab}=2,8$

substrate	Model parameters					
	b_0 / Iuml^{-1}	b_1 /Jmol^{-1}	b_2	b_3/Jmol^{-1}	b_4	b_5
sucrose	144.008	38.396 10^{-6}	0	8.5063	1479.98	114,27 10^{-6}
inulin	34.987	0.06293	3.3057 10^{3}	23.618 10^{3}	0.9863 10^{3}	93.735 10^{-6}

SUCROSE			
	DF	Sum of squares	Mean Square
Model	6	72716.1	12119.3
Error	16	19934.8	1245.92
Uncorrected total	22	92650.9	
Corrected total	21	31012	
INULIN			
	DF	Sum of squares	Mean Square
Model	6	381.12	63.52
Error	16	114.8	7.17
Uncorrected total	22	495.9	
Corrected total	21	168.2	

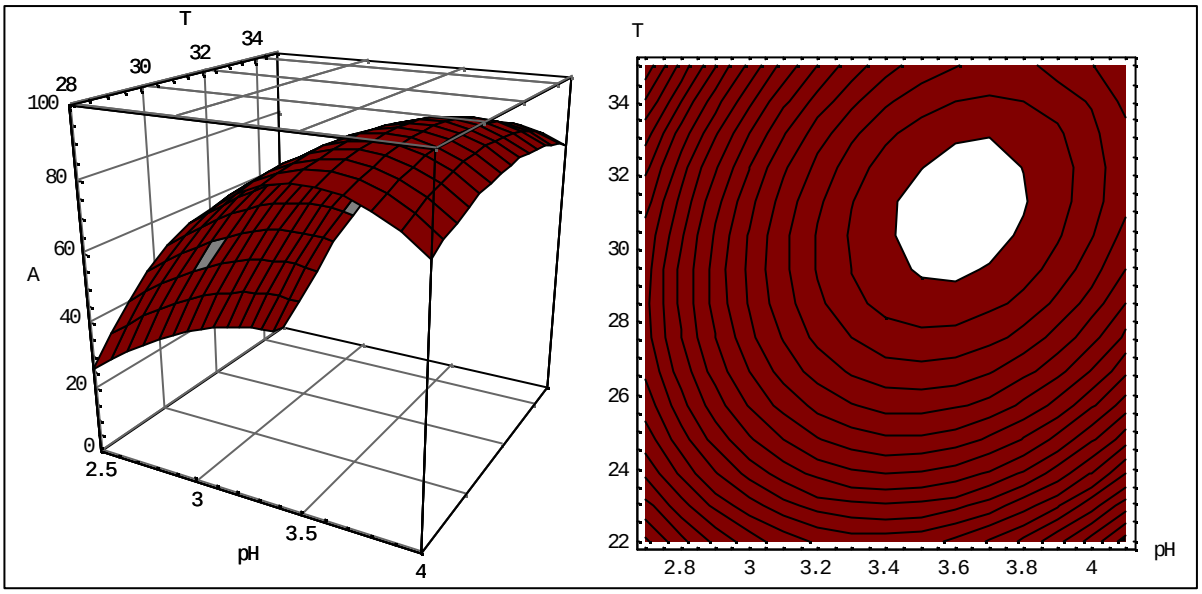

Fig. 4. Response surface and contour diagram for enzyme activity (A) for sucrose as substrate.

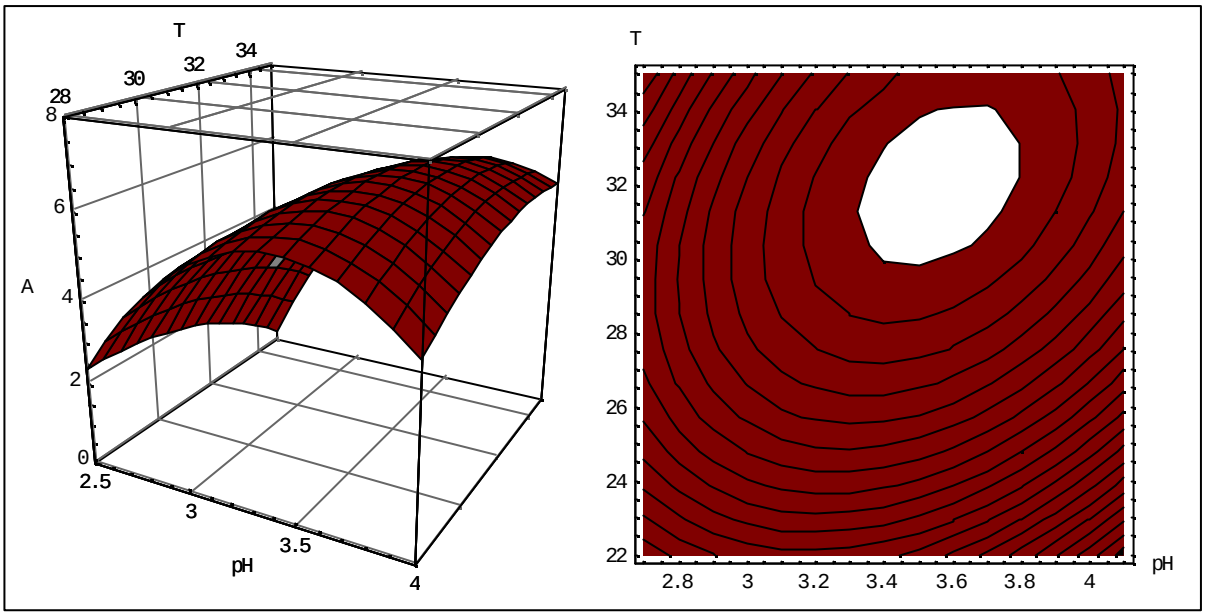

Fig. 5. Response surface and contour diagram for enzyme activity (A) for inulin as substrate.

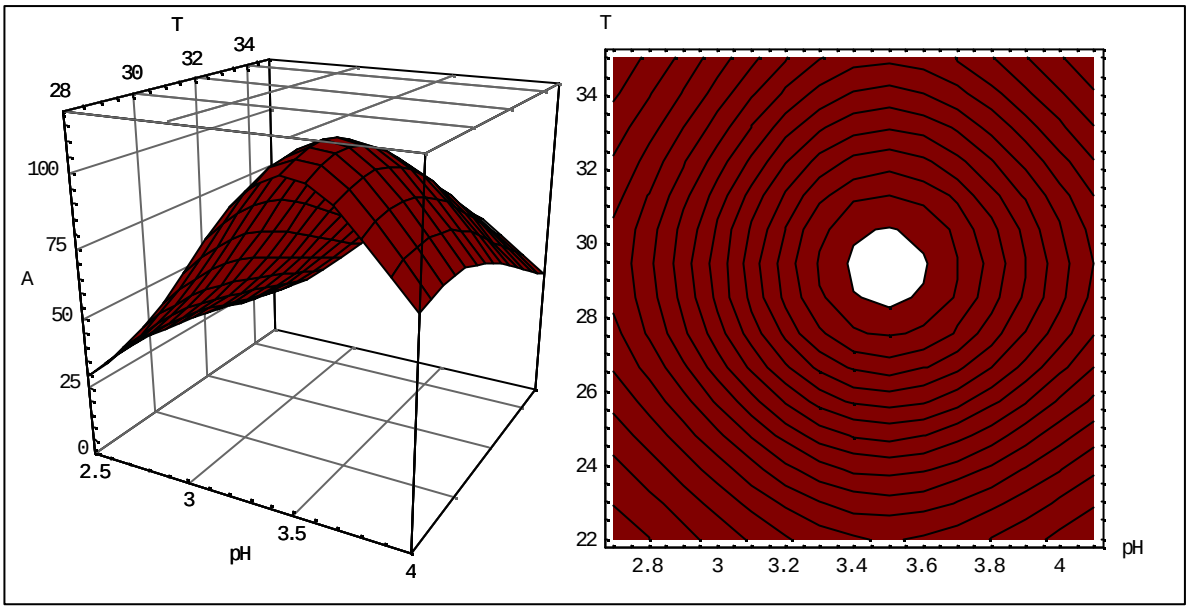

Fig. 6. Response surface and contour diagram achieved using theoretical model of activity (A) and software "Wolfram Research *Mathematica* 4.0", substrate is sucrose

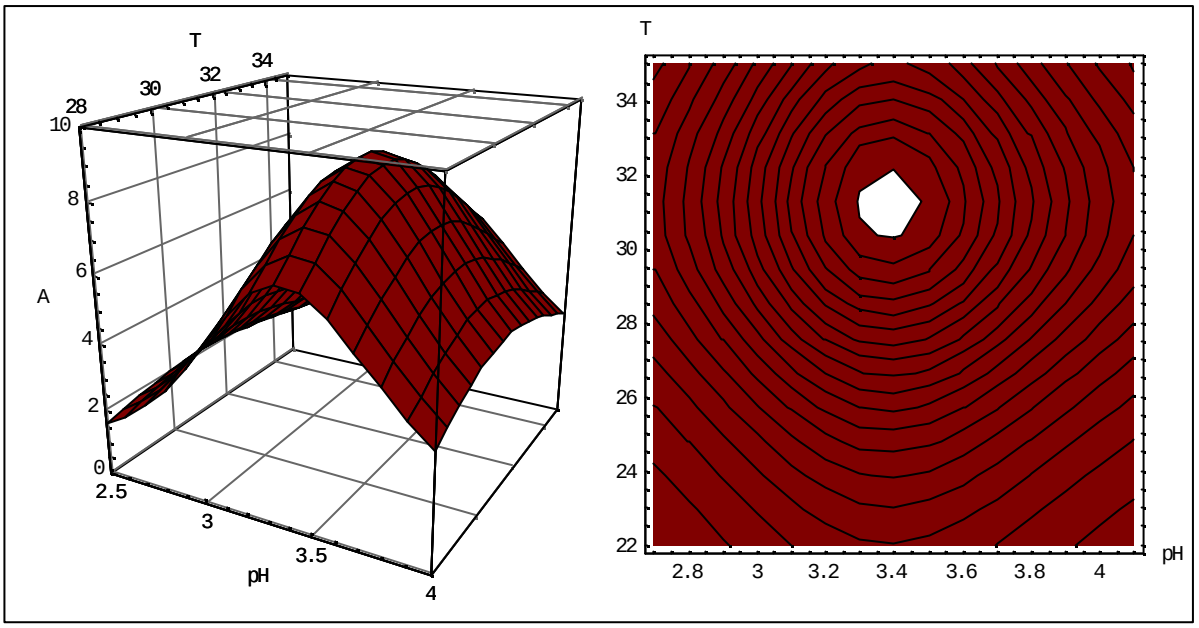

Fig.7. Response surface and contour diagram achieved using theoretical model of activity (A) and software "Wolfram Research *Mathematica* 4.0", substrate is inulin