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Optimisation of Inulinase Production by *Kluyveromyces bulgaricus*

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Summary

The present work is based on observation of the effects of pH and temperature of fermentation on the production of microbial enzyme inulinase by *Kluyveromyces marxianus* var. *bulgaricus*. Inulinase hydrolyzes inulin, a polysaccharide which can be isolated from plants such as Jerusalem artichoke, chicory or dahlia, and transformed into pure fructose or fructooligosaccharides. Fructooligosaccharides have great potential in food industry because they can be used as calorie-reduced compounds and noncariogenic sweeteners as well as soluble fibre and prebiotic compounds. Fructose formation from inulin is a single step enzymatic reaction and yields are up to 95 % the fructose. On the contrary, conventional fructose production from starch needs at least three enzymatic steps, yielding only 45 % of fructose.

The 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 of 3.6. The effect of temperature was slightly lower and optimal values were between 30 and 33 °C. 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. The 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.

As a comparison to this method, optimisation of a physiological enzyme activity model depending on pH and temperature was also studied.

Key words: Kluyveromyces marxianus var. *bulgaricus*, inulin, inulinase, optimisation, response surface analysis

Introduction

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

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Microbial enzyme inulinase (EC 3.2.1.7) hydrolyzes plant polymer inulin into fructooligosacharides and 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 polyfructosans, consisting of linear β-2,1-linked polyfructose chains displaying a terminal glucose unit. The average length of an inulin chain varies depending on the function of the plant and season. Theoretically, inulin should contain 30 sugar units at a minimum. Oligosaccharides are compounds with great potential of use in food industry. Particularly interesting are fructooligosaccharides (FOS), 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)*.

There is a great interest in adding FOS to dairy products, because prebiotic inulin enhances the absorption of calcium *(2)*. Inulin can be considered as dietary fibre, a substitute for fat and low calorie sweetener *(3)*. The use of prebiotic ingredients in combination with probiotics (*e.g.* high quality synbiotic yoghurts) offers an exciting possibility to enhance the health effects *(4)*. 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 beneficial bacteria from genera *Bifidobacterium* and *Lactobacillus* in the colon, thereby promoting gut health. The fermentation products from these groups of bacteria 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 *(5)*. 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. 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 *(6)*. 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 *(7–9)*.

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

Materials and Methods

Microorganism and growth conditions

Kluyveromyces marxianus var. *bulgaricus* ATCC 16045 was the microorganism used for the production of inulinase. First, microorganism was grown in test tubes filled with culture medium containing agar for 24 hours, and afterwards in the 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 °C, at 150 rpm for 24 hours. The duration of fermentation was 48 hours for the first series of experiments and 42 hours for the second series of experiments. Inulinase was produced in 1000-mL flasks containing 300 mL of culture medium. Fermentation was carried out with 10 % of inoculum on a rotary shaker and at different temperatures, depending on the experiment. Fermentation medium contained: sucrose 14 g/L, yeast extract 10 g/L, peptone 20 g/L, K_2HPO_4 1 g/L *(10)*. The range of pH was from 2.3 to 3.7, and the range of temperature was from 24 to 36 °C 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 °C.

Determination of inulinase activity

The determination of inulinase activity is based on the rate of liberation of free sugar units in controlled conditions. The activity was assayed as follows: 1 mL enzyme solution was mixed with 5 mL of sucrose or inulin, 2.5 mL of acetate buffer and 1.5 mL of distilled water. Usually the enzyme is appropriately diluted. The mixture was maintained at 50 °C in an incubator and the rate of appearance of fructose was determined by the dinitrosalicylic acid (DNS) method *(11)*. One unit of inulinase activity is defined as the amount of enzyme catalysing the liberation of 1 μmol of fructose/min under the specified conditions.

Experimental design

The major difference from a classical methodology (univariable analysis) is that this method enables to vary all of the 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 factorial design of 2^2 trials.

$$v(pH,t) = b_o + b_1\,\mathrm{pH} + b_2\,t \qquad /1/$$

Later, a full factorial design plus star configuration with four central point replications was used for two variables – pH and t – having invertase and inulinase activities as responses.

$$v(\mathrm{pH},t) = b_o + b_1\,\mathrm{pH} + b_2\,t + b_3\,\mathrm{pH}^2 + b_4\,\mathrm{pH}\;t + b_5\,t^2 \qquad /2/$$

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

Coded variable levels	pH		t/°C	
	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 factorial design plus star configuration – first (I) and second (II) series of experiments

Coded variable levels	pH		*t*/°C	
	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

Physiological (theoretical) model of activity

Apart from optimisation of the fermentation parameters by the first and second order polynomial approximation /1–2/ an approximate physiological model may be applied. In this work a physiological dependence of maximum enzyme reaction rate on temperature and pH /3/ is applied.

$$\nu(\mathrm{pH},t) = b_0 \frac{Exp\left(-\frac{b_1}{R\cdot t}\right)}{1+b_2\cdot Exp\left(-\frac{b_3}{R\cdot t}\right)} \cdot \frac{1}{1+\frac{b_4}{\mathrm{pH}}+b_5\cdot \mathrm{pH}} \qquad /3/$$

The expression is derived on the assumption of temperature dependence of thermodynamic equilibrium between active and inactive states of the enzyme, and pH dependence of equilibrium between ionisation states of the enzyme *(12, 13)*. The use of approximate physiological 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 the parameters and the 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 and high precision numerical evaluation provided with *Mathematica* software *(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 values are presented in Table 3. The analysis by response surface method was applied for the results obtained at 48 hours of fermentation. Activity varied according to the fermentation con-

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

Number of trial	Level		Value		Enzyme activity IU/mL				
					Sucrose				Inulin
	pH	*t*/°C	pH	*t*/°C	24 *h*	30 *h*	36 *h*	48 *h*	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 the factorial design for the second series of experiments

Number of trial	Level		Value		Enzyme activity IU/mL		
					Sucrose		Inulin
	pH	*t*/°C	pH	*t*/°C	24 *h*	42 *h*	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

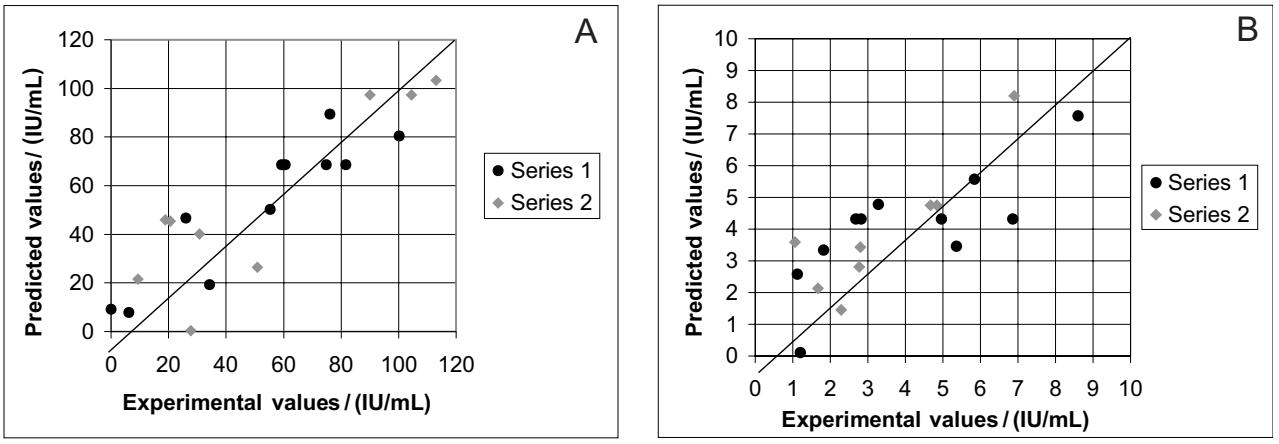

Fig. 1A. Values of enzyme activities predicted by surface response method versus experimental data achieved with sucrose as substrate
Fig. 1B. Values of enzyme activities predicted by surface response method versus experimental data achieved with inulin as substrate

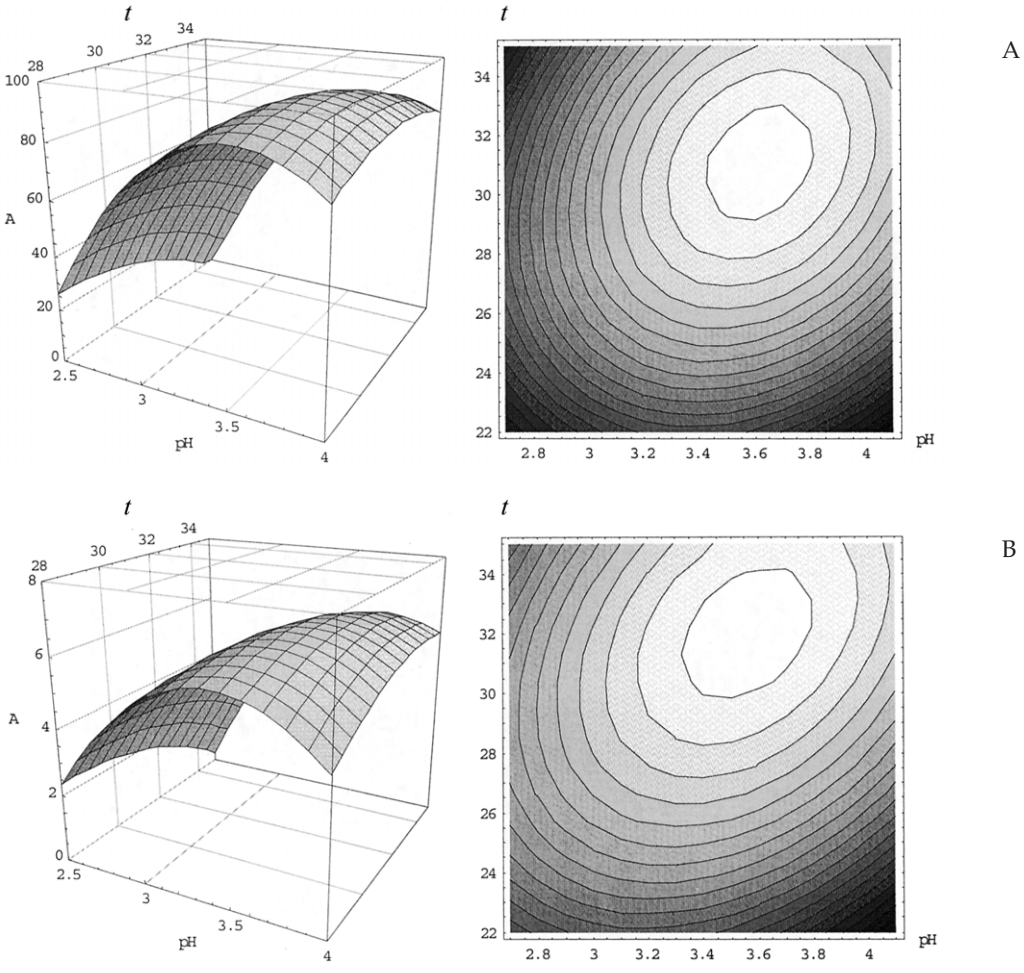

Fig. 2A. Response surface and contour diagram for enzyme activity (A) for sucrose as substrate
Fig. 2B. Response surface and contour diagram for enzyme activity (A) for inulin as substrate

ditions. 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 of 30 °C, while with inulin as a substrate the highest value of enzyme activity was 8.6 IU/mL.

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 the values are given in Table 4. Analysis (response surface method) was applied using the results at 42

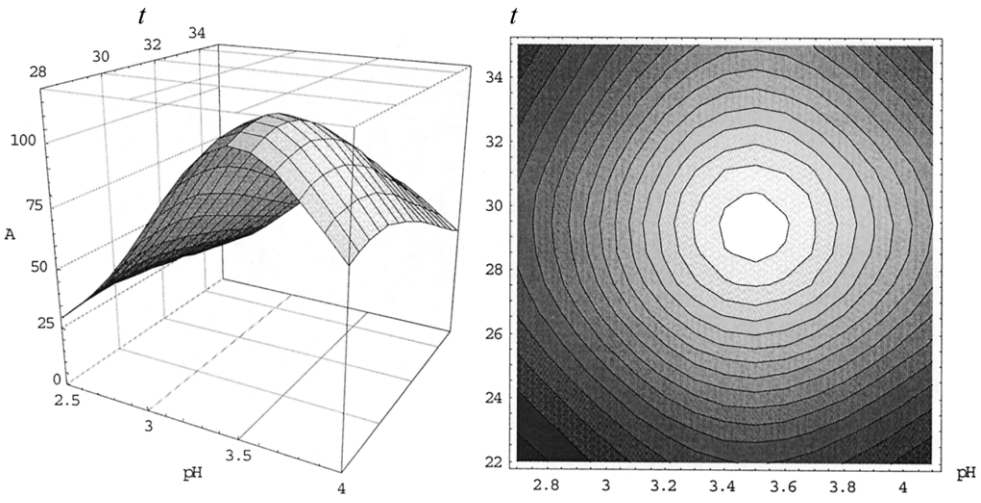

Fig. 3. Response surface and contour diagram achieved using physiological model of activity (A) with sucrose as substrate

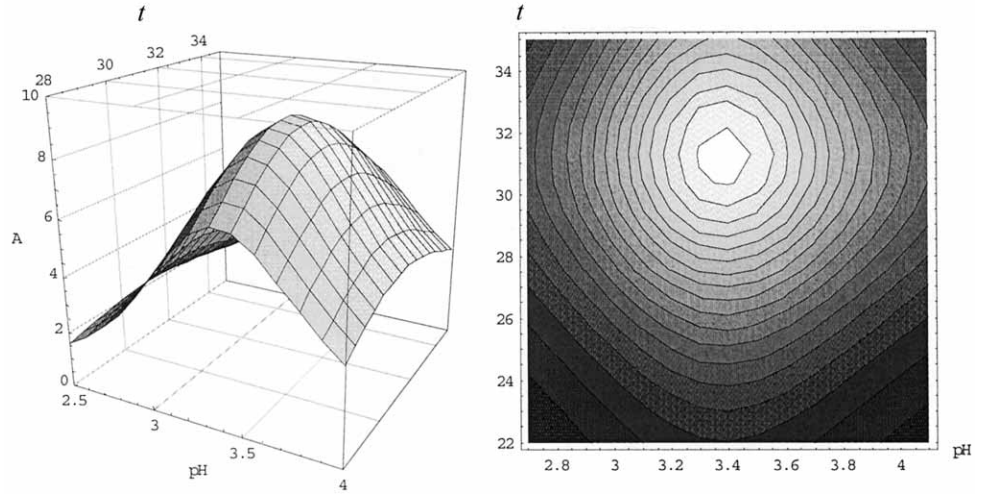

Fig. 4. Response surface and contour diagram achieved using physiological model of activity (A) with inulin as substrate

hours of fermentation. The highest values of 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 of 30 °C.

Comparisons between experimental data and predictions by surface response method, given by the equation /2/, for the first and second series of experiments with sucrose and inulin as substrates are offered in Figs. 1A and 1B. Coefficients of correlation, R^2, were: 0.81 (for the first series of experiments) and 0,88 (second series of experiments) for sucrose as substrate, and 0.67 (I) and 0.80 (II) for inulin as substrate. For both series of experiments, the errors of model predictions are randomly distributed around the line of symmetry, which indicates random character of the experimental error.

The response surfaces and contour diagrams for enzyme activities with the substrates are shown in Figs. 2A and 2B. The surfaces are computed and graphically presented by the use of *Mathematica* software *(15)*. From the surfaces, higher sensitivity of enzyme activity on pH compared to temperature for the selected range of experimentation can be deduced. Optimal conditions can be deduced from the corresponding contour diagrams.

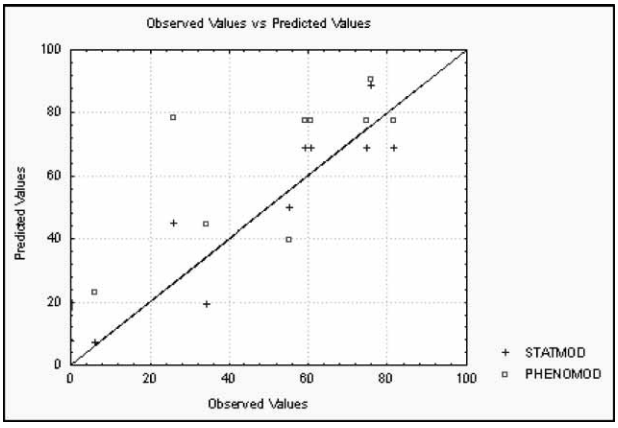

Fig. 5. Predicted versus experimental enzyme activities achieved with sucrose as substrate determined by statistical and physiological models

Optimal conditions of enzyme activity can be recalculated by the use of physiological model of maximum rate dependence on pH and temperature /3/. The determination of activity surfaces requires nonlinear iterative procedure, and the results obtained by Levenberg – Marquardt algorithm with *Mathematica* software are depicted in Figs. 3 and 4. The comparison of the two models is depicted in Figs. 5 and 6.

Predicted versus experimental enzyme activities achieved with sucrose as a substrate determined by the statistical and physiological models are given in Fig. 5, and deviations by the statistical and physiological models of the enzyme activities achieved with sucrose as substrate are given in Fig. 6.

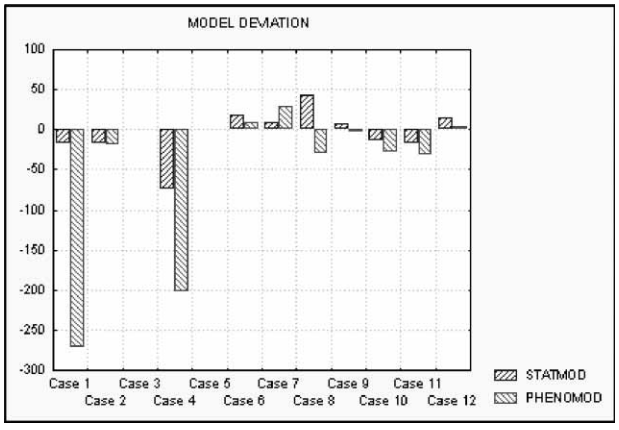

Fig. 6. Deviations by statistical and physiological models of the enzyme activities achieved with sucrose as substrate

Optimisation based on physiological model is numerically more complex compared to the surface response method (statistical model), but it may provide better interpolation and more accurate prediction of optimal conditions when experimental data are sufficiently accurate. Physiological models enable the interpretation of parameters, such as values of energies of activation and equilibrium constants, which can be related to physical and biochemical mechanisms.

Conclusions

The main achievement of this work is optimisation of the inulinase production by *Kluyveromyces marxianus* by the use of sucrose instead of inulin as a carbon source.

pH value of the medium showed to be more important parameter in the experiments when sucrose is used for determination of enzyme activity, while temperature has a slightly weaker influence. The optimal range for pH is 3.4–3.6, and for temperature 29–31 °C.

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

Statistical model has lower deviation than the physiological one. This is due, from one point of view, to the fact that statistical model can adapt itself to almost any experimental result, and, from another point of view, either the physiological model is not as suitable for this process as desired, or the experimental results are not so good. Experimental design can be performed not only with polynomial models but also with physiological models, which is a contribution of this work.

However, application of physiological 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 the mechanism of enzyme activity.

Symbols

b_i	model parameters
pH	acidity
t	temperature (°C)
V	volume (L)
v	enzyme activity (IU / mL)

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Optimiranje proizvodnje inulinaze s *Kluyveromyces bulgaricus*

Sažetak

Ovaj se rad temelji na ispitivanju utjecaja pH i temperature na proces biosinteze enzima inulinaze s kvascem *Kluyveromyces bulgaricus* (prije *Kluyveromyces marxianus*). Mikrobni enzim inulinaza hidrolizira inulin, polisaharid izoliran najčešće iz biljaka (jeruzalemska artičoka, cikorija i dalija), do fruktoze ili fruktooligosaharida.

Fruktooligosaharidi se uvelike primjenjuju u prehrambenoj industriji jer se mogu koristiti kao niskokalorični i zaslađivači koji sprečavaju karijes, te kao topljiva vlakna i prebiotički spojevi. Potpuna je hidroliza inulina jednostupanjska enzimska reakcija koju katalizira inulinaza uz 95 %–tni prinos fruktoze. Nasuprot tome, konvencionalna proizvodnja fruktoze iz škroba zahtijeva barem tri stupnja, s tri različita enzima, a prinos je tek 45 % fruktoze.

Proces proizvodnje inulinaze optimiran je metodom planiranja pokusa (»experimental design«). pH-vrijednost hranjive podloge pokazala se kao najvažniji parametar u proizvodnji inulinaze i optimalna je vrijednost iznosila 3,6. Temperatura je imala nešto slabiji utjecaj, a optimalne su vrijednosti bile između 30 i 33 °C. Pri niskoj pH-vrijednosti podloge mikroorganizam nije uspijevao proizvesti dovoljnu količinu enzima, pa su i enzimske aktivnosti male. Slično djelovanje uzrokovano je i pri visokim temperaturama uzgoja. Najviše vrijednosti enzimske aktivnosti, postignute pri optimalnim uvjetima, iznosile su: 100,16 do 124,36 U/mL (sa saharozom kao supstratom za određivanje enzimske aktivnosti) ili 8,6 do 11,6 U/mL (s inulinom kao supstratom).

Metode planiranja pokusa i analize odzivnih ploha omogućuju promatranje utjecaja nekoliko parametara istodobno, te utvrđivanje intenziteta tih utjecaja, a i njihovih mogućih interakcija.

Usporedo je provedeno optimiranje fiziološkog modela enzimske aktivnosti ovisno o pH i temperaturi.