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Optimisation of Inulinase Production by *Aspergillus niger* Using Simplex and Classical Method

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Summary

The simplex method has been used to optimise the inulinase production by *Aspergillus niger* strain 13/36 with respect to 5 components of the medium. The extracellular enzyme activity was almost 80 U/mL, and over 1.6-fold higher than the results obtained by the classical »one-factor-at-a-time« method. The greatest advantage of the simplex method is that the optimum factor levels were determined in only 12 iterative steps, shortening a typical optimum search process considerably. This is the first use of the simplex method for optimising inulinase production.

Key words: simplex optimisation, inulinase, *Aspergillus niger*

Introduction

Inulin is hydrolysed by two types of inulinases: exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) and endoinulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7). Inulinases are used in the production of high-fructose syrups (*1–4*), which have gained importance as sweetening agents. Inulinases have also been used for the production of inulo-oligosaccharides – low caloric saccharides acting as growth factors for beneficial microorganisms in the intestinal flora (*5–7*). Another application of inulinases is the production of ethanol from inulin (*4,8*).

The optimisation of the medium by the classical method involves changing one independent variable (such as the nutrient, temperature, pH, *etc.*) while fixing others at certain levels. This one-dimensional search is laborious and time-consuming, especially for a large number of variables, and frequently does not guarantee the determination of optimal conditions. Hence a more practical method, simplex, was adopted to study the effect of parameters affecting inulinase production. A number of reports concerning the optimisation of inulinase production by microorganisms has appeared (*5,9–12*). However, to our knowledge, no research on the use of the simplex method for this purpose has yet been performed.

Materials and Methods

Microorganism and media

The strain of *Aspergillus niger* 13/36, producing high inulinase activity in shaken cultures, was used. *A. niger* strain 13/36 was obtained from the Department of Food Technology and Storage, Agricultural University, Lublin, Poland. During the experiments, the culture was maintained at 4 °C on malt agar slants and sub-cultured every month. The composition of the basal culture medium used for screening the strains for inulinase was as follows (g/L): sucrose 50, yeast extract 20.0, $NaNO_3$ 20.0, K_2HPO_4 5.0, $MgSO_4 \cdot 7H_2O$ 1.0 (*13*).

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Growth conditions

The strain *A. niger* 13/36 was incubated in 500-mL conical flasks, each containing 100 mL of the medium. The media were inoculated with 2 mL of spore suspensions (about $2 \cdot 10^6$ spores) and cultured at 30 °C for 4 days on a rotary shaker (220 rpm). Then mycelium was separated by filtration through Miracloth quick filtration material (Chicopee Mills, Inc., New York, USA) and inulinase activity was measured in the filtrate.

Classical one-factor-at-a-time method

Different sucrose contents (0.5–8.0 %) were added individually instead of its initial value (5 %) in the basal medium. The 1.5 % of sucrose, which gave the highest inulinase activity, was used in further experiments for optimisation of other medium components, according to the scheme:

1. sucrose 0.5–8.0 %, yeast extract 2 %, $NaNO_3$ 2 %, K_2HPO_4 0.5 %, $MgSO_4 \cdot 7H_2O$ 0.1 %;
2. K_2HPO_4 0.05–2.0 %, sucrose 1.5 %, yeast extract 2 %, $NaNO_3$ 2 %, $MgSO_4 \cdot 7H_2O$ 0.1 %;
3. $NaNO_3$ 0.05–3.0 %, sucrose 1.5 %, yeast extract 2 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, K_2HPO_4 0.5 %;
4. $MgSO_4 \cdot 7H_2O$ 0.005–0.25 %, sucrose 1.5 %, yeast extract 2 %, K_2HPO_4 0.5 %, $NaNO_3$ 0.2 %;
5. yeast extract 0.1–4.0 %, sucrose 1.5 %, K_2HPO_4 0.5 %, $NaNO_3$ 0.2 %, $MgSO_4 \cdot 7H_2O$ 0.025 %.

As it is shown in the scheme, each component of the medium at optimised m/V ratio was used in the subsequent experiments.

Experimental design

The simplex method was utilised for the optimisation of 5 variables (concentration of yeast extract, sucrose, $NaNO_3$, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$) to maximise inulinase production by *A. niger* strain 13/36. The software MultiSimplex AB (v. 2.1.1), Karlskrona, Sweden, 1998, was used for this purpose.

The simplex optimisation method is a modification of efficient sequential optimisation techniques called the basic simplex method and the modified simplex method (*14,15*). The simplex methods can handle many variables with only a few trials, and do not require any assumptions with regard to the underlying model. The simplex algorithms are based on an initial design of k+1 trials, where k is the number of examined factors. A simplex is a k-dimensional geometric figure with k+1 vertices. The initial trials form the first simplex, which is modified and directed to more favorable conditions after each step. The optimisation process ends when the optimisation objective has been reached or when the responses cannot be improved further. Table 1 gives the matrix of the experiments suggested by simplex.

Analytical procedure

The reaction mixture contained 0.1 mL of appropriately diluted culture filtrate and 0.9 mL m/V ratio of 0.5 % inulin (from dahlia tubers, Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.1 M acetate buffer (pH=5.0) and it was incubated at 50 °C. After the incubation for 20 min, the increase in reducing sugars was estimated by the 3,5-dinitrosalicylic acid method (*16*). Absorbance was measured at 550 nm. One unit (U) of inulinase activity was defined as the amount of enzyme which produces 1 μmol of reducing sugars per min under the above conditions. The protein content in the medium and in the culture filtrate was determined by the method of Schacterle and Pollack (*17*). After cultivation, the mycelium dry mass was determined by washing and drying it at 105 °C. Fermentations were performed in culture flasks in triplicate, and the analyses were carried out in duplicate. The data given here are the mean values of the measurements. The mean standard error for inulinase was ±0.23 and ranged from ±0.003 to ±0.38 U/mL.

Table 1. Matrix of the experiments suggested by simplex method for optimisation of inulinase production by *Aspergillus niger* strain 13/36

Trial no.	Sucrose	Yeast extract	$NaNO_3$	$MgSO_4 \cdot 7H_2O$	K_2HPO_4
	γ/(g/L)				
1	40.0	25.0	15.0	0.5	4.0
2	40.0	25.0	25.0	1.5	6.0
3	60.0	25.0	15.0	0.5	6.0
4	60.0	15.0	15.0	1.5	4.0
5	60.0	25.0	25.0	0.5	4.0
6	40.0	15.0	25.0	0.5	6.0
7	64.0	31.0	13.0	1.3	3.6
8	45.6	37.4	22.2	0.2	5.4
9	67.8	32.4	11.1	0	3.2
10	79.0	35.3	19.5	0.4	4.9
11	66.6	39.4	7.3	0.3	5.2
12	63.6	36.8	17.0	0	6.3

Results and Discussion

The first step of simplex consists in the definition of the optimisation project, which includes the control variables, their reference values and variation step as well as the response variables, their optimisation objectives, their influence on the joint response, and finally how the membership function is transformed. After the initial trials, the simplex method is sequential, with the addition of one new trial at a time and an evaluation of the control variables. The optimisation procedure includes a revaluation rule, which means that every time after a certain number of experiments the previous trial is repeated experimentally. Therefore, the effect of other sources of variation might be considered in this way. The optimisation process ends when the optimisation objective has been reached (*18*).

As a starting point for the optimisation of the medium, basal medium for *A. japonicus*, chosen as the best among 5 others, was used (*13*). The variables considered in the optimisation were the concentration of yeast extract, sucrose, $NaNO_3$, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$. The experiments suggested by the simplex and the obtained results are given in Tables 1 and 2, respectively. After 12 runs the simplex started to suggest the already given experiments, while the membership function showed al-

Table 2. Inulinase activity of *Aspergillus niger* strain 13/36 grown in optimised media by simplex method. Experimental results of the design presented in Table 1

Trial no.	Inulinase activity		γ*	γ (protein)	γ (reducing sugars)
	U/mL	U/mg protein	g/L	mg/mL	mg/mL
1	45.7	10.4	17.1	4.4	0.4
2	37.3	7.5	18.3	5.0	0.4
3	50.4	9.7	19.3	5.2	0.6
4	28.4	8.9	20.1	3.2	0.6
5	46.9	8.8	18.8	5.3	0.7
6	27.5	8.6	17.6	3.2	0.3
7	47.5	7.8	21.6	6.1	0.8
8	57.2	7.5	19.6	7.6	0.4
9	60.8	6.0	20.8	10.2	0.7
10	60.0	10.3	23.6	5.8	0.6
11	79.8	10.1	20.1	7.4	0.4
12	37.9	4.8	19.5	7.8	0.6

*dry mass (weight)

most constant values, which means that the simplex was around the objective. Therefore, it was decided to stop the experimentation at run number 12 since the conditions for the experiment number 13 were similar to those of the experiment number 9.

According to the obtained results (Table 1 and 2) it could be concluded that the optimum conditions for the production of inulinase were achieved in the medium consisting of (%): yeast extract 3.94, sucrose 6.66, $NaNO_3$ 0.73, K_2HPO_4 0.52 and $MgSO_4 \cdot 7H_2O$ 0.03 (trial no. 11). Other proposed media proved much less effective regarding the amount of inulinase activity.

In subsequent studies, the medium was optimised by the classical »one-factor-at-a-time« method. The relationship between the concentration of sucrose and the amounts of inulinase produced by *A. niger* strain 13/36 is shown in Fig. 1A. Maximum production of the enzyme occurred in the medium containing 1.5 % of sucrose. Among mineral salts present in the medium the highest inulinase activity was obtained at $NaNO_3$ 0.2 %, $MgSO_4 \cdot 7H_2O$ 0.025 % and K_2HPO_4 0.5 %. The synthesis of inulinase was also significantly influenced by the increasing concentration of yeast extract. In this case the activity of the enzyme in the culture filtrate showed a maximum of 49.3 U/mL (at 2 % content of yeast extract). As shown in Fig. 1E, rapid increase in enzyme activity of this strain is accompanied by a simultaneous increase in dry weight of the mycelium and protein content.

Five optimised parameters producing the highest response were compared with optimum conditions obtained by the classical »one-factor-at-a-time« method, where the same parameter ranges were tested. The parameter values and the activities of the two different methods are compared in Table 3. The optimum provided by the simplex software differs considerably from the one obtained in the »one-factor-at-a-time« experiment. The sucrose concentration was over 4-fold higher, while yeast extract and $NaNO_3$ were about 2- and 3-fold higher, respectively, than those determined in the »one--factor-at-a-time« experiment. A possible explanation for this observation may concern the significantly higher concentration of sucrose and yeast extract in the basal medium and the limited number of 12 runs. It is possible that an increased number of runs might lead to reaching the second peak of higher inulinase activity with the lower concentration of these components. There were no significant changes in $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 concentrations. Inulinase activity under optimised culture conditions was 79.8 U/mL, over 2.5-fold higher than that in the basal medium. This response was about 1.6-fold better than the one obtained by the classical »one-factor-at-a-time« method (Table 2, Fig. 1). Similar results (1.5-fold increase of inulinase activity) were obtained by Poorna and Kulkarni (*19*), using fractional factorial design.

The addition of $MgSO_4 \cdot 7H_2O$ did not stimulate inulinase production. An inhibition of the production of enzymes at a higher concentration of this compound was observed (Fig. 1D). A similar effect was observed when $NaNO_3$ was used as nitrogen source – the highest activity was obtained at the m/V ratio of 0.2 % (the classical »one-factor-at-a-time« method) (Fig. 1C) or 0.73 % (the simplex method). This may indicate that $NaNO_3$ inhibits the production or secretion of inulinase. Inulinase production is affected by the kind of nitrogen source employed (yeast extract, peptone, corn steep liquor) (*9*, *19*,*20*).

Among the substrates, inulin and sucrose have been used as the preferred carbon source. In general, if the microbial strain showed only inulinase activity, inulin served as the best substrate (*4*,*19*,*21*), but if the microorganism exhibited inulinase activity coupled with invertase activity, sucrose served as a better source for enzyme production (*3*,*4*,*22*). Cruz *et al.* (*23*) used glycerol for inulinase production by yeast culture *K. marxianus* and found that inulinase yields were over 3-fold higher than in the medium with inulin only. Since inulinase was produced in glycerol medium without an inducer, it was thought that enzyme production was partially constitutive. A similar constitutive nature of inulinase production by *A. niger* was suggested by Derycke and Vandamme (*9*).

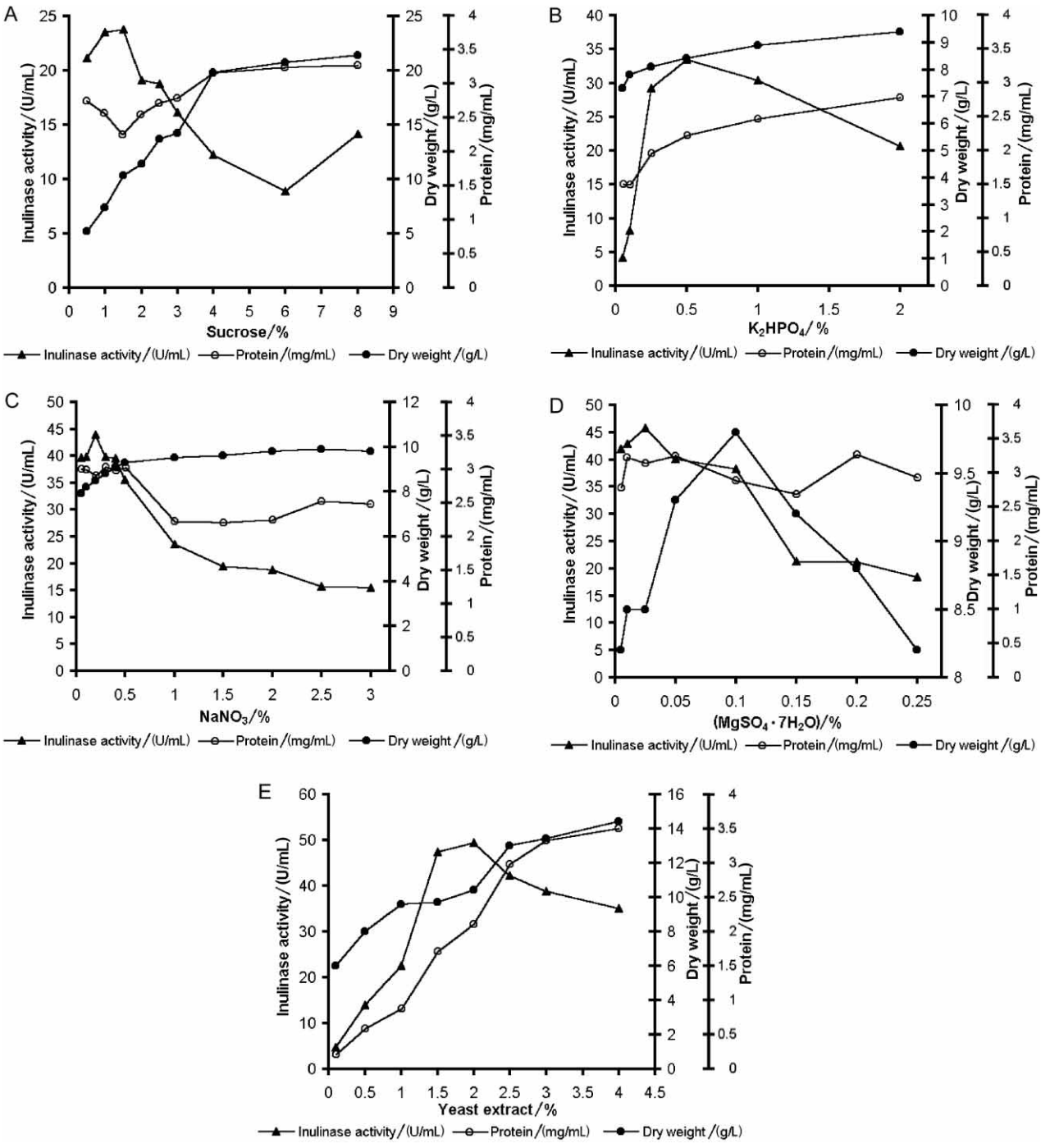

Fig. 1. Factors affecting inulinase production by *Aspergillus niger* strain 13/36

Poorna and Kulkarni (*19*) carried out a study of the use of various carbon sources, individually or in combination for inulinase production, using fractional factorial design. Inulin was found to be the most suitable substrate for enzyme production. The results suggested that inulinase production by fungal culture was probably inducible and was subject to catabolic repression. With inulin (2 %) as the sole carbon source, maximum inulinase level (80 U/mL) was obtained at 60 h and thereafter there was a decrease in the activity of the enzyme to about 28 U/mL in 120 h. The increase in inulinase activity was parallel to the growth.

This is in agreement with our studies regarding the highest inulinase activity (37.1 U/mL) obtained on the basal medium with 5 % of inulin as the carbon source. Insignificantly lower (31.2 U/mL) activity was reached using sucrose (instead of inulin) at the same concentration. For this reason, and because the higher price of inulin in comparison with sucrose, the latter was used for the optimisation study. This is quite an interesting alternative since sucrose is highly soluble, cheap, and it is the major carbon source in molasses, which is an attractive feedstock for large-scale fermentation, whereas inulin is only available in limited quantities and at high cost.

Table 3. Comparison of the reaction parameters of optimal inulinase activity determined by the two different methods

Method	Number of iterative steps	Sucrose	Yeast extract	$NaNO_3$	$MgSO_4 \cdot 7H_2O$	K_2HPO_4	Inulinase activity
		%					U/mL
One-factor-at-a-time method	39	1.5	2.0	0.2	0.025	0.5	49.3
Simplex	12	6.66	3.94	0.73	0.03	0.52	79.8
Basal medium	0	5.0	2.0	2.0	0.1	0.5	31.2

The results of further studies with the optimised medium by the simplex method were compared to the values in the literature for inulinase production in other media. The activity of inulinase produced by *A. niger* strain 13/36 was within the values reported for *A. niger* (*8*,*9*,*19*) and *Kluyveromyces marxianus* (*24*) or significantly higher in comparison with the enzyme synthetised by *Streptomyces* sp. (*11*). Moreover, the medium we propose for inulinase production has a simple chemical composition in comparison with the complex media reported by other authors (*11*,*19*). The main disadvantage concerns the higher cost of the medium arising from a high concentration of yeast extract and sucrose.

Conclusion

In conclusion, it appears that the performance of the simplex optimisation has proved satisfactory in the medium-screening exercise. Compared with the classical »one-factor-at-a-time« method the amount of experiments required to reach a significant increase of enzyme activity is lower. In the case of classical »one-factor-at--a-time« method the amount of experiments was at least 42 and when the simplex method was introduced the number of experiments decreased to 12. In any case, none of the »one-factor-at-a-time« methods has given the optimum conditions. The increase in the inulinase activity (over 2.5-fold in comparison with the initial enzyme activity in basal medium) demonstrates the efficiency of the simplex method.

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Optimiranje proizvodnje inulinaze s pomoću *Aspergillus niger* simpleks i klasičnom metodom

Sažetak

Simpleks metoda upotrijebljena je prvi put za optimiranje proizvodnje inulinaze s pomoću soja *Aspergillus niger* 13/36 koristeći 5 komponenata podloge. Ekstracelularna aktivnost enzima iznosila je skoro 80 U/mL, a bila je 1,6 puta veća od one dobivene klasičnom metodom. Najveća prednost simpleks metode bila je u tome što su se optimalne količine sastojaka u podlozi odredile u samo 12 uzastopnih pokusa, skraćujući bitno vrijeme u usporedbi s klasičnom metodom.