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The optimization of some extracellular enzymes biosynthesis by *Aspergillus niger* 377-4

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The effect of initial solid and moisture contents, temperature and time of incubation on the production of polygalacturonase, phytase, acid phosphatase, xylanase and β-glucanase by *Aspergillus niger* 377-4 during solid state fermentation was studied. Parameters of enzyme synthesis were optimized using statistical experimental designs. It was shown that the capacity of strain to synthesize the aforementioned enzymes could be modified within a wide range by culture parameters selection. The optimal polygalacturonase production efficiency was achieved with the initial medium mass of 19.9 g and humidity of 59.9%, after 77.7 h of incubation at 28.9°C. The best combination of culture parameters for phytase synthesis was: initial medium mass 19.9 g, moistures 50%, temperature 33°C and incubation time 83.9 h. The highest activity of acid phosphatase was obtained after 81.3 h of incubation at 27°C, with initial substrate mass of 17.8 g and moistness content of 60%. The initial solid and moisture contents to synthesize xylanase were 19.9 g and 50%, respectively, with incubation time of 73 h at 29.6°C. The highest efficiency of β-glucanase biosynthesis was obtained when *A. niger* 377-4 was cultivated for 80.4 h at 27°C on a initial medium mass of 20 g and initial level of moistness 59.9%.

Keywords: *Aspergillus niger*, solid-state fermentation, enzymes biosynthesis, optimization

Introduction

Aspergillus niger has great, genetically conditioned ability to synthesize industrially important enzymes. It is necessary, however, to select optimal bioprocess conditions to make the maximum use of this potential. Sometimes even slight changes in cultivation parameters can significantly differentiate the amount and profile of enzyme biosynthesis [1,2]. It was shown that proper aeration of the medium [3] and the length of the cultivation period [2] may influence the phytase synthesis by *Aspergillus* strains. The intensity of biosynthesis of polygalacturonase, pectin lyase and β-glucanase can be modified by changing water activity in the medium [1,4,5]. The capacity of *Aspergilli* to produce pectinolytic enzymes is strongly dependent on the interaction of physical cultivation parameters (pH, water activity, the length of cultivation period) [6,7].The primary aim of the study was to examine the sensitivity of *Aspergillus niger* 377-4 to changes in culture parameters including: the mass of the medium, initial moisture content, temperature and time, and also to determine the influence of the interactions between these parameters on the strain capability to biosynthesize polygalacturonase, phytase, acid phosphatase, xylanase and β-glucanase. Simultaneously, an attempt was made to optimize the parameters of mold cultivation so as to obtain the most effective biosynthesis of the aforementioned enzymes without changes in the medium composition. This could significantly widen the profile of *A. niger* 377-4 applications as a producer of commercial enzymatic preparations.

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Material and methods

Microorganism

Aspergillus niger 377-4 was purchased from the Fruit and Vegetable Industry Factory Pektowin, Poland. Mother cultures were grown on an agar slants (20 g/l) with the addition of unhopped wort (8°Blg) and NaCl (3 g/l), at 30°C for 12 days. To obtain the inoculum, spores were suspended in saline solution with 0.01% Tween 80.

Fermentation condition and preparation of crude enzyme solution

The strain cultivation was conducted on a solid medium composed of beet pulp, wheat bran and

ammonium sulphate (the medium composition is the factory's secret) in 500ml capacity flasks. The medium was sterilized (121°C, 1 atm, 45 min) and inoculated with the spore suspension (9 x 10^6 spores per 1 g).

In subsequent cultivations, the temperature (27, 30, 33°C), duration (60, 72, 84 h), dry mass (15, 17.5, 20 g) and moistness (50, 55, 60%) of the medium were differentiated. As a result, a complete 4-value (4^3) plan of the experiment was obtained, which was complemented with one central point, which rendered 65 experimental schemes. Six repetitions were performed for each scheme.The cultivations were discontinued by pouring sterile distilled water (7°C). The amount of the introduced water was sevenfold higher than the final mass of the cultivation. The closed flasks were shaken at 100 rpm, at room temperature for 90 min and then centrifuged (6000 rpm, 20 min at 4°C). In the supernatants the activity of extracellular polygalacturonase, phytase, acid phosphatase, xylanase and β-glucanase was determined.

Enzyme assay

All measurements were made in 6 repetitions.The activity of polygalacturonase (EC 3.2.1.) was determined in the presence of 0.68% pectin solution at pH 4.5 and 30°C, over a 10 min time period. One unit of polygalacturonase activity (PGU) was defined as the amount of enzyme that liberated 1 μmol of D-galacturonic acid per min. Phytase (EC 3.1.3.8) activity was determined according to the Gulati *et al*. method [8] at pH 5.0 and 30°C, over a 30-minute time period with 5 mM sodium phytate (Sigma Chemical Co) as the substrate. One unit of phytase activity (FTU) was defined as the amount of enzyme that liberated 1 μmol of phosphorus per min. Acid phosphatase (EC 3.1.3.2) activity was determined according to the Agoreyo *et al*. method [9] at 30°C, by incubating the enzyme with 5.5 mM p-nitrophenylophosphate sodium salt (Sigma Chemical Co) at pH 4.5 for 15 min. One unit of acid phosphatase activity (AcPU) was defined as the amount of enzyme which released 1 μmol of p-nitrophenol under reaction conditions. Xylanase (EC 3.2.1.8) and β-glucanase (EC 3.2.1.6) activity was determined at pH 5.0 and 30°C, for 30 min with 1% solutions of AZCL-xylan and AZCL-curdlan (Megazyme International, Bray, Irland) [10]. One unit of xylanase activity (XU) and β-glucanase activity was defined as the amount of enzyme that solubilizes 1 μg of the substrate per min under these assay conditions.

Experimental design and optimization

To determine the optimal conditions for biosynthesis of test enzymes the Experimental Planning and Analysis Statgraphics Plus statistics package was used. The models of the polygalacturonase, phytase, acidic phosphatase, xylanase and β-glucanase biosynthesis were verified by calculating determination coefficients. In order to determine the optimal parameters of biosynthesis only models exhibiting $R^2 > 50\%$ were used.

Results and discussion

The alteration of the cultivation conditions caused statistically significant changes in the profile of enzymes synthesis by *A. niger* 377-4. The level of polygalacturonase biosynthesis changed from 6 620 to 25 887 PGU per cultivation flask and depended mostly on the medium mass and humidity (Fig. 1(a)). The mathematical model of the polygalacturonase biosynthesis by *A. niger* 377-4 had the following form: $18006.80 + 3301.02x_1 + 1148.68x_2 - 827.68x_3 + 445.40x_4 - 870.58x_1^2 + 860.15x_1x_2 - 634.15x_1x_3 + 1024.91x_1x_4 + 462.34x_2^2 - 185.18x_2x_3 - 340.55x_2x_4 - 1734.55x_3^2 + 100.83x_3x_4 - 1345.32x_4^2$ ($R^2 = 78.5\%$). The standardized values of input quantities ensuring the maximum biosynthesis of the enzyme ($x_1 = 0.99$, $x_2 = 0.99$, $x_3 = -0.36$, $x_4 = 0.47$) corresponded to the following real values: solid content (x_1) 19.9 g; initial medium moistness (x_2) 59.9%; cultivation temperature (x_3) 28.9°C; duration of the cultivation (x_4) 77.7 h. Patil and Doyanand [4] indicated 34°C as the optimal temperature for polygalacturonase biosynthesis by *A. niger* growing on a solid medium of 65% humidity for 96 h. These researchers, however, used a completely different medium composition. Castillo *et al*.[11] considered a 72-hour *A. niger* cultivation at 30°C as optimal for polygalacturonase synthesis. The optimal cultivation period which we suggest is longer (77.7 h), which is probably the consequence of twice as high medium mass as the one used in the quoted study. The biosynthesis efficiency of phytase by *A. niger* 377-4 was greatly differentiated (19 - 313 FTU per cultivating flask), which depended mostly on the incubation temperature. Its rise within the test range induced the enzyme synthesis. Also changes in the

cultivation time and the medium mass for which it was possible to determine the optimal regions (Fig. 1(d)) had a significant influence. A differentiation of the medium moistness with the remaining conditions unchanged did not significantly influence the phytase biosynthesis level. This fact may be surprising as the initial water content in the medium constitutes a critical factor determining the mycelial growth rate and enzyme production [2]. However, there are studies showing that the optimal medium humidity to synthesize phytase by *A. niger* can even fluctuate between 50% and 60% [12,13]. The mathematical model of phytase synthesis by *A. niger* 377-4 with 73% probability had the following form: $192.44 + 19.32x_1 - 0.96x_2 + 25.09x_3 + 20.50x_4 - 25.59x_1^2 - 5.09x_1x_2 + 21.85x_1x_3 + 3.17x_1x_4 + 0.71x_2^2 - 5.26x_2x_3 - 8.07x_2x_4 + 6.57x_3^2 - 2.45x_3x_4 - 10.86x_4^2$. The maximum phytase synthesis by the test strain was achieved by 84-hour cultivation at 33°C on a medium with the initial mass of 19.9 g and 50% moistness. The ability of *A. niger* 377-4 to synthesize acid phosphatase ranged from 251.5 to 712.6 AcPU per cultivating flask and depended significantly on each of the test factors and their interactions (Fig. 1(e)). The increasing content of water in the medium stimulated the synthesis of acid phosphatase, while a rise in the cultivation temperature induced a significant decrease in the enzyme activity. Thus, it appears that acid phosphatase produced by *A. niger* 377-4, as compared to phytase, proved to be more sensitive to limited oxygen access and the hindered heat release related to it. Different oxygen and temperature requirements for the biosynthesis of both phosphomonoesterases by *Aspergilli* are also confirmed by other authors' studies [3]. The polynomial illustrating with 68% probability the optimal conditions for the effective biosynthesis of acid phosphatase by *A. niger* 377-4 had the following form: $535.25 + 25.29x_1 + 41.14x_2 - 33.66x_3 + 41.79x_4 - 59.13x_1^2 - 20.88x_1x_2 + 14.75x_1x_3 + 4.78x_1x_4 - 7.25x_2^2 - 27.88x_2x_3 - 13.54x_2x_4 + 0.95x_3^2 - 18.45x_3x_4 - 30.29x_4^2$. The following real values corresponded to the standardized values ($x_1 = -0.07$, $x_2 = 1$, $x_3 = -1$, $x_4 = 0.77$) of the input quantities ensuring the maximum biosynthesis of acid phosphatase: medium mass 17.8 g; initial medium moistness 60%; cultivation temperature 27°C; cultivation period 81.3 h.The effectiveness of the xylanase biosynthesis by *A. niger* 377-4 ranged from 9 807 to 33 850 XU per cultivating flask, which along with the biosynthesis of acid phosphatase, constituted the weakest response to cultivation parameters modifications. It was shown that the level of the enzyme production significantly depended on the medium mass and moistness. This is consistent with results obtained by Bhatt *et al.* [14]. During the differentiation of the medium mass, the growing value of the test factor was accompanied by a constant rise in the efficiency of xylanase synthesis. An increase in the medium humidity, in turn, limited the strain's capacity to synthesize this enzyme (Fig. 1(b)). The mathematical model describing the significance of the individual parameters of *A. niger* 377-4 cultivation for xylanase biosynthesis with 85% probability had the following form: $20842.30 + 3239.85x_1 - 3334.18x_2 - 1710.34x_3 - 99.52x_4 - 936.65x_1^2 - 354.89x_1x_2 + 1516.35x_1x_3 + 494.11x_1x_4 + 712.38x_2^2 + 248.11x_2x_3 + 283.23x_2x_4 - 1867.39x_3^2 - 59,86x_3x_4 - 694.38x_4^2$. The optimal standardized input values ($x_1 = 0.99$, $x_2 = -1$, $x_3 = -0.12$, $x_4 = 0.08$) after the conversion into real values were the following: initial medium mass 20 g; initial moistness 50%; bioprocess temperature 29.6°C; cultivation period 73 h. These parameters are consistent with the results obtained by the Senthilkumar *et al.*[15] (a 3-day *A. niger* cultivation at 30°C). The level of β-glucanase biosynthesis by *A. niger* 377-4 was significantly dependent on the initial medium mass, the temperature and time of incubation. During the differentiation of the medium mass and cultivation time, the test ranges clearly encompassed the distinct optimal regions for the enzyme biosynthesis. No such region was determined for the bioprocess temperature where the lowest one was simultaneously the best for β-glucanase production (Fig. 1(c)). The efficiency of β-glucanase synthesis under experimental conditions ranged from 10 309 to 42 117 GlU per cultivating flask and the model illustrating the most optimal conditions for this synthesis ($R^2 = 82\%$) was: $23123.1 + 3357.11x_1 - 143.21x_2 - 2576.36x_3 + 1458.71x_4 - 3118.55x_1^2 + 1348.92x_1x_2 - 3314.88x_1x_3 + 313.97x_1x_4 + 602.53x_2^2 - 533.69x_2x_3 - 359.30x_2x_4 + 6539.63x_3^2 - 469.25x_3x_4 - 1340.69x_4^2$. The conversion of the obtained, standardized values ($x_1 = 1$, $x_2 = 0.99$, $x_3 = -1$, $x_4 = 0.70$) of the input quantities into real values allowed to determine the parameter settings which enabled *A. niger* 377-4 to synthesize β-glucanase with maximum efficiency. The settings are the following: medium mass 20 g; initial medium moistness 59.9%; cultivation temperature 27°C; cultivation period 80.4 h.

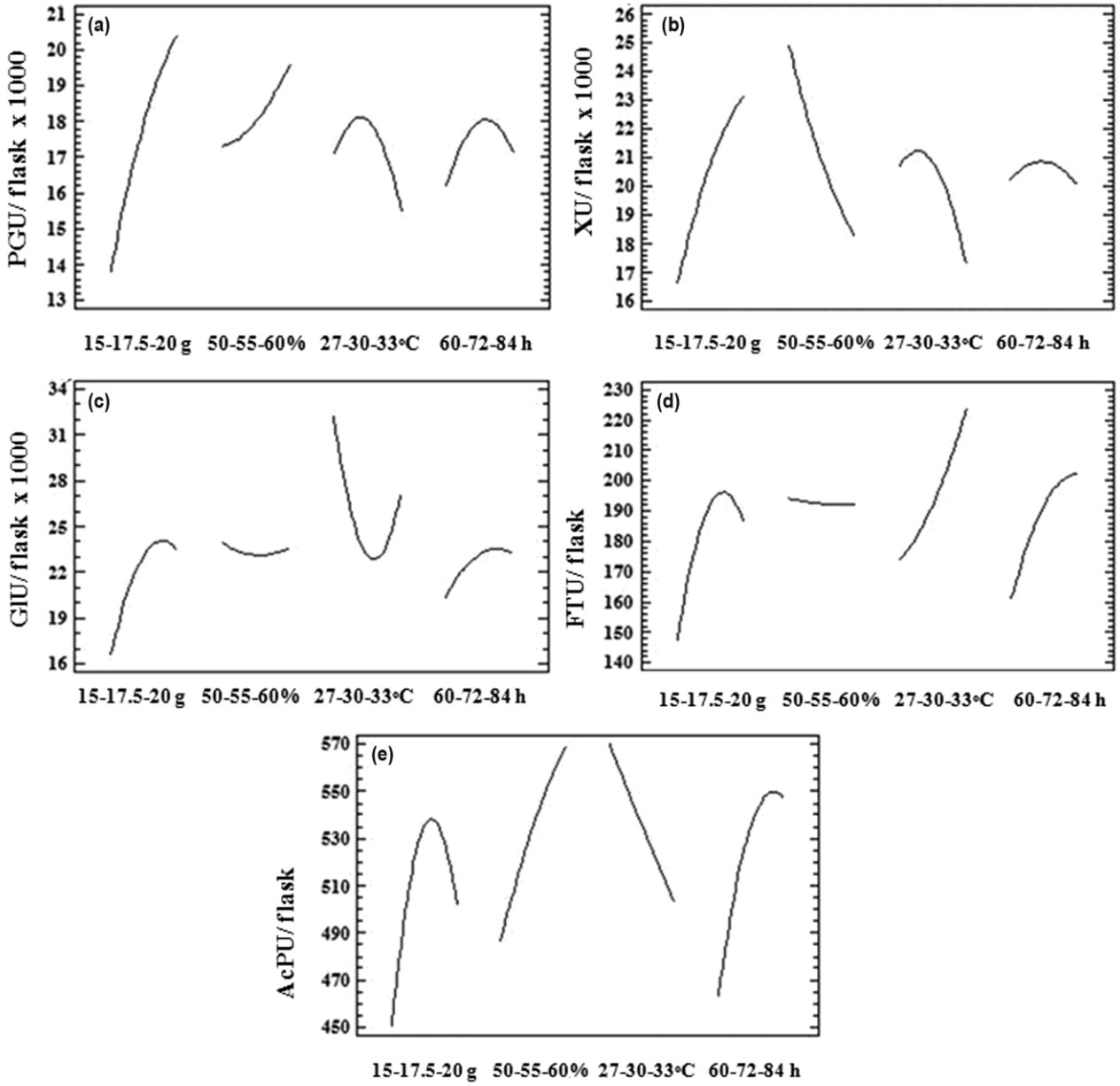

Fig. 1—Influence of solid content and moisture, temperature and incubation time for (a) polygalacturonase, (b) xylanase, (c) β-glucanase, (d) phytase and (e) acid phosphatase production by *A. niger* 377-4.

Conclusions

The capacity of *A. niger* 377-4 to biosynthesize various enzymes can be modified within a wide range by selecting appropriate physical cultivation parameters. A high cultivation temperature (33°C) stimulates *A. niger* 377-4 to biosynthesize phytase, whereas a lower temperature (27°C) facilitates the biosynthesis of acid phosphatase and β-glucanase. Prolonging the cultivation period to over 80 h causes *A. niger* 377-4 to secrete more phytase and acid phosphatase into the medium, but it limits the biosynthesis of polygalacturonase, xylanase and β-glucanase. The cultivation of *A. niger* 377-4 on a low-moistness medium (50%) creates favorable conditions for the biosynthesis of phytase and xylanase, but hinders the biosynthesis of polygalacturonase, β-glucanase and acid phosphatase.

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