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Research Article

Inulinolytic activity of broths of *Aspergillus niger* ATCC 204447 cultivated in shake flasks and stirred tank bioreactor

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It is the first detailed study of an inulinolytic fungus *Aspergillus niger* ATCC 204447 since its discovery, covering submerged cultivations both in shake flasks and a stirred tank bioreactor. Various carbon sources were applied to induce the inulinolytic activity in shake flask cultures. The highest volumetric and specific (per gram of biomass) activities (respectively 0.68 U/mL and 184 U g/X) were observed for the initial inulin and sucrose concentrations equal to 20 g/L. The fungus grew as large (>3 mm) spherical pellets. The influence of inoculum density and application of microparticle-enhanced cultivation (MPEC) were studied in the batch bioreactor cultivations. Inoculum density moderately affected the inulinolytic activities, whose highest values were 0.7 U/mL and 165 U g/X at the lowest studied spore density of $3.33 \cdot 10^8 \text{ L}^{-1}$. Dispersed hyphae evolved in the bioreactor made the broth difficult to aerate due to high apparent viscosity (exceeding 200 Pa s^n at shear rate about 0.05 s^{-1}) and shear thinned properties (flow behavior index below 0.2). In MPEC (10 μm talc microparticles) the pellets of diameter between 1 and 2 mm were formed, which facilitated the aeration of the broth and increased the specific inulinolytic activity 3.5-fold.

Keywords: Bioreactor / Inulinase / Microparticles / Rheology / Shake flasks

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1 Introduction

Inulinases are fructofuranosyl hydrolases, which depolymerise inulin, a polydispersed fructan, consisting mainly of β -2,1-fructofuranosyl moieties terminated by glucose. There are two types of inulinases, exo- and endoinulinases (BRENDA, www.brenda-enzymes.org). Exoinulinase (β -D-fructan fructohydrolase, EC 3.2.1.80) splits off the terminal monosaccharide units. Endoinulinase (2,1- β -D-fructan fructanohydrolase, EC 3.2.1.7) randomly breaks down the internal bonds of inulin into fructooligosaccharides. Inulin hydrolysis products are used in the preparation of fructose syrups, the efficient, and healthy sweeteners.

The microbial sources of inulinases are bacteria (*Bacillus* sp.), yeasts (mainly *Kluyveromyces marxianus*), and fungi of genus *Aspergillus* and *Penicillium*. *Kluyveromyces marxianus* have been widely studied for its inulinolytic properties [1–4] but filamentous fungi (*Aspergilli*) are believed to be the best producers of inulinases [5].

Aspergilli are the widely used producers of various enzymes for industrial applications [6] including inulinases [7] but in few publications more or less systematic studies on the cultivation conditions to produce inulinases were presented. The production of inulinases by *A. niger* van Teighem was studied with the use of a statistical medium optimization technique. Out of many independent variables (medium components) the influence of the variety of carbon sources (inulin, glucose, fructose, and sucrose) on the inulinolytic activity of this fungus was studied [8]. Inulinases are induced enzymes and inulin was claimed to be the only inducer for the production of inulinases [9]. Actually, pure inulin is the best inducer of the inulinolytic activity but various natural inulin sources (from plants) in *Aspergilli* cultures were also tested [9–11]. Other than inulin pure carbohydrates like glucose induced the inulinolytic activity in *K. marxianus* [12].

Hardly any data are available about the production of fungal inulinases in the larger than that of shake flasks scale, e.g. in the stirred tank bioreactors. Dinarvand et al. studied the impact of intrinsic and extrinsic parameters on inulinase production by *A. niger* ATCC 20611 in shake flasks [13]. So did Trivedi et al. for another *Aspergillus* species, optimizing various cultivation

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media [14]. Only Skowronek and Fiedurek used a stirred tank bioreactor in the various operation modes for *A. niger* mutant strain [15]. Most recent research also concerned solid-state fermentation [16,17]. Since its discovery [18] the inulinolytic strain *A. niger* ATCC 204447 has never been studied in any bioreactor system.

Thus, this pioneering and preliminary, with regard to *A. niger* ATCC 204447, work is aimed at studying the production of inulinases in the submerged cultivations in shake flasks and stirred tank bioreactor. First, various pure carbon sources and inducers (glucose, fructose, sucrose, and inulin) were used at various concentrations and inulinolytic activities of the obtained broths were analyzed by a novel liquid chromatography method. Second, *A. niger* ATCC 204447 cultivations were conducted in the bioreactor and the inulinolytic activity was studied in the function of the number of spores used for inoculation. Third, microparticle-enhanced cultivation was applied in the bioreactor experiments to test, whether it leads to the increase of the inulinolytic activity.

2 Materials and methods

2.1 Microorganism

Aspergillus niger ATCC 204447 designated as A42 purchased from LGC Standards was used in this research. It was isolated from dry figs in Ismir (Turkey) and described for the first time by Ongen-Baysal et al. [18]. It is, according to our best knowledge, the first use of this strain for the detailed study of the production of inulinases. Conidiospores were grown at 25°C on slants containing peptone and malt extract (ATCC Medium no. 325, Blakeslee's Formula).

2.2 Shake flask experiments

A one-stage cultivation without any preculture was applied. Much care was devoted to start the cultivations with the identical initial conditions. For the individual experiment conidiospores were washed from four agar slants into a sterile culture medium (without carbohydrates), then the averaged spore suspension was dispensed into four 150-mL working volume flasks. If more than one experiment was made in parallel, the number of slants and flasks was proportionally multiplied. A separately presterilized carbohydrate solution of the valid volume and concentration was added to the flasks with averaged suspension of spores. As a result of these operations, around 10^9 spores per liter (each time enumerated) were present in the averaged medium and the concentrations of media components were as indicated below. *A. niger* ATCC 204447 was grown at 30°C, at the constant rotational speed of 110 min^{-1} for 96 h. The culture medium contained 10 g/L yeast extract, 2 g/L NaNO_3 , 1 g/L K_2HPO_4 , 0.5 g/L MgSO_4 , 0.027 g/L $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 0.018 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 g/L KCl and 0.25 g/L NaCl. The initial pH was set to 5.5. Inulin (INU) from chicory (purchased from Sigma), glucose (GLU), and sucrose (SUC) at various concentrations and combinations were used as the carbon sources (Table 1). For two selected shake flask experiments the reproducibility was tested. It covered three parallel runs with

Table 1. Carbon sources applied in the culture media for the shake flask cultivation of *A. niger* ATCC 204447 to induce its inulinolytic activity

Experiment	Initial inulin concentration, c_{INU}^0 (g INU/L)	Second carbon source	Concentration of the second carbon source; c_{GLU}^0 (g GLU/L) or c_{SUC}^0 (g SUC/L)
E5	2.5	Glucose	35
E6	5		
E7	10		
E8	20		
E9	40		
E10	2.5	None	0
E11	5		
E12	10		
E13	20		
E14	40		
E15a	10	Sucrose	10
E15b	20		20
E16a	10	Glucose	20
E16b	20		40

the same medium compositions (the ones applied in the experiments E15 and E16). The deviations of carbon substrate concentration values and inulinolytic activity did not exceed, on average, 5%, however for biomass content it was even 12%.

2.3 Bioreactor experiments

Two series of bioreactor experiments were conducted. Six batch processes in the thermostated stirred tank bioreactor Biostat B-Plus (Sartorius, Germany) of 5.3 liters working volume were carried out. It was equipped with three Rushton turbines on the shaft, steel air sparger, and standard sensors: pH probe, oxygen probe, redox potential probe, and foam probe. The culture medium had the same composition as the ones in shake flasks, excluding the amount and type of carbon sources. Inulin (INU) from chicory at 5 g/L and sucrose (SUC) at 20 g/L were used. Carbohydrates were sterilized separately. Antifoam A (purchased from Sigma) was used to struggle against foaming. About 5 mL was poured into the bioreactor before inoculation and then it was automatically added to the bioreactor dependent on the needs. One-stage cultivation was used. The fungus grew at 30°C and oxygen saturation level ($p\text{O}_2$) was set at 15% and controlled by air flow rate and stirring speed. Process parameters like pH, $p\text{O}_2$, stirring speed, air flow rate were monitored on-line and controlled with the use of MFCS software (Sartorius, Germany). For each experiment conidiospores were washed from the slants into a 200 mL of sterile physiological NaCl solution and pumped into the bioreactor. In the first series (four runs) the number of spores (inoculum density) varied. The experiments were denoted as D, E, F, and G, and bioreactor was then inoculated with $9.00 \cdot 10^9$, $5.00 \cdot 10^9$, $1.00 \cdot 10^9$, and $3.33 \cdot 10^8$ spores per liter, respectively. The second independent series consisted of two batch processes (experiments H and J). In these experiments inoculum contained $3.33 \cdot 10^8$ spores per liter. To the inoculum in

Figure 1. Changes of (A) and (B) inulinolytic activity, (C) and (D) specific inulinolytic activity in the filtered culture medium with the initial glucose concentration of 35 g/L and various inulin concentration (experiments from E5 to E9) and with the sole inulin (experiment from E10 to E14); initial inulin concentration in the respective experiments was in the range from 2.5 to 40 g INU/L.

experiment H 10 μm talc microparticles were added at concentration of 12 g/L to perform microparticles-enhanced cultivation (MPEC).

In bioreactor runs reproducibility was similar for carbon sources and inulinolytic activities (two nonparallel test runs with the same medium composition and inoculum density) but slightly worse for biomass (on average 14% deviation).

2.4 Rheological measurements

Measurements of the rheological properties of the culture broth in the bioreactor experiments were carried out with the use of a rotational rheometer Bohlin CVO 120 (Malvern). The rheometer was thermostated ($\pm 0.5^\circ\text{C}$). In order to determine the rheological properties of the culture broth two different measuring systems were used. The properties of the sterile medium with antifoam agent (without biomass) were determined in the cone-plate system. The diameter of cone was equal to 40 mm and its angle equal to 1 degree. The distance between the cone and the plate was set in accordance with manufacturer's settings and equal to 30 μm . The determination of the properties of the culture broth with fungal biomass was performed with the use of the plate-plate measuring system. The gap between these sensing elements was established upon the size of the agglomerates and ranged from 1500 to 3000 μm . Each time the change of its width was taken into account in the algorithm determining the average shear rate in the measuring gap. Additionally, the plates were equipped with the walls to avoid the loss of the broth during the measurements. The distance between the wall and the edge of the plate was equal to about 10 mm and had no influence on the values of the measured torque.

2.5 Assay of enzymatic activity

The new assay for inulinolytic activity was elaborated. It was made as follows. A 1 mL of the filtered (to remove biomass) culture medium (crude enzyme) and 4 mL of a 2% solution of inulin in phosphate buffer (pH = 7) were incubated at 50°C . After a predetermined time (30 or 60 min dependent on the predicted level of the inulinolytic activity) the enzymatic reaction was stopped by adding a 15 mL of anhydrous acetonitrile. The constant pH value of the assay was applied to obtain the comparable values of the inulinolytic activity of the broth sampled in various moments of the experiment as pH level in the consequent runs changed in time even by 2.5 units. The pH level of the assay fitted in the best analytical range for inulinases, which is between 5 and 8 [19]. It also assured that inulin used as a substrate for this assay was thermally stable and did not hydrolyze on its own during the incubation [20]. Acetonitrile served as diluting agent preparing the sample for the chromatographic analysis too. Glucose (GLU), fructose (FRU), and sucrose (SUC) concentrations were determined in the postreaction solution with the use of liquid chromatography. As residual fructose and glucose concentrations in the culture medium had to be taken into account in order to find the amount fructose and glucose released in the enzymatic reaction only, the following dilution procedure was applied. For the determination of sucrose, glucose, and fructose concentrations in the culture medium distilled water (instead of inulin solution) and acetonitrile were identically added to the samples so as to produce the same dilution as in the case of the inulinolytic activity assay. It assured the similar analytical signals and minimized any error prone to the chromatographic analysis. The amount of released fructose was found by the subtraction of their concentrations in the postreaction solution from the detected residual concentrations in the medium. The inulinolytic activity (volumetric) of the crude filtered cultivation broth was

Figure 2. Changes of glucose and fructose concentrations in the cultivation broth in the experiments from E5 to E9 (initial glucose concentration 35 g GLU/L; initial inulin concentration from 2.5 to 40 g INU/L).

expressed in U/mL, which means that 1 μmol of fructose per 1 mL of the broth was released from inulin within one minute. The accuracy of the assay for triplicate samples was estimated at ± 0.002 U/mL. In order to evaluate the efficiency of the used strain to produce inulinases the volumetric inulinolytic activities were divided by the amount of biomass (X) and thus specific inulinolytic activity (biomass-related) was expressed in U/gX.

2.6 Determination of carbohydrates and biomass content

Liquid chromatography (UPLC[®], Aquity Waters, USA) analysis was performed in the following conditions. Aquity Amide BEH chromatographic column was thermostated at 35°C and

mobile phase flow rate was equal to 0.29 mL/min. Elution was made with 75% CH_3CN supplemented with 1% triethylamine. Carbohydrates, i.e. fructose, glucose, and sucrose were detected with the evaporative light scattering detector. Under these analytical conditions carbohydrates were eluted at the following retention times (t_R): fructose at 5.80 min, glucose at 4.47 min, and sucrose at 6.96 min. The accuracy of the determination of carbohydrates concentration was on average ± 0.02 g/L. Liquid chromatography coupled with mass spectrometry (Waters, Synapt G2) was used to find fructooligosaccharides in the postre-action solution after the assay of the inulinolytic activity and in the cultivation broth. To produce M-H ions, electrospray ionization in the negative mode (ESI-) was applied. The following voltages, temperatures and flows rates were set: capillary voltage 3 kV, sampling cone voltage 40 V, extractive cone voltage 4 V;

source and desolvation temperatures were 120 and 300°C, respectively. Cone and desolvation gas (nitrogen) flow rates were 50 L/h and 500 L/h, respectively.

In order to determine biomass as dry weight a 40 mL of suspension was filtered under atmospheric pressure through a paper filter (Filtrak 389) of the predetermined mass. The filter with biomass was dried at 105°C and after 24 h weighed. The accuracy of the determination of biomass was ± 0.4 g/L.

3 Results

3.1 Detection of endoinulinolytic activity of *A. niger* ATCC 204447

At the beginning the presence of inulinases in the filtered cultivation broth of the studied *A. niger* ATCC 204447 was checked and the analytical method to assay the enzymatic activity was tuned. In this study the most popular method for the determination of the inulinolytic activity by the reaction of released reducing sugars with the DNS reagent was rejected [21]. First of all, it took into account all reducing moieties in the molecules and even this reaction in the solution of pure inulin was observed. Secondly, it was found that media components present at the residual concentrations (glucose, fructose, and other components) in the postreaction mixture were also prone to this assay. This method detected all reducing sugars and other chemicals in the sample overstating the assay of the inulinolytic activity. Therefore, fructose, glucose, and sucrose concentrations were analyzed in the postreaction solutions in the culture media by means of liquid chromatography.

The endoinulinolytic activity of the broths was confirmed by the presence of fructooligosaccharides in the postreaction solution. They were tracked after their mass to charge ratio (m/z). The absolute error of m/z determination [$\Delta(m/z)$] was always not higher than 10^{-2} , which allowed for the unequivocal identification of the ions. The following M-H ions were detected: trisaccharide as the ion of $m/z = 503.1573$ [$\Delta(m/z) = -0.0039$] ($t_R = 13.187$ min), tetrasaccharide as the ion of $m/z = 665.2162$ [$\Delta(m/z) = -0.0022$] ($t_R = 23.834$ min) and pentasaccharide (with water molecule attached) as the ion of $m/z = 845.2809$ [$\Delta(m/z) = +0.0035$] ($t_R = 28.321$ min). These fructooligosaccharides were also detectable in the culture medium.

3.2 Influence of carbon source on inulinolytic activity of *A. niger* ATCC 204447

In the experiments from E5 to E9 inulin concentrations in the culture medium ranged from 2.5 to 40 g INU/L. The second carbon source was glucose at 35 g GLU/L (Table 1). It was found that the inulinolytic activity (Fig. 1A) rose with the increase of inulin concentration in the culture medium. In the experiments E5 and E6, in which inulin concentration was low, the inulinolytic activity increased slowly to 48 h and subsequently declined. In the experiments E7, E8, and E9, in which higher inulin concentrations were used, the increase of the inulinolytic activity in the broth was more rapid. In addition, it did not ultimately vanish in 96 h of the run but only slightly decreased. In

the experiment E9 the inulinolytic activity increased throughout the whole duration of the process.

In the series of the experiments from E10 to E14 inulin concentration varied from 2.5 to 40 g INU/L but no glucose was introduced as the second carbon source. Inulin is theoretically the best substrate for the inulinolytic microorganisms. In these experiments with inulin as the sole carbon source the inulinolytic activity decreased or remained more or less constant after 24 or 48 h of the run. Despite using inulin at the highest concentration (40 g INU/L) in experiment E14 the inulinolytic activity reached only 0.20 U/mL (Fig. 1B). Generally, the obtained results were slightly worse than those in the experiments from E5 to E9. Especially faster decrease of the inulinolytic activity in the experiments E13 and E14 is worth mentioning. It indicated that the presence of the other than inulin carbohydrate was required to sustain the inulinolytic activity of *A. niger* ATCC 204447. The values of the specific inulinolytic activity, i.e. referred to the amount of biomass grown were also different (Fig. 1C and D) in the runs with and without glucose as the second carbon source. The general trend, namely more carbon substrate induces higher inulinolytic activity, was saved in this case too. But faster decrease of the specific inulinolytic activity was observed in the runs with the highest amounts of inulin (experiments E13 and E14).

The levels of the inulinolytic activity were strictly associated with glucose and fructose concentrations in the broth (Fig. 2). In the experiments, in which low inulinolytic activity was detected (from E5 to E7) inulin was slowly hydrolyzed and the increase of fructose concentration was not too high. It forced *A. niger* ATCC 204447 to the rapid consumption of glucose from the beginning of the experiment. In the experiments E8 and E9 with high inulinolytic activities the situation was different. Fructose was quickly released from inulin, became the preferred carbon source and, thereby, glucose was either slower consumed or even its consumption was delayed. In the experiment E9, in which the highest initial inulin concentration in the culture medium was used, the release of fructose from inulin and its subsequent uptake by the fungus was sufficient for its demands so that glucose concentration in the first day of the experiment remained on its initial level.

The changes of carbon substrate concentration were completely different in the experiments with inulin as the sole carbon source (experiments from E10 to E14). In the culture broth (Fig. 3) the presence of fructose hydrolyzed from inulin by inulinases was observed but its concentrations were much lower than those in the previous experiments from E5 to E9. In the experiments E10, E11, and E12 in the last hours of the runs fructose was fast consumed by the fungus (in the experiment E10 only 0.25 g FRU/L was observed at 48 h). In the experiments E11 and E12 the total exhaustion of fructose occurred already in 48 h of the run. In the experiments E13 and E14 high initial inulin concentration was used and the inulinolytic activity of the broth was high too (Fig. 1B). As a result, fructose concentration in the medium also increased (Fig. 3). Only then the changes of fructose concentrations in the culture medium resembled the profiles from the previous series of experiments, in which glucose was used as the second carbon source. In the experiment E14, even in 96 h fructose was still detected. In the experiments from E10 to E14, no glucose was detected in the cultivation broth, probably due to two reasons. First, endoinulinolytic activity dominated, which

Figure 3. Changes of fructose concentration in the cultivation broth in the experiments from E10 to E14 (initial inulin concentration from 2.5 to 40 g INU/L).

was proved upon the qualitative analysis of postreaction mixture by MS. Second, in the individual molecule of inulin there are about 30-fold more fructose moieties than those of glucose and so small amount of glucose was hard to detect. Even released, glucose must have been immediately consumed.

In Fig. 4A changes of biomass concentration in the experiments from E5 to E9 are shown. Biomass concentration increased with the increase of the initial concentration of inulin. The first 24 h of the experiments from E5 to E9 covered trophophase (fungal biomass growth phase). Then the rapid biomass growth was accompanied by fast utilization of the available carbon substrates: glucose and fructose from the hydrolyzed inulin (Fig. 2). After 24 h of the experiments in the idiophase (fungal biomass stationary phase) biomass concentration began to decrease. It was especially seen in these runs, in which the highest inulinolytic activities were observed. This decline was observed despite the presence of sugars (Fig. 2) and organic nitrogen (data not shown) in the culture medium. Actually, only half of the available organic nitrogen was assimilated in the first 24 h of the run. That is why, it rather could not be a limiting substrate. Neither could carbon substrate. It was probably for oxygen availability inside the formed large pellets (diameter above 3 mm) that fungal growth and activity that have been somewhat limited. When inulin was the sole carbon source in the culture medium in the experiments E10–E14, which meant less available carbon substrate, biomass concentration was significantly lower (more than twice) compared to the previous series of experiments (Fig. 4B). Also the temporal profiles of biomass concentration were different in comparison to the experiments carried out with two carbon sources. When inulin was used as the sole carbon source, biomass grew slower, linearly up to 48 h of the experiment. Also in these experiments, the decrease of biomass concentration in the idiophase was observed but it was not as steep as in the experiments from E5 to E9. In all these shake flask experiments, the pelleted morphology was always observed and pellet was large and their diameters even exceeded 3 mm.

Figure 4. Changes of biomass concentration in the experiments (A) from E5 to E9 (initial glucose concentration 35 g GLU/L) and (B) from E10 to E14; initial inulin concentration in the respective experiments varied from 2.5 to 40 g INU/L.

As the levels of the inulinolytic activity in the cultivations of *A. niger* ATCC 204447 with inulin as the sole carbon source were generally lower and less stable than those in the cultivations with glucose and inulin, the two various cosubstrates, sucrose and glucose, were tested for inducing inulinolytic activity in the following experiments. In the experiments E15a and E15b apart from inulin, sucrose was used at 10 and 20 g SUC/L as the second carbon source and in the experiments E16a and E16b glucose at 20 and 40 g GLU/L (see Table 1). These experiments were made in parallel with the same spore suspension to find, which cosubstrate, glucose, or sucrose, is better for the production of inulinases by *A. niger* ATCC 204447. This time the amount of carbon substrate was exactly the same in the respective experiments. The selection of sucrose was justified by the earlier reports [8, 22, 23] and the fact that this sugar can probably induce the inulinolytic activity by means of its structural similarity to inulin and is a substrate for inulinases (BRENDA, www.brenda-enzymes.org). However, the addition of sucrose made the process more difficult to analyze due to the fact that it was not possible to distinguish fructose released respectively from inulin and sucrose in the cultivation broth. All glucose found in the broth was attributed to be

Figure 5. Effect of the use of sucrose (experiments E15a and E15b, $c_{\text{SUC}}^0 = 10$, and 20 g SUC/L, respectively) and glucose (experiments E16a and E16b, $c_{\text{GLU}}^0 = 20$, and 40 g GLU/L, respectively) as the second substrates (apart from inulin, $c_{\text{INU}}^0 = 10$ and 20 g INU/L in the respective E15a, E16a, and E15b, E16b runs) on (A) changes of the inulinolytic activity, (B) changes of the specific inulinolytic activity, (C) changes of glucose and fructose concentration in the cultivation broth (experiments E15a and E15b), (D) changes of glucose and fructose concentration in the cultivation broth (experiments E16a and E16b).

originated from sucrose. In Fig. 5 the results of these experiments are shown.

From Fig. 5A it is clearly seen that due to doubling of inulin and glucose concentrations in the culture medium (compare experiments E16a and E16b) the inulinolytic activity increased twice. What is more, when sucrose was the second carbon source, it resulted in over threefold increase in the inulinolytic activity (compare experiments E15a and E15b). In the experiment E15b, in which the second carbon source was sucrose and the medium was the most concentrated, the highest inulinolytic activity reached 0.68 U/mL. Similar results were observed for the specific inulinolytic activity (Fig. 5B), whose highest value was 184 U/g/X in the experiment E15b. Similar to the previous experiments with sole inulin, specific inulinolytic activity decreased after 24–48 hours. The replacement of sucrose as the second carbon source with glucose (compare experiments E15b and E16b) resulted in the drastic decrease of the inulinolytic activities. In these experiments, the highest inulinolytic activities reached only 0.24 U/mL and 22 U/g/X (Fig. 5A and B). The choice of the second carbon source influenced on glucose and fructose concentrations in the broth (Fig. 5C and D). In the experiment E15b fructose concentration was increasing rapidly reaching the highest value 45 g/L at 48 h and declined sharply to 19 g/L afterwards. In the experiment E15a fructose concentration quickly increased to 23 g/L at 24 h and ultimate utilization of this monosaccharide occurred in 72 h. In the experiments E16a and E16b the temporal changes of fructose concentration were different. Until 48 h its concentrations increased slower than those in the experiments E15a and E15b reaching 6 g/L and 17 g/L for the experiments

E16a and E16b, respectively. In the experiment E16a fructose was completely utilized in 96 h while in the experiment E16b its concentration in the end of the run was still at the level of 16 g/L. Glucose concentration trends were similar for all the experiments E15 and E16. However, its concentrations were higher in the experiments E16a and E16b than those in the experiments E15a and E15b, which is understandable, as glucose, not sucrose, was the second carbon source thereof.

3.3 Influence of inoculum density on inulinolytic activity in the batch bioreactor culture

In the first series of bioreactor experiments the number of spores per liter (inoculum density) used for the inoculation ranged from $9 \cdot 10^9$ to $3.33 \cdot 10^8$. In the bioreactor experiments the inulinolytic activity of the culture medium (Fig. 6A) occurred relatively late, in 48 h of the runs. In the experiment D (with the highest number of spores) even bigger delay was observed. The inulinolytic activity was detected after 72 h but ultimately in this run its value was the highest and reached 0.75 U/mL. In all bioreactor experiments the inulinolytic activity was increasing rapidly within the following 24 h from its occurrence. Next, this increase slowed down and the highest values of inulinolytic activities was usually observed about 120 h of the runs. In the experiment G the inulinolytic activity reached 0.70 U/mL and it was the second highest value obtained. The trend of the specific inulinolytic activity (Fig. 6B) in the experiment G stood significantly out from the other experiments. Starting from 0 U/g/X at 24 h of this

Figure 6. Changes of (A) inulinolytic activity, (B) specific inulinolytic activity in the batch bioreactor experiments D, E, F, and G, (C) changes of glucose and fructose concentration in the cultivation broth in the batch bioreactor experiments D and E, (D) changes of glucose and fructose concentration in the cultivation broth in the batch bioreactor experiments F and G and (E) changes of biomass concentration in the batch bioreactor experiments D, E, F, and G.

experiment the specific inulinolytic activity rose rapidly until the end of the cultivation reaching the highest value out of all bioreactor experiments equal to 158 U g^{-1} . The second highest specific inulinolytic activity equal to 100 U g^{-1} was observed in 120 h of the experiment D. In the experiment E the highest specific inulinolytic activity was 49.34 U g^{-1} and it was observed in 96 h of the experiment. In the experiment F the specific inulinolytic activity fluctuated.

Changes of glucose, fructose, and sucrose concentrations in the culture broth are presented in Fig. 6C and D. Sucrose was completely hydrolyzed until 24 h in each experiment, which, in consequence, caused the increase of glucose and fructose concentrations within this period of experiment. The increase of fructose concentration was also attributed to the hydrolysis of inulin. Later on, concentrations of these both monosaccharides began to decline in the following 24 h. In 72 h of the experiments all sugars were ultimately utilized. The changes of fructose

concentration in all these runs are very similar, while glucose was slower consumed in experiments D and E, in which the highest numbers of spores was used for the inoculation.

Until 24 h biomass concentration was closely associated with the introduced number of spores (Fig. 6E). More spores used simply resulted in higher biomass concentration. As expected the lowest biomass concentrations were observed in the experiment G and the highest in the experiment D. Naked eye observations proved that within the first 24 h mixing and aeration in the bioreactor remained undisturbed. Later on, it was difficult to find any trend for biomass concentration as mixing was not so ideal as earlier due to the high amount of biomass grown and, above all, its dispersed morphology and consequent rheological properties (see Section 3.6). For example it reached maximum in 72 h of the experiment F. Its value was 13.5 g L^{-1} and decreased rapidly later on. In the experiment D with the highest inulinolytic activity observed there was no sudden changes biomass concentration.

Figure 7. Oxygen saturation level of the culture broth (A), redox potential (B), stirring speed, and air flow rate (C), pH (D) in the batch bioreactor experiments D, E, F, and G.

In 48 h of this experiment it was equal to 8.66 g/L and remained approximately at this level until the end of the experiment.

3.4 Rheological parameters of the culture broth

The most important finding with regard to biomass of *A. niger* ATCC 204447 was that in each of these runs dispersed morphology evolved in the bioreactor irrespective of the number of spores used for inoculation. It was a completely different morphology from the pelleted one observed in shake flasks. Dispersed morphology was the reason that the broth was highly viscous. High viscosity of media in these experiments actually prevented from the efficient mixing and aeration but despite this the inulinolytic activity was detected. It must be also mentioned that it was impossible to hold the set oxygen saturation level ($pO_2 = 15\%$) within the majority of the duration of the experiments (Fig. 7A) because to these adverse effects. In order to quantify them the rheological measurements were made for the experiment G to better explain the runs of these experiments. Rheometric measurements revealed that the broth was strongly non-Newtonian and at low values of shear rate its apparent viscosity was extremely high (Table 2).

The culture broth satisfied Ostwald-de Waele power law and consistency index and the flow behavior index were found for it. Very low values of n shown in Table 2 (even below 0.2) clearly indicated that the studied fluid was strongly shear thinned (pseudoplastic). At the beginning of the experiment G apparent viscosity at shear rate 0.01 s^{-1} was 0.0392 Pa s^n and at 500 s^{-1} was 0.00966 Pa s^n . However, these values increased

Table 2. Rheological parameters of the culture broth of *A. niger* ATCC 204447

Cultivation time (h)	Apparent viscosity (Pa s^n)	Shear rate for apparent viscosity measurement (s^{-1})	Consistency index ($\text{N m}^{-2} \text{ s}^n$)	Flow behavior index (–)
0	0.0392 0.00966	0.010 500	0.0009	1.025
24	121 18	0.0273 337	8.440	0.127
48	673 0.0272	0.0334 494	3.400	0.197
72	214 0.0335	0.0462 665	16.15	0.137
96	135 0.0757	0.0270 149	2.702	0.262
120	36.0 0.0510	0.0262 99.5	1.662	0.426

dramatically and in 48 h of the experiment viscosity and at shear rate equal to 0.0334 s^{-1} reached 673 Pa s^n . In that moment of the experiment at high shear rate equal to 494 s^{-1} apparent viscosity was equal to 0.0272 Pa s^n . All in all, it is worth mentioning that the naked eye observations of the experiment G inoculated with the lowest number of spores indicated for the most efficient mixing and aeration in the bioreactor, compared to the other experiments.

3.5 Aeration, oxygen saturation, and pH of the culture broth

The effect of the varied number of spores together with the impact of high non-Newtonian viscosity of the broths was clearly reflected by the on-line measurements. Oxygen saturation level (pO_2) of the culture broth (Fig. 7A) was meant to be kept at the level of 15% but, as mentioned above, this value was hardly reached due to high viscosity of the broth (Table 2). It was caused by inefficient mixing and aeration. The latter was confirmed by the close-to-zero pO_2 levels that were measured during all bioreactor experiments. Nevertheless, the pO_2 values measured in the early stages of the processes supplied the following important information about the run of the experiments. Above all, the duration of lag-phase (actually spore germination time) could be evaluated upon the abrupt decrease of pO_2 from initial 100% level. Spores are known not to require any oxygen in its early stages of germination [24]. In the experiments D and E lag-phase ended after 3.5 h. From 9.5 h pO_2 declined sharply, which was probably caused by the exponential fungal growth (early trophophase). Afterwards, in about 14 h of the experiments the period of slow decrease of pO_2 level was observed as long as pO_2 level of the culture broth reached the close-to-zero value. In this moment the first signs of inefficient mixing were also visible and *A. niger* must have utilized all supplied oxygen and probably oxygen deficiency took place. In the experiments F and G lag-phase lasted longer than in the other experiments (10 hours). Then rapid decline of pO_2 level was observed. It reached the close-to-zero level after about 24 h. As the culture broth in all these runs was very nutritious, oxygen availability was the main growth limiting factors within at least next 12–48 h. In the experiment F the period of the close-to-zero pO_2 level was the shortest and after 36 h of the experiment pO_2 increased rapidly. In the experiments D and G the increase of pO_2 started after 84 h when fungal biomass was hardly active due to nutrient deficiency. In the experiment E the increase of pO_2 level was not observed.

It is clearly seen that redox potential (Fig. 7B) was closely related to the number of spores introduced. At the lowest number of spores introduced (experiment F and G) its values showed high peaks reaching even +300 mV, at about 24 h, which indicated on highly oxidative conditions in the bioreactor. On the other hand in the experiment D, with the highest number of spores negative values (−34 mV at 54 h) were observed. In the experiment E the lowest redox potential was 0 mV.

The upper and the lower setting values of air flow rate and stirring speed to control oxygen saturation were defined at the beginning of each experiment. Due to very high viscosity of culture medium and related problems with aeration and mixing the upper setting values had to be manually increased when they were reached due to the action of the control loop and the target pO_2 level was not achieved. All the actions described below showed, how difficult task was to aerate the bioreactor, in which biomass of *A. niger* ATCC 204447 fast grew in the form of dispersed morphology. In the experiment D the lower setting values for stirring speed and air flow rate were 200 min^{-1} and 1.5 L/min, respectively. However, after 18 h of the experiment mixing became inefficient and stirring speed and air flow rate achieved their upper setting values: 350 min^{-1} and 4 L/min, re-

spectively (Fig. 7C). As the desired pO_2 level was not reached, these values had to be changed to 500 min^{-1} and 5 L/min, respectively but even then never was the set pO_2 level achieved, unless the fungus lost its activity in the late idiophase. In the experiment E the lower setting values for the stirring speed and the air flow rate were higher than before: 250 min^{-1} and 2.5 L/min. Again after 18 h of the experiment E parameters also reached the upper setting values stirring speed and air flow rate at 500 min^{-1} and 5 L/min, respectively but until the end of the experiment and there was no possibility to keep pO_2 level at 15%. That is why, at 32 h of the next experiment F the upper setting value for stirring speed value was even increased from 500 min^{-1} to 600 min^{-1} and again no successful aeration was achieved. In the experiment G, in which the lowest number of spores was used. The upper setting values of stirring speed and air flow rate of 600 min^{-1} and 5 L/min were reached only at 24 h (Fig. 7C). Despite the insufficient aeration and stirring the inulinolytic activity was detected in the broth, nevertheless it is impossible to say what would be its value, if the required oxygen saturation was achieved.

In Fig. 7D changes of pH values are shown. They were not controlled in all experiments in any way. As in the case of redox potential it was closely related with introduced number of spores. Within first 12 h of the experiments F ($1 \cdot 10^9$ spores per liter) and G ($3.33 \cdot 10^8$ spores per liter) pH level raised slightly from its initial value of 5.5–6, while in the experiment D ($9 \cdot 10^9$ spores per liter) and the experiment E ($5 \cdot 10^9$ spores per liter) to 5.75. Later on, pH value began to decline. In the experiments D and E this decline was moderate and the lowest pH value observed was 4.5. It rose again from this point up to 6.25 and 6, respectively. In the experiments F and G pH level declined more abruptly. Its lowest pH value reached 3.75 and raised to 5.25 in experiment F. In the experiment G pH declined even to 3.25, which was the lowest pH value noted. Later on, a small increase of pH level to 3.75 was noticed.

3.6 Application of MPEC to facilitate *A. niger* ATCC 204447 cultivation in the stirred tank bioreactor

In the second series of the bioreactor experiments micro particles-enhanced cultivation (MPEC) was applied to act on *A. niger* morphology. Two experiments, i.e. control and MPEC ones, were conducted. This series of experiments was independent of the previous four runs and performed several months later. That is why, the results of these experiments will not be compared with the experiments from D to G. Talc microparticles of average 10 μm diameter were used.

The highest values of inulinolytic activity (Fig. 8A) in the experiment H (MPEC) were equal to 0.18 U/mL in 60 h and 0.19 U/mL in 90 h of the experiment. In the comparative experiment J conducted without talc microparticles addition, the highest inulinolytic activity was lower and reached 0.11 U/mL in 60 h. The temporal changes of the inulinolytic activity in the experiments H and J were different. In the control experiment J the inulinolytic activity occurred after 36 h and increased until 60 h of the experiment. Later on, it gradually decreased. In the experiment H (MPEC) the occurrence of inulinolytic activity took place later, in 48 h of the run and started to quickly rise

Figure 8. Changes of the inulinolytic activity (A) and specific inulinolytic activity (B) in the batch bioreactor experiments H and J, (C) changes of glucose and fructose concentration in the cultivation broth in the batch bioreactor experiments H and J, and (D) changes of biomass concentration in the batch bioreactor experiments H.

until 60 h. Having reached the maximum value of 0.18 U/mL, which was almost a twofold increase compared to the control run, it fluctuated between 0.16 and 0.18 U/mL. In the case of the specific inulinolytic activity (Fig. 8B) in the experiment H with talc microparticles the obtained values were even over threefold higher than those in the experiment J, which indicates that fungal biomass was more efficient in the production of inulinases in the presence of microparticles. In the experiment H it was 32.35 U g/X, while in the experiment J only 9.97 U g/X. Subsequent (from 60 h of the run) decline of the specific inulinolytic activity was also observed.

Changes of glucose, fructose, and sucrose concentrations in the culture medium are depicted in Fig. 8C. As in the previous experiments, at the beginning of experiments sucrose and inulin were hydrolyzed by the fungus causing the increase of glucose and fructose concentrations. Sucrose vanished from the medium within 36 h in both runs. Simultaneously, fructose concentrations reached their maxima of 9.14 g/L in the experiment H and 8.84 g/L in the experiment J. Fructose was depleted after 72 h in the experiment H and after 60 h in the experiment J, so talc microparticles did not contribute to faster fructose utilization. In the case of glucose the same relationship was noticed. It was completely utilized after 72 h of the experiment J and after 90 h of the experiment H. Talc microparticles addition slowed down biomass growth and decreased the amount of biomass formed too (Fig. 8D). The highest biomass concentration was obtained in 90 h at the level of 8.72 g/L in the experiment H and 12.58 g/L in the experiment J.

Oxygen saturation level of the culture broth (Fig. 9A), as previously, was to be kept at the level of 15%. The lag-phase and the

slight decrease of pO₂ level of the culture broth occurred after 2 h of the experiment J. Afterwards, pO₂ level dropped down to close-to-zero levels within 15 h, which was associated with the exponential fungal growth in early trophophase. In the experiment H (with microparticles) lag-phase and trophophase lasted longer and the close-to-zero pO₂ level was reached only after 65 h. Furthermore, between 24 and 65 the upper setting values of stirring speed and air flow rate (600 min⁻¹ and 5 L/min) were not achieved. Due to the presence of microparticles the pellets of 1–2 mm diameter were formed, what much facilitated aeration and stirring (to a lower extent) of the broth. The destruction of pellet after about 65 h of the run regained dispersed morphology and the properties of the suspension began to be similar to the ones in the control run.

Initial redox potential (Fig. 9B) in experiments H and J was about 187 mV and peaked at 273 mV and 300 mV, respectively after 24 h. In the experiment H the lowest redox potential value was 30 mV and it occurred in 84 h. In the experiment J redox potential went down to 58 mV in 48 h. Afterwards its value increased to 175 mV. Negative values of redox potential were observed in the run with the microparticles in the end of the run.

In the experiments H and J the lower and the higher setting values of air flow rate were 2.5 L/min and 5 L/min, while of stirring speed 250 and 600 min⁻¹, respectively. In the experiment H in 27 h air flow rate peak reached 4.86 L/min and stirring speed peak reached 416 min⁻¹. The upper setting values of these parameters were obtained only in 62 h. In the experiment J air flow rate fluctuated around the upper setting value since 18 h of the experiment. Stirring speed peaked in 17 h reaching 544 min⁻¹. Afterwards its value decreased to 418 min⁻¹ in 25 h and

Figure 9. The saturation level of the culture broth (A), redox potential (B), stirring speed, and air flow rate (C), pH (D) in the batch bioreactor experiments H and J.

achieved upper set value 600 min^{-1} in 45 h. All in all, it seemed that in the case of MPEC run it was easier to aerate the medium.

In the early stages of the runs the temporal changes of pH level in the experiments H and J (Fig. 9D) were roughly the same. The initial pH level of 5.8 decreased rapidly until 30 h reaching the value of 4.11. Subsequently, in the experiment H pH value decreased slowly to about 3.7 and after 88 h it increased up to 4.2. In the experiment J, the drop of pH after 30 h was more rapid and the lowest pH value was even 2.9 in 75 h. It increased later up to 3.7.

4 Discussion

The values of the inulinolytic activities obtained by other researchers vary in the wide range and strongly depend on the microorganism used, i.e. strain, medium composition, and cultivation conditions. Therefore, it is difficult to directly compare the data included in this work with them. For example, upon the review by Ricca et al. [6] it can be found that for *A. niger* 817 the inulinolytic activity was only 0.0685 U/mL , while for *A. niger* mutant 817 it reached 160 U/mL . With regard to the *A. niger* ATCC 204447 strain used in this study it is only possible to cite the preliminary data from its discoverers. Ongen-Baysal et al. reported the maximum inulinolytic activity at 4600 U per gram of substrate [18]. It is difficult to recalculate these units as these authors used Jerusalem artichoke (*Helianthus tuberosus* L.), undefined medium component, as the carbon substrate and changed its concentration in the cultivation medium from 0.5 to 6%. The inulinolytic activity unit was not explicitly defined in their work either. In the other work from these authors a

mixed culture of *A. niger* and *K. marxianus* was used for the production of inulinases and these data are useless for the purpose of comparison [22]. For another fungus *Penicillium sp.* TN-88 growing in the liquid medium with inulin as the carbon source the endoinulinolytic activity was 9.9 U/mL [25]. For yeasts *K. marxianus var. bulgaricus* out of three carbon sources tested, i.e. glucose, fructose, and sucrose, the latter substrate resulted in the highest inulinolytic activity of 15.4 U/mL at 30 g/L and 208 U/mL at 20 g/L [23]. Although it was a different microorganism, these data at least confirmed the present results about the usefulness of sucrose for the production of inulinases. What is also interesting, Ge and Zhang studied the influence of octadecanoylsucrose added to the basal medium with sucrose. It enhanced the inulinolytic activity nearly sevenfold [26]. Also the addition of the natural compounds like dandelion (*Taraxacum officinale* L.) root extract induced the production of endoinulinase in *A. niger* NK-126 and in 96 h of cultivation the activity reached 55 U/mL , which was fourfold higher than that in the medium with pure chicory inulin [10]. Poorna and Kulkarni tested the production of inulinases by *A. niger* van Teighem using the variety of carbon sources [8]. Although the highest inulinolytic activities (80 U/mL) were obtained for pure 2% inulin, the addition of 0.1% sucrose also resulted in the satisfactory inulinolytic activity (75 U/mL). Worse results were obtained when the mixtures of inulin (2%) and glucose (0.1%) and inulin (2%) and fructose (0.1%) were used, i.e. 48 and 54 U/mL , respectively. Sole fructose and glucose as the carbon sources did not induce inulinolytic activity. It was found in the present work that it was sucrose that in the combination with inulin allowed for achieving the highest inulinolytic activity in the broth of *A. niger* ATCC 204447 out of

all studied media compositions. It can be thus anticipated that the valid combination of sucrose and inulin may assure the potentially utilizable level of the inulinolytic activity in this strain. There are hardly any experimental data from stirred tank bioreactors for the inulinolytic strains to compare with the ones in this study. In the bioreactor study of highly productive *A. niger* mutant strain by Skowronek and Fiedurek even 22.1 U/mL inulinolytic activity can be found [15]. To sum up, it must be mentioned, all values of the inulinolytic activities presented above were determined upon the assay of reducing sugars, not fructose concentration alone as made here.

As the majority of *Aspergilli* in the media of weakly acidic and neutral pH [27, 28], *A. niger* ATCC 204447 formed macroscopic pellets in the shake flask cultivation. These pellets were of large diameter (above 3 mm) and they may have influenced the run of the experiments due to intrapellet oxygen limitation. Diffusive oxygen transfer in such large pellets is extremely inefficient [29, 30].

In the batch cultivation in the stirred tank bioreactor the strongest factor, i.e. the number of spores used for inoculation, influencing the evolution of the fungal cultures was applied to study its effect on inulinolytic activity, specific inulinolytic activity, biomass concentration, redox potential, and pH [27]. Upon these experiments it ultimately occurred that *A. niger* ATCC 204447 is a relatively fast-growing strain, which under high shearing stress form dispersed morphology in the studied range of inoculum densities. It is somewhat unusual as there are *Aspergilli* that save their pelleted morphology in stirred tank bioreactors despite higher stirring speeds (even reaching 800 min⁻¹) than those in this study [31]. As a result of the morphological form evolved in the bioreactor experiments culture broth was strongly pseudoplastic (flow behavior index even about 0.2) and had extremely high apparent viscosity reaching even 672 Pa sⁿ for low shear rate 0.0334 s⁻¹) Rarely are so viscous and shear thinned fungal suspension met [31, 32]. As a result of fungal morphology and consequent rheological properties inefficient mixing and aeration was observed. Despite the aeration rate amounted to almost 1 vvm oxygen saturation level of the culture broth defined at the level of 15% was not achieved. It seems that for such fungus as *A. niger* ATCC 204447 growing in the carbon substrate rich medium aeration with air enriched with oxygen can be suggested to at least increase the driving force of convective mass transfer [31].

Another observed adverse effect (from the point of view of the inulinolytic activity) of the relatively strong decrease of pH level was also connected with the aforementioned oxygen deficiency as organic acid are then formed by fungi of genus *Aspergillus* [33].

In order to influence fungal morphology microparticles-enhanced cultivation (MPEC) was applied for the bioreactor cultures. Normally, the addition of inorganic microparticles is used to decrease pellet size or to change pelleted morphology into dispersed one [34–36]. In spite of it, talc microparticles were applied in *A. niger* ATCC 204447 culture upon the previous premises that microparticles played the stabilizing role for the formation pellets under high mechanical stress in the stirred tank bioreactor [37]. Astonishingly, the addition of 10 µm talc microparticles forced the formation of pellets. It contributed to the almost twofold increase of the volumetric inulinolytic activity and 3.5-fold increase of the specific inulinolytic activity, com-

pared to the control run. Unfortunately, it is not as much as the increases reported previously for other enzyme producing fungal systems: 3.7-fold increase of β-fructofuranosidase activity and 9.5-fold α-glucoamylase activity for various enzyme-producing strains of *Aspergillus niger* [35]. Nevertheless, this positive change of the inulinolytic activity may be attributed to the formation of pellets, which positively influenced oxygen saturation level of the culture broth. In the experiment with talc microparticles added pO₂ fell down to close-to-zero level only after disintegration of pellets about 65 h of the run.

To sum up, the experimental data presented above allow for the following conclusions. The presence of fructooligosaccharides in the mixture after enzymatic reaction with inulin confirms the endoinulinolytic activity of *A. niger* ATCC 204447, which makes this strain worth further research as a producer of inulinases. *A. niger* ATCC 204447 is a fast-growing strain, which may form troublesome morphologies from large pellets in the shake flask culture to highly dispersed morphology in the stirred tank bioreactor. The first causes the diffusive mass transfer resistance. The latter makes the broth highly viscous of pseudoplastic rheological properties. Both aggravate the access of oxygen to fungal cells. With the increase of inulin concentration in the culture medium the inulinolytic activity of *A. niger* ATCC 204447 increases, nevertheless the second sugar carbon substrate, especially sucrose, is useful to achieve higher inulinolytic activities of the cultivation broth. In the batch bioreactor generally comparable or even higher volumetric inulinolytic activities are obtained to those in shake flasks, however they do not significantly depended on the number of spores that served as the inoculum. Due to high biomass growth in the bioreactor the inulinolytic activities referred to biomass content (the specific ones) are usually lower than those in shake flasks. Application of microparticles-enhanced cultivation (10 µm talc microparticles) leads to the formation of medium-size pellets for the certain period of the cultivation time in the batch stirred tank bioreactor, nevertheless the increase of the inulinolytic activity of the broth attributed to these microparticles can be estimated as moderate.

Practical application

The main application of inulinases (enzymes that hydrolyze a polyfructan called inulin) is the production of highly sweet syrups. Due to the facts that inulin consists of 36 fructose moieties per one glucose moiety (on average) and that endoinulinase is capable of transforming inulin into much sweeter than sucrose and fructose fructooligosaccharides (containing three, four, or five fructose moieties), the syrups obtained by hydrolysis of inulin are a healthier and sweeter replacement of corn-originated syrups containing far too much glucose. As they are sweeter than sucrose they can be applied in lower amounts. What is more, their taste is more consumer-friendly than that of other sucrose replacements. *Aspergillus niger* ATCC 204447 is a yet untested in the laboratory bioreactor scale strain possessing inulinolytic activity and that is why it can be potentially applied as another efficient producer of inulinases.

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5 References

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