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Original scientific paper

Enhancement of amylase production by *Aspergillus* sp. using carbohydrates mixtures from triticale

BILJANA DOJNOV[1]*, MARICA GRUJIĆ[2], BOJANA PERČEVIĆ[2] and ZORAN VUJČIĆ[2]

[1]*Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, Belgrade, Serbia and* [2]*Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 12–16, Belgrade, Serbia*

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Abstract: With the purpose of finding a suitable available inducer in combination with starvation, carbohydrate mixtures from triticale were used and compared with well-known amylase inducers in fungi. Carbohydrate mixtures from triticale induced the production of an amylase cocktail (α-amylase and glucoamylase) in *Aspergillus niger*, unlike induction with well-known inducers that induce only glucoamylase, shown by zymography and TLC analysis of the carbohydrate mixtures before and after fermentation. Glucoamylase production by *A. niger* was the highest in the presence of the extract obtained after autohydrolysis of starch from triticale (95.88 U mL^{-1}). Carbohydrate mixtures from triticale induced the production of α-amylase in *A. oryzae*. More α-amylase isoforms were detected when using a complex carbohydrate mixture, compared to induction with maltose or starch. A 48-h induction was the most efficient using a triticale extract (101.35 U mL^{-1}). Carbohydrates from triticale extracts could be used as very good cheap amylase inducers. Triticale, still not fully utilized, could be taken into consideration as an inducer in amylase production by *Aspergillus* sp, and in such a way, it could be used as the sole substrate in fermentation.

Keywords: α-amylase; glucoamylase; maltose; starch; enzyme production; fungi.

INTRODUCTION

The fungi *Aspergillus* sp. are well-known producers of amylases, which are industrially important enzymes. Filamentous fungi produce hydrolytic enzymes in the form of enzymes mixtures – cocktails. Glucoamylase and α-amylase are produced concomitantly in fungal fermentations.[1–4]

Maximal production of the enzymes may be achieved by using appropriate inducer molecules. Induction is the main controlling mechanism in the pro-

* Corresponding author. E-mail: bdojnov@chem.bg.ac.rs
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duction of amylases in fungi. The effects of small molecules on amylase production were examined even when using complicated bioreactors.[5] It is known that α-amylase and glucoamylase are inducible enzymes in *Aspergillus* sp.[6] The induction mechanism of amylase production in *Aspergillus* sp. has been successfully studied.[7,8] Induction study of α-amylase production in *A. oryzae* began in 1961.[9] Starch and its hydrolysis products, mostly studied is maltose, are known inducer of α-amylase in *A. oryzae*.[10–12] Maltose is also a good glucoamylase inducer in *A. niger*.[13] Starvation launches a special mechanism leading to increased amylase production in *Aspergillus* sp.[14]

Considering all the above known, the induction mechanisms and the fact that *Aspergillus* sp. produce concomitantly α-amylase and glucoamylase, a cost--effective fermentation could be optimized by using inducers for the production of special amylase cocktail. This brought about the idea to examine the possibility of using a carbohydrate mixture from triticale (x *Triticosecale*, Wittmak). Triticale is an important industrial crop insufficiently utilized yet. Triticale cultivation has many benefits compared to other crops and its production and use have been intensively studied.[5,15] Triticale contains high amounts of starch (about 60 %) and protein (from 12 to 15 %).[16] It also contains higher amounts of major mineral elements (K, P and Mg) and nutritionally important minor elements (Na, Mn, Fe, Cu and Zn) than wheat.[17]

The opportunity of using cost effective and available carbohydrate mixtures from triticale as inducers of amylase in the two most important fungal producers of amylase, *A. niger* and *A. oryzae*, were examined in this study. Two kinds of triticale extracts, starchy extract and the extract obtained after starch hydrolysis by endogenous amylases were used as the sole fermentation substrates in submerged fermentation (SmF) and were compared with synthetic media containing the known inducers maltose and starch. The induction mechanisms were combined with mycelial starvation to cover all known methods of induction.

EXPERIMENTAL

Reagents

All used reagents and solvents were of the highest purity and purchased from Merck and Sigma–Aldrich. Triticale (x *Triticosecale* sp.) "Rtanj" line was obtained from the "Center for Small Grains Kragujevac", Kragujevac, Serbia.

Microorganisms and fermentation conditions

Aspergilus niger ATCC 10864 and *A. oryzae* ATCC 56747 strains were cultivated while obtaining matured spores. Spore suspensions were prepared in a 0.1 % Tween 80 solution at a concentration of 5.9×10^5 spores mL^{-1}.

Fermentations

Two parallel fermentations were performed with *A. niger* and *A. oryzae*. Submerged fermentations (SmF) were performed for 73 h at 30 °C and 210 rpm. The spore suspensions (10 vol. %) were inoculated in Czapec solution with 0.5 % yeast extract. Fungal mycelia

obtained in 18 h (5 %) were transferred into induction or non-induction media, as shown in experiments scheme in Fig. 1. Fungal mycelia were washed twice with water and dried with filter paper between the experimental phases shown in schematic diagram. Fermentations were stopped after scheduled time (Fig. 1) by centrifugation of the biomass for 15 min at 5000×*g*.

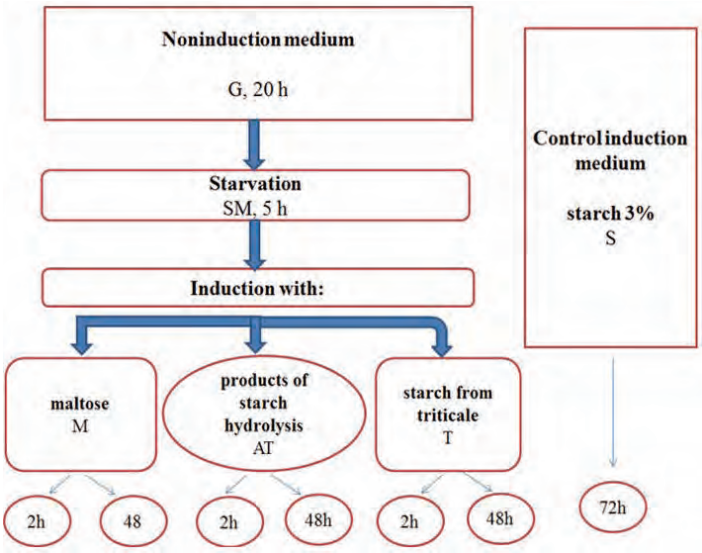

Fig. 1. Schematic presentation of amylase induction in *A. niger* and *A. oryzae*. MG – medium with glycerol, SM – starvation medium, MM – medium with maltose, AT – autohydrolysate of triticale extract, T – Triticale extract, MS – Medium with starch. Compositions of specified substrates are given in Table I.

TABLE I. The compositions of the media used in the fermentations by *A. niger* and *A. oryzae* following the scheme shown in Fig. 1

Medium		Composition
MG	Medium with glycerol	Peptone 20 g L^{-1}; glycerol 30 g L^{-1}; KH_2PO_4 5 g L^{-1}; $MgSO_4$ 2.5 g L^{-1}
SM	Starvation medium	KH_2PO_4 5g L^{-1}; $MgSO_4$ 2.5 g L^{-1}
MM	Medium with maltose	Peptone20 g L^{-1}; maltose 30 g L^{-1}; KH_2PO_4 5g L^{-1}; $MgSO_4$ 2.5 g L^{-1}
AT	Autohydrolysate of triticale extract	Decanted extract obtained after incubation of milled triticale (x *Triticosecale* sp.) and water in 1:3 ratio, autohydrolysis at 60 °C for 3 h[a]
T	Triticale extract	Decanted extract obtained from mixing milled triticale (x *Triticosecale* sp.) and water in 1:3 ratio without auto hydrolysis of starch
MS	Medium with starch	Peptone 20 g L^{-1}; raw starch 30 g L^{-1}; KH_2PO_4 5 g L^{-1}; $MgSO_4$ 2.5 g L^{-1}

[a]Preparation of autohydrolysate of the triticale extract is described in the Experimental, *Media preparation*

Media preparation

The compositions of the media used in the examination of the induction of amylase production by *Aspergillus* sp. are given in Table I.

Triticale was finely ground to flour using a "Bragal" mill. The triticale flour was suspended in water (1:3 *w*/*V* ratio), mixed and strained through a strainer to obtain the triticale extract (T). The autohydrolysate of triticale (AT) was prepared by incubation of suspension of triticale flour and water (1:3 *w*/*V* ratio) for 3 h at 60 °C.[18] The obtained suspension was strained through a strainer to separate the liquid from solid part. The liquid phase was used as the AT. The other media were prepared by mixing the individual components. All media were autoclaved under standard conditions prior to use.

Amylase activity assay

The amylase activity was assayed at pH 5.0 according to the dinitrosalicylic acid (DNS) procedure[19] using of 1.0 % (*w*/*V*) soluble starch as substrate, for 30 min at 35 °C. Maltose was used as the standard. Each data point represents the mean of three independent assays (standard error, SE, values were less than 5 % of the means). One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 μmol of maltose in 1 min at 35 °C.

Glucoamylase activity assay

Glucoamylase activity was assayed at pH 5.0 using 1.0 % (*w*/*V*) soluble starch as the substrate in 30 min at 35 °C. Glucose (final product of the reaction) was detected in the reaction mixture by coupled reaction with glucose oxidase and horseradish peroxidase (HRPO, Trinder reagent). Each data point represents the mean of three independent assays (*SE* values were less than 5 % of the means). One unit of glucoamylase activity was defined as the amount of enzyme required to produce 1 μmol of glucose in 1 min at 35 °C.

Zymographic detection of α-amylase and glucoamylase

α-Amylase and glucoamylase were detected simultaneously using zymography.[4] α-Amylase was detected in a PAA (polyacrylamide) gel with copolymerized β-limit dextrins, stained by iodine solution. The α-amylase activity appeared as clear bands on a purple background. Both amylases were detected as clear bands on a blue background, using soluble starch as substrate and iodine solution for staining, in the native EF (electrophoresis) PAA gel after printing. Glucoamylases were detected on an NC (nitrocellulose) membrane using a substrate solution (1.0 % (*w*/*V*) starch in buffer) and a reaction mixture for glucose detection (glucose oxidase, HRPO and 4-Cl-α-naphthol as substrate). Specific reaction product, purple and insoluble, appeared on the NC in bands corresponding to glucoamylase.

Starch, reducing sugar and glucose concentrations

The concentrations of starch in triticale extract and triticale autohydrolysate were determined by the iodine dextrine color (IDC) method by measuring the absorbance at 590 nm.[20] Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method[19] using maltose as the standard, while glucose concentration was measured by Trinder reagent.

TLC analysis of carbohydrates in triticale extract and triticale autohydrolysate

Carbohydrates were detected by thin layer chromatography (TLC) on silica plates, 4.5 cm×6 cm (Silica gel 60 F-254, Merck, Darmstadt, Germany), using a Camag development chamber in the tank configuration. The plates were developed by the double-ascending method in a solvent system consisting of butan-1-ol, ethanol, water and glacial acetic acid (5:3:2:0.5 volume ratio). Standard solution of the oligosaccharides mixtures (1.0 mg mL^{-1}

each) was prepared in water and they consisted of: glucose (C1), maltose (C2), maltotriose (C3), maltotetraose (C4), maltopentaose (C5), maltohexaose (C6) and maltoheptaose (C7) (Across and Sigma Aldrich, USA). All separations were performed at ambient temperature (22±2 °C). The carbohydrates were detected by spraying the plates with an ethanolic solution containing 0.5 % (*w*/*V*) *α*-naphthol and 5 vol. % H_2SO_4, followed by heating for 10 min at 120 °C.

RESULTS AND DISCUSSION

Induction of amylase cocktails in *A. niger* and *A. oryzae* were examined by submerged fermentations (SmF) according to scheme showed in Fig. 1. The impact of two kinds of triticale extracts were compared with the impacts of known amylase inducers using various media, the compositions of which are given in Table I.

Carbohydrate composition of triticale extract and triticale autohydrolysate

Triticale extract (T) and triticale autohydrolysate (AT) differed in their carbohydrate contents, especially in their starch contents, Table II. Triticale grains contain more than 60 % starch, classifying it as a starchy cereal.[16,21] The concentration of starch in the triticale extract used in this research was 10 mg mL^{-1}, which represents the quantity of starch available to the fungi during fermentation. Only a trace of starch was detected in triticale autohydrolysate because of starch hydrolysis during the autohydrolysis process by the *α*-amylase contained in the triticale. The amount of reducing sugars was increased 12.5 times after autohydrolysis, which corresponds to the decreased starch content.

TABLE II. Starch, reducing sugar and glucose contents in triticale extract and triticale autohydrolysate

Sample	Starch, mg mL^{-1}	Reducing sugars, mM	Glucose, mM
Triticale extract (T)	10.24	16.60	11.04
Triticale autohydrolysate (AT)	0.75	201.09	146.18

The TLC analysis revealed that the triticale extract contained a wide range of carbohydrates, Fig. 2C, lane T. Maltose was the most abundant carbohydrate, apart from glucose and maltotriose, in triticale extract after autohydrolysis, Fig. 2C, lane AT. These differences suggest potential different induction of *α*-amylase and glucoamylase in *Aspergillus* sp.

Induction of amylase production in Aspergillus *sp.*

Non-growing mycelia of *Aspergillus* sp. are a good model system of amylase induction.[11] For this reason, 20-h cultures of both *Aspergillus* species were used. Starvation before addition of carbohydrates is well known as good method for enzyme induction.[11,14,22] Starvation of *Aspergillus* mycelia for 5 h was applied before adding the inducers to the medium, Fig. 1. This enables fungi to meta-

bolize all ingredients present in growth medium and to maximalize the uptake of new molecules added after starvation. All inductions were monitored after 2 h (rapid induction) to compare the impact of maltose and starch[11] with the impact of the triticale extract and of the triticale autohydrolysate. The inductions were further monitored and production levels were determinate in 48 h, which is actually 72 h after the start, because it is commonly used fermentation time for fungi SmF.

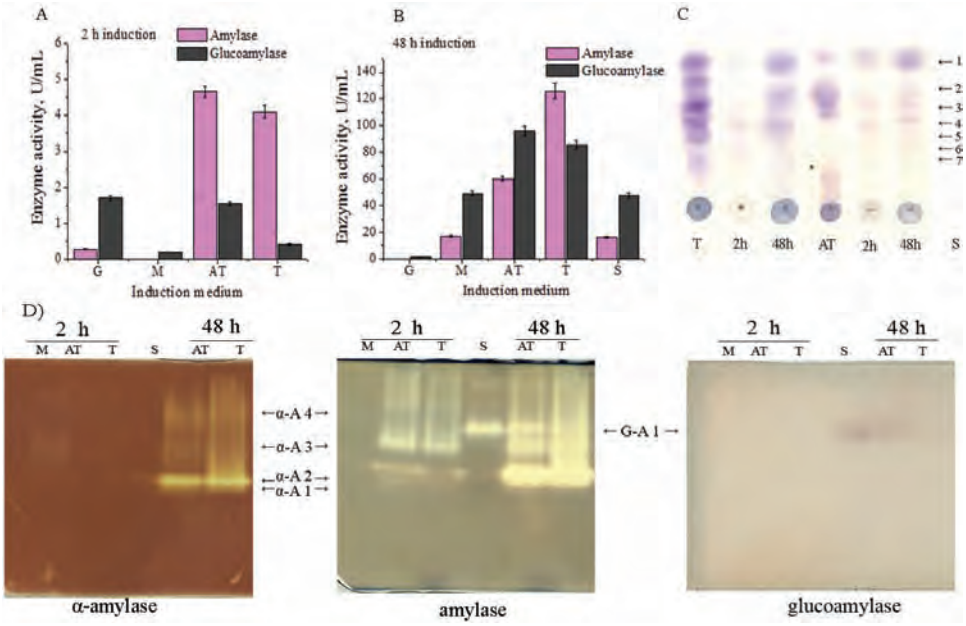

Fig. 2. Glucoamylase and *α*-amylase production by *A. niger* depending on the type of inducers (carbohydrate source) after 2 and 48 h. A) Enzymatic activities, U mL^{-1}, after 2 h of induction; B) enzymatic activities, U mL^{-1}, after 48 h of induction; C) TLC analysis of the carbohydrates in the SmF samples; S – standard carbohydrates: 1 – glucose, 2 – maltose, 3 – maltotriose, 4 – maltotetraose, 5 – maltopentaose, 6 – maltohexaose and 7 – maltoheptaose; D) zymographic detection of *α*-amylase and glucoamylase in the SmF samples. The arrows indicate the positions of the *α*-amylase isoforms (*α*-A1 to *α*-A4) and the glucoamylase isoform (G-A1). G – glycerol, MM – medium with maltose, AT– autohydrolysate of the triticale extract, T – triticale extract and MS – medium with starch.

Induction of glucoamylase and α*-amylase production in* A. niger

The impact of all inducers on *A. niger* amylases production were monitored by enzymatic assays, TLC analysis of the obtained carbohydrates, and zymogram detection of *α*-amylase and glucoamylase in the fermentation extracts and the results are presented in Fig. 2.

Rapid induction (after 2 h) with triticale extract and triticale autohydrolysate led only to a noticeable increase in the α-amylase production, as evidenced by the enzymatic assay and zymogram, Fig. 2A and D. Low levels of both amylases were detected in all the examined extracts, indicating that 2 h was too short for production.

The glucoamylase level was lower in the medium with maltose than in medium with glycerol, which is contrary to literature results.[11] However, as this was not the case after the 48 h of fermentation with induction (Fig. 2B), it could be because the mycelia had not started to express the induced enzymes within 2 h. Levels of enzymes detected after 2 h in medium with glycerol originated from the standard enzyme pool. The results obtained for *A. oryzae* confirmed this assumption, Fig. 3A and B. The observation indicated that it is necessary to monitor the fermentation for 48 h.

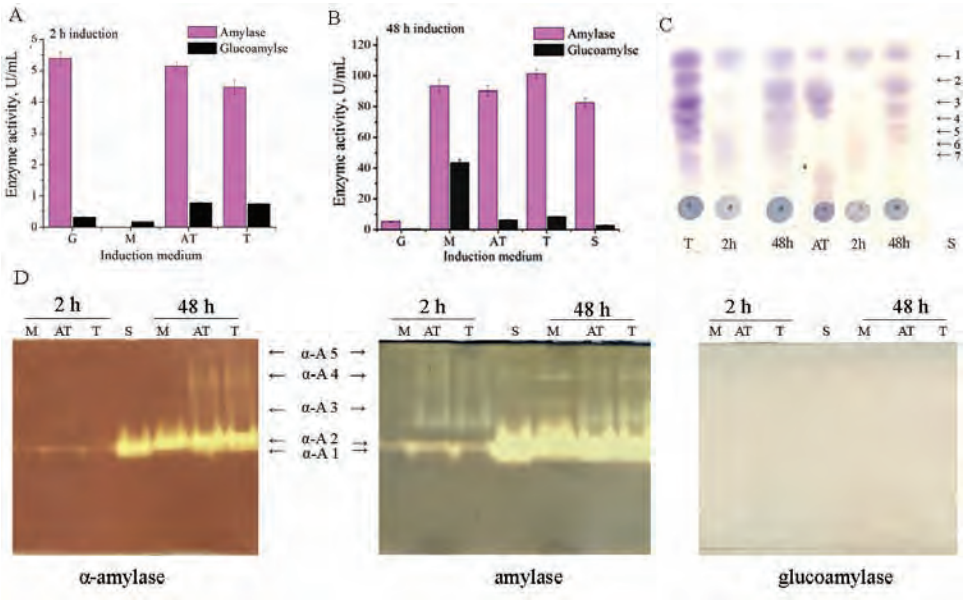

Fig. 3. Production of α-amylase by *A. oryzae* in dependence on the type of inducer (carbohydrate source) after 2- and 48-h fermentation. A) and B) Enzymatic activities, U mL^{-1}, after 2 and 48 h of induction, respectively; C) TLC analysis of carbohydrates in the SmF samples; S – standard carbohydrates: 1 – glucose, 2 – maltose, 3 – maltotriose, 4 – maltotetraose, 5 – maltopentaose, 6 – maltohexaose and 7 – maltoheptaose; D) zymographic detection of α-amylase and glucoamylase in the SmF samples. The arrows indicate the positions of the α-amylase isoforms (α-A1 to α-A5). G – glycerol, MM – medium with maltose, AT– autohydrolysate of the triticale extract, T – triticale extract and MS – medium with starch.

The fact that glucoamylase and α-amylase were produced concomitantly is often ignored and *A. niger* was shown as a producer of glucoamylase solely.[23–25]

The results prove that *A. niger* produce glucoamylase and α-amylase in different ratio, depending on the applied inducers. Maltose was a strong inducer of glucoamylase after 48 h, Fig. 2B and D, which was shown by using maltose and AT as inducers. Maltose was the most abundant carbohydrate in AT according to TLC analysis, Fig. 2C. The high level of glucoamylase in the fermentation with AT was confirmed by enzymatic assay, specific zymogram detection and TLC, as well the detection of a high quantity of glucose. Maltose is a well-known glucoamylase inducer.[13,26] The obtained results confirmed this, and showed that maltose was a better glucoamylase inducer when used in a mixture with the other carbohydrates (maltotriose and glucose) in AT. *A. niger* produced glucoamylase when cultivated on starch as a carbon source – control medium, and with T. This proves that starch is a good amylase inducer and the starch hydrolysis products formed during fermentation are especially good amylase inducers. The use of T as an inducer favors the production of α-amylase in *A. niger*, Figs. 2B and D. The choice of the carbohydrate mixture as inducer affects various amylase complexes leading to enrichment with glucoamylase if AT was used or α-amylase if T was used.

Induction of α*-amylase production by* A. oryzae

The impacts of all the examined carbohydrates as inducers on the production of amylases by *A. oryzae* were monitored in the same way as for *A. niger* and the results are shown in Fig. 3.

A. oryzae produced only α-amylase isoforms in all the examined fermentations, Fig. 3D. The period of 2 h was too short for production according to the obtained low level of amylase, Fig. 3A. T and AT proved to be better α-amylase inducers than maltose and starch after 48 h induction. The triticale extract, containing a mixture of carbohydrates C1 to C7 (Fig. 3C lane T), induced the highest amount of α-amylase production after 48 h (Fig. 3B). Carbohydrate profile of starch hydrolysis products corresponded to typical fungal α-amylase profiles after 48 h fermentation with T and AT inducers (Fig. 3C).[14]

Starch and its hydrolysis products are well known inducers of α-amylase.[10,11] This was also shown in the presented results obtained using starch as the carbon source (control medium) and, particularly, the triticale extract in the fermentation. The best-known and most studied inducers of α-amylase in *Aspergillus* sp. are maltose and isomaltose, arising from maltose during fermentations.[7,9,11] Moreover, the obtained results confirmed that maltose is a good inducer for α-amylase in *A. oryzae*, using maltose and AT, which contained a high amount of maltose (Fig. 3C line AT).

lead to increase in α-amylase production after 48 h, of which the triticale extract was the most effective, Fig. 3B. The TLC analysis (Fig. 3C) showed that the T extract contained a spectrum of carbohydrates from C1 to C5, responsible

for the highest level of α-amylase production. Both types of triticale extracts induced as many as five α-amylase isoforms, Fig. 3D. Major α-amylase isoform (α-A2) was presented in all tested samples. However, only α-A1 and α-A2 were present when *A. oryzae* was cultivated on starch. This further favors the usage of the triticale extracts as the α-amylase inducer in *A. oryzae* because the presence of more enzyme isoforms in an enzyme preparation provides easier adaptation to the required industrial conditions of starch hydrolysis.

CONCLUSIONS

The presented results satisfied the aims set out in the Introduction section, *i.e.*, improving amylase production levels and allowing the use of a single fungal strain and a cheap and accessible inducer for the production of specific amylase complexes that might give different product profiles of starch hydrolysis depending on the industrial requirements. This could open a new chapter in triticale utilization. It could be considered as a universal means, as was proven for the two most widely used fungal amylase producer strains. The benefits derived from the consequences of the presented results might be a greater use of triticale, otherwise insufficiently used, as well as higher fungal amylase production.

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И З В О Д

ПОВЕЋАЊЕ ПРОДУКЦИЈЕ АМИЛАЗА СМЕШОМ УГЉЕНИХ ХИДРАТА ИЗ ТРИТИКАЛА КОРИШЋЕЊЕМ *Aspergillus* sp.

БИЉАНА ДОЈНОВ[1], МАРИЦА ГРУЈИЋ[2], БОЈАНА ПЕРЧЕВИЋ[2] и ЗОРАН ВУЈЧИЋ[2]

[1]*Центар за хемију, Институт за хемију, технологију и металургију Универзитета у Београду, Његошева 12, Београд и* [2]*Катедра за биохемију, Хемијски факултет, Универзитет у Београду, Студентски трг 12–16, Београд*

У циљу проналажења одговарајућег лако доступног индуктора гљивичних амилаза у комбинацији са гладовањем, смеша угљених хидрата из тритикала је испитана и упоређена са већ описаним и познатим индукторима. Смеша угљених хидрата из тритикала је код *Aspergillus niger* индуковала продукцију амилазног коктела (α-амилазе и глукоамилазе), за разлику од индукције са добро познатим индукторима који индукују само глукоамилазу, што је показано зимограмом и TLC анализама угљених хидрата смеша пре и после ферментације. Продукција глукоамилазе *A. niger* је била највећа у присуству екстракта добијеног после аутохидролизе скроба из тритикала (95,88 U/mL). Смеша угљених хидрата из тритикала је код *A. oryzae* индуковала продукцију α-амилазе. Значајно више α-амилазних изоформи је детектовано коришћењем комплексних смеша угљених хидрата као индуктора, у поређењу са малтозом или скробом. Индукција у трајању од 48 h је најефикаснија када се користи екстракт тритикала (101,35 U mL^{-1}). Угљени хидрати из екстраката тритикала могу да се користе као веома добри и јефтини индуктори амилазе. Тритикале, житарица која још увек није у потпуности искоришћена, може се узети за разматрање као индуктор у производњи амилаза коришћењем *Aspergillus* sp., и то тако да

се користи као једини супстрат у подлози за ферментације без додатка других нутритивних елемената.

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