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Highly efficient production of *Aspergillus niger* amylase cocktail by solid-state fermentation using triticale grains as a well-balanced substrate

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Abstract: Triticale (x *Triticosecale*, Wittmack), an important industrial crop, with high grain yield, containing high amounts of starch, proteins and also major and minor mineral elements, is not yet sufficiently utilized. The simultaneous production of α-amylase and glucoamylase isoforms by *Aspergillus niger* on triticale grains, without any nutritive supplements, was developed, optimized and scaled up 10 fold for the first time. The specific combination of the examined effects led to the production of a novel glucoamylase isoform. Reduction of particle size, increase in oxygen availability and substrate height lead to an increase of 30 % in the production of amylases. Reduction of the relative humidity from 65 to 30 % increased glucoamylase production 2 fold and α-amylase production by 30%. The peak production of α-amylase (158 U g^{-1}) and glucoamylase (170 U g^{-1}) were obtained in Erlenmeyer flasks and in scaled-up trays. The obtained *A. niger* amylase cocktail was more efficient in raw starch hydrolysis from wheat flour, 29 % more efficient in glucose formation and 10 % more efficient in total reducing sugar formation, than the commercially available amylase cocktail SAN Super 240L, which is widely used in industry.

Keywords: α-amylase; glucoamylase; fungi; enzyme production; solid-state fermentation, raw starch.

INTRODUCTION

Solid-state fermentation (SSF) is widely used for fungal amylase production. It has many advantages over submerged fermentation (SmF), such as lower cost, higher efficiency, reduced catabolic repression, easier maintenance, easier scale-up, *etc.*[1–3] Production of important industrial enzymes requires testing of various types of substrates and optimization of the fermentation conditions.

Natural materials, mostly wheat, are in use as substrates for the production of industrial enzymes and bioethanol, especially in Europe.[4,5] Triticale (x *Triticosecale*, Wittmak) is an important industrial crop that is not yet sufficiently utilized. Cultivation of triticale has many benefits when compared to other crops, such as high biomass and grain yield in a wide range of soils (it is particularly resistant to low pH) and climatic conditions, with yields from 3 to 6 t ha^{-1} depending on the geographic region.[6] It is also cultivated in Serbia and gives high yields,[7] especially in the region near Valjevo. Triticale contains high amounts of starch (about 60 %) and proteins (from 12 to 15 %).[8] It also contains higher amounts of the major mineral elements (K, P and Mg) and nutritionally important minor elements (Na, Mn, Fe, Cu and Zn) than wheat.[9] It could be a good choice as a substrate for fungal amylase production for several reasons: it is cheaper than other crops, not part of the human diet, and the obtained fermentation waste could be used as well balanced animal fodder. The use of triticale as substrate for *A. niger* amylase production, without any additional supplements, is described for the first time herein.

Glucoamylase and α-amylase are likely to be produced concomitantly in fungal fermentations.[10–12] This fact is often ignored and *A. niger* was shown to be a producer of solely glucoamylase.[13–15] In fungal enzyme production, the most efficient combination of the genetic properties of the fungus and substrate composition should be applied under optimal cultivation conditions. It would be far more efficient to use *A. niger* for the simultaneous production of both α-amylase and glucoamylase and to optimize the fermentation process in terms of enhancement of the production of both enzymes. Conventional techniques for raw starch hydrolysis involve two phases: liquefaction and saccharification. The first step (gelatinization and liquefaction) requires a high temperature (over 100 °C) at pH 6.5, as well as bacterial thermostable α-amylases.[16] Fungal α-amylase or glucoamylase are used in the final phase (saccharification) depending on desired products – maltose or glucose.[16] Saccharification requires a temperature of 55 °C at pH 5.5 in order to obtain maltose syrups, or 60 °C at pH 4.5 for glucose syrups.[17] Implementation of different reaction conditions and several types of amylase makes starch hydrolysis difficult and expensive. An amylase cocktail (containing α-amylase and glucoamylase) could be used for starch hydrolysis in only one hydrolysis step, which would reduce the costs and/or time required for complete starch hydrolysis. Significant cost reduction could be achieved by avoidance of pH changes during starch hydrolysis, which are necessary in conventional methods. Cost reduction could also be achieved by saccharification using "*in situ*" produced enzymes, which avoids transportation expense, and which is trending in the development of bioethanol processing nowadays.

The aim of this work was to develop and optimize the simultaneous production of highly efficient *A. niger* α-amylase and glucoamylase using triticale

grains as substrate for SSF without any additional supplements; and to test the efficiency of the obtained enzyme cocktail in raw starch hydrolysis. Moreover, tests were performed with wheat flour as the starting material for raw starch hydrolysis.

EXPERIMENTAL

Reagents

All employed reagents and solvents were of the highest available purity and purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA). Triticale (x *Triticosecale* sp.) "Odisej" line was obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. SAN Super 240L was received from Novozyme as a gift. Raw starch from wheat and wheat flour were produced in "Fidelinka", Subotica, Serbia.

Microorganisms

The *A. niger* ATCC 10864 strain was cultivated on potato dextrose agar (PDA) at 28 °C for 7 days to obtain mature spores. A spore suspension was prepared in 0.1 % Tween 80 solution at a concentration of 5.9×10^5 spores mL^{-1}.

Optimization of SSF

Triticale, of three different particle sizes, was used for SSF. Equal quantities of triticale and water were mixed in a 250-mL Erlenmeyer flask, incubated at 60 °C for 3 h,[18] and autoclaved. The spore suspension was spread on substrates in 1:0.19 ratio, *w*/*V*. SSF was performed at 28 °C for 120 h. The initial external relative humidity (*RH*) was 65 or 30 % during fermentation. The fermentations were terminated at 24, 29, 33, 57, 81, 96, 105 and 120 h.

The influence of particle size on α-amylase and glucoamylase production

Triticale grains were coarsely ground in a Mulinex mill, whereby obtaining mixture of particles with different sizes (30 % 1mm, 28 % 2–3 mm, 18 % 4 mm, 20 % 5–6 mm, 4 % 7 mm) was obtained. Three solid-state supports were each made of 16 g of coarse ground triticale (mixture of particles with different sizes) and 16 g of water. Another set of three solid-state supports was prepared, each mixed with 8 g of coarse ground triticale, 8 g of whole grain triticale and 16 g of water (combined grains). A third set of three solid-state supports was prepared each with 16 g of whole triticale grains (size 8 mm) and 16 g of water. Fermentations were performed in Erlenmeyer flasks (250 mL) closed off with microbiological cotton wool caps at 28 °C, *RH* 30 % for 96 h.

The influence of aeration on α-amylase and glucoamylase production during SSF

Each SSF in these experiments was performed on 16 g of coarse ground triticale grains mixed with 16 g of water. Oxygen availability was varied using Erlenmeyer flasks covered either with microbiological cotton wool caps, or with metal microbiological caps. Both SSFs were realized in duplicate at 28 °C and *RH* 30 %.

The influence of relative humidity (RH) on α-amylase and glucoamylase production during SSF

Each SSF in these experiments was performed on 16 g of coarse ground triticale grains mixed with 16 g of water. The first SSF was realized at an initial external *RH* 30 % and the second was performed at an initial external *RH* of 65 %. The *RH* was measured using a humidity meter. Humidity was kept constant on *RH* 65 % using mist maker immersed in sterile distilled water. Both SSFs were performed in duplicate at 28 °C in Erlenmeyer flasks with metal microbiological caps.

The influence of substrate height on α-amylase and glucoamylase production during SSF

Three different quantities of coarse ground triticale grains (16, 32 and 48 g) were used in SSF to examine the influence of the substrate height on amylase production. Equal quantities of triticale grains and water were mixed in Erlenmeyer flasks of identical size and shape. SSF on different quantities of triticale grains was realized in duplicate at 28 °C and at an initial external RH of 65 %, using 250 mL Erlenmeyer flasks with metal microbiological caps.

Scale up

Coarse ground triticale grains (up to 160 g) were placed in trays (dimensions 20.5 cm×14.0 cm× 6.0 cm) and soaked with equal volume of water (160 mL). The trays were incubated at 60 °C for 3 h,[18] and autoclaved. Spore suspension was spread on the substrates in 1:0.19 ratio, w/V. SSF was performed in duplicate at 28 °C for 120 h. The initial external *RH* was 30 % during fermentation. SSF appearance on the tray is shown in Fig. 1.

Fig. 1.SSF appearance on the tray after 36 h.

Enzyme extraction after SSF

Fermentation was terminated by the addition of a cold buffer solution (50 mM acetate buffer pH 4.5, 0.1 % Tween 80) in a 1:5 ratio (w/V) and homogenized with an Ikaturrax homogenizer in 3 cycles of 15 s. Extraction was performed at room temperature for 1 h. The obtained mixture was centrifuged at 5000 g for 15 min. The crude fermentation extracts were released from small sugars, which could interfere with the assays, by gel filtration on 2 mL Sephadex G25 columns. Acetate buffer pH 5.0 was used for the elution of proteins from the crude fermentation extracts. The eluted fractions were concentrated by ultrafiltration and then used for further analysis.

Amylase activity assay

The amylase activity was assayed at pH 5.0 according to the dinitrosalicylic acid (DNS) procedure[19] using 1.0 % (w/V) soluble starch as substrate at 35 °C for 30 min. Maltose was used as a standard. Each data point represents the mean of three independent assays (standard errors of measurement (*SEM*) were less than 5 % of the means). One unit of *α*-amylase activity, U mL^{-1}, was defined as the amount of enzyme required to produce 1 µmol of maltose in 1 min at 35 °C. The specific amylase activity, U g^{-1}, was calculated as the amylase units per 1 g of dry substrate in SSF (triticale).

Glucoamylase activity assay

The glucoamylase activity was assayed at pH 5.0 using 1.0 % (w/V) soluble starch as substrate at 35°C for 30 min. Glucose (the final product of the reaction) was detected in the reaction mixture by coupled reaction with glucose oxidase and horseradish peroxidase (HRPO, Trinder reagent, Pointe Scientific, USA). Samples (10 µL) were mixed with Trinder reagents (1 mL),[20] incubated for 30 min and the absorbance was measured at 520 nm, according to the manufacture's instructions. Each data point represents the mean of three

independent assays (the *SEM* values were less than 5 % of the means). One unit of glucoamylase activity, U mL^{-1}, was defined as the amount of enzyme required to produce 1 μmol of glucose in 1 min at 35 °C. The specific glucoamylase activity, U g^{-1}, was calculated as the amylase units per 1 g of dry substrate in the SSF (triticale).

Zymographic detection of α-amylase and glucoamylase

α-Amylase and glucoamylase were detected simultaneously on a zymogram.[21] After electrophoresis (EF) separation of the crude fermentation extracts on native 10 % polyacrylamide (PAA, 30 % T, 2.7 % C) gel, the gel was printed simultaneously on a PAA gel with copolymerized *β*-limit dextrin (0.8 %) and on a nitrocellulose membrane (NC). *α*-Amylase was detected in the PAA gel with copolymerized *β*-limit dextrins after staining with iodine solution. The *α*-amylase activity appeared as clear bands on a purple background. In the native EF PAA gel after printing, both amylases were detected as clear bands on blue background using soluble starch as the substrate, and iodine solution for staining. Glucoamylases were detected on NC membrane using starch and a reaction mixture for glucose detection (Trinder's reagent and 4-Cl-1-naphthol as substrate). The NC was incubated in buffered substrate solution (1.0 % (*w*/*V*) starch, 50 mM acetate pH 5.0, 2.0 mM NaCl and 0.1 mM CaCl$_2$) and reaction mixture for glucose detection in a 9:1 ratio, after 30 min at 30 °C. A specific reaction leading to an insoluble purple product occurred on the NC membrane in the bands corresponding to glucoamylase. Positions and relative activities (representations) of the obtained bands were quantified using a GelAnalyzer 2010.

Hydrolysis of raw starch by the amylase cocktail

Raw starch from wheat and wheat flour (per 30 mL of 2 % mixtures in acetate buffer, pH 5.0) were soaked at 65 °C. Thereafter, equal quantities (in enzyme units, 106.3 U mL^{-1} reaction mixtures) of commercial glucoamylase product (SAN Super 240L) or *A. niger* amylase cocktail were added to both substrates. Hydrolysis was performed at 50 °C overnight with agitation. Starch mixtures were analyzed for presence of starch, dextrins, reducing sugars and glucose prior to and after hydrolysis. The concentrations of starch and dextrin were determined by the iodine dextrin color method (IDC method), measuring the absorbance at 590 nm and 540 nm, respectively.[22] Reducing sugars were determined by the DNS method, while the glucose concentration was measured using Trinder's reagents.

RESULTS AND DISCUSSION

Optimization of SSF

α-Amylase and glucoamylase activity is expressed in U/g of dry substrate that could be compared to each other in optimization processes. Each point on the graphs is a mean of the enzyme activities obtained from two independent fermentations ± *SEM*.

Use of triticale as substrate for amylase production in SSF was justified, since it resulted in peak amylase concentrations higher than amylase concentrations obtained by SSF on wheat flour, without additional supplements.[23] Moreover, it is in range of the amylase produced on rice waste materials without additional supplements.[24] These results indicate that triticale could be taken into consideration when choosing a substrate for amylase production. Triticale is an

accessible, cheap natural substrate, and the remaining biomass could be used as fodder. Simultaneously, SSF is an environmental friendly method.

The influence of particle size on α-amylase and glucoamylase production. Production levels of α-amylase and glucoamylase by *A. niger* on three forms (sizes) of triticale grains, *i.e.*, coarsely ground grains (particles of different sizes: 30 % 1 mm, 28 % 2–3 mm, 18 % 4 mm, 20 % 5–6 mm, 4 % 7 mm, combined grains – 50 % of whole grains (8 mm) and 50 % of coarsely ground grains, and whole grains (8 mm), are shown in Fig. 2. The highest α-amylase and glucoamylase activities were obtained on coarsely ground grains, 133.02 and 91.71 U g^{-1}, respectively. The activities were 30 % higher than those obtained on whole triticale grains, Fig. 2.
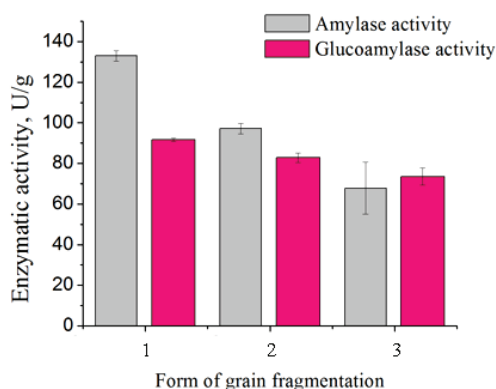

Fig. 2. Impact of particle size on amylase production by SSF using 16 g, in 96 h, RH 30 % in Erlenmeyer flasks with cotton cups. 1 – Coarse ground triticale, 2 – combined grains and 3 – whole grains.

Particle size is an important factor affecting enzyme production by SSF and it should optimized.[25] Coarse ground triticale grains were of the most suitable particle size because they ensure better accessibility of fungi to the substrate inside the particles since the height of the penetrating hyphae layer is lower and, consequently, the restrictions for the diffusion of nutrients and products are reduced. Moreover, smaller particle sizes involve higher specific surface areas and, consequently, larger areas of external mycelium with better oxygenation. These were previously shown to be a critical factor affecting enzyme production.[25,26] On the other hand, larger particles (whole grains) provide a limited surface for *A. niger* attack, which is known to be a limiting factor for enzyme production in SSF.[27] Smaller particles of triticale – bran, give homogenous and impervious substrate for fungal SSF because low particle size involve particle aggregations that reduce the interparticular space and thus, the volume and removal of the air phase; therefore they have not been tested here. The use of coarse ground triticale grains also increased the availability of starch and starch hydrolysis products (dextrin chains and maltose) formed during incubation of triticale at 60 °C for 3 h.[18] This further increases the production of amylase cocktail. It is

known that maltose and even some dextrins are major inducers of amylase production in *A. niger*.[28–30]

The influence of aeration on α-amylase and glucoamylase production during SSF. The use of cotton wool microbiological caps (higher oxygen) resulted in better fungal growth and higher production of α-amylase and glucoamylase than the use of metal microbiological caps (lower oxygen), Fig. 3. Sporulation of *A. niger* was delayed when SSF was performed in an Erlenmeyer with a metal microbiological cap.
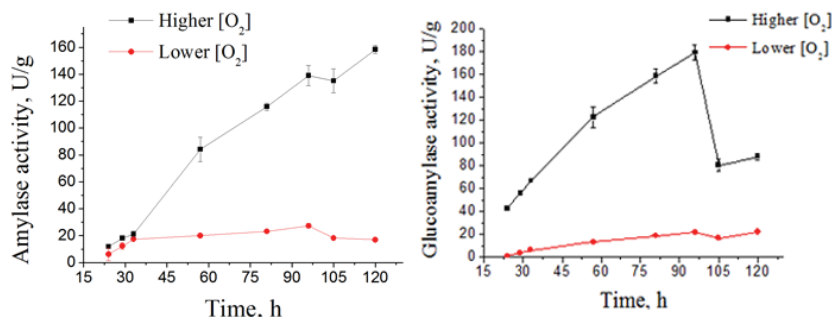

Fig. 3. Impact of aeration on amylase production in SSF with cotton wool caps and metal caps on 16 g of coarse grounded triticale, on *RH* 30 %.

Use of cotton wool microbiological caps results in better fungal growth and higher production of α-amylase and glucoamylase than the use of metal micro-biological caps. Prolonged production of α-amylase to the highest level in SSF under higher oxygen availability compared with the decreased production in SSF under lower oxygen availability seems to be more coherent with the fact that more aerated conditions generally accelerate the culture of aerobic microorg-anisms. The use of cotton wool caps provides for better aeration, higher oxygen consumption and CO_2 evaporation, thereby facilitating enhanced removal of metabolic heat. All these factors are important for the process of enzyme pro-duction in SSF.[3] Fungal growth and amylase production under low oxygen con-centrations were also reduced in *A. oryzae*.[26] Sporulation of *A. niger* was delayed when SSF was performed in an Erlenmeyer flask with a metal microbiological cap, maybe due to the slower evaporation of water under these terms.

As part of further investigations of the influence of individual factors on the production of amylases, metal microbiological caps were nevertheless used because the results showed that the *SEM* was lower than the *SEM* obtained when cotton wool microbiological caps were used.

Influence of humidity on α-amylase and glucoamylase production during SSF. *A. niger* production of α-amylase and glucoamylase during SSF on 16 g of coarse ground triticale grains at *RH* 65 % and *RH* 30 % was compared, Fig. 4.

The production of both α-amylase and glucoamylase was higher at *RH* 30 % but neither was in correlation with *A. niger* growth. SSF under conditions of high *RH* is followed by extensive biomass growth but lower production of amylases. The sporulation of *A. niger* was accelerated under conditions of lower *RH*, which could be associated with higher production of both enzymes.
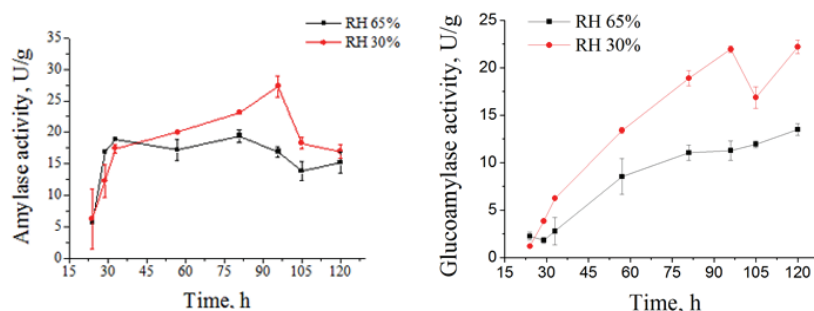

Fig. 4. Impact of RH amylase production in SSF at *RH* 30 % and at *RH* 65 % on 16 g of coarse ground triticale in Erlenmeyer flasks with metal cups.

Fungal SSF can occur in the *RH* range from 20 to 80 % with the optimal *RH* depending on the type of substrate and the microorganism.[25] Maintenance of the optimal *RH* is important because it determines the water activity, which decreases during the fermentation part of the hydrolytic process in substrate and also because of increasing temperature in the system.[25] Reduction of bed porosity causes slower transport of oxygen at high *RH* values. Additionally, a low water content in SSF increases the concentration of nutritive elements in the substrate,[28] which could have contributed to higher production of *A. niger* amylases during SSF at the low RH value. The obtained results were not in correlation with results previously published on *A. niger*.[31] However, it is difficult to observe the impact of *RH* independent of other factors affecting enzyme production in SSF.[32] Acceleration of sporulation at the lower *RH* was due to a higher dryness of the substrate, which implies a higher oxygenation of the mycelium that would explain the better amylase production.

The influence of substrate heights on α-amylase and glucoamylase production during SSF. The impact of the substrate height was examined by using different quantities of coarse ground triticale grains, Fig. 5. Fungal growth was enhanced on higher quantities of substrate. The highest α-amylase activity was detected on 32 g of triticale grains (medium substrate height) during SSF, Fig. 5. Glucoamylase production was in correlation with fungal growth and was the highest on 48 g of triticale grains (highest substrate height), Fig. 5.

A higher substrate height increases the total surface area that may affect the slower evaporation of water from the lower layers of the substrate, thus main-

taining a better water activity, which would enhance fungal growth and amylases production. This may explain the higher amylase production when *A. niger* was cultivated on higher substrate heights.
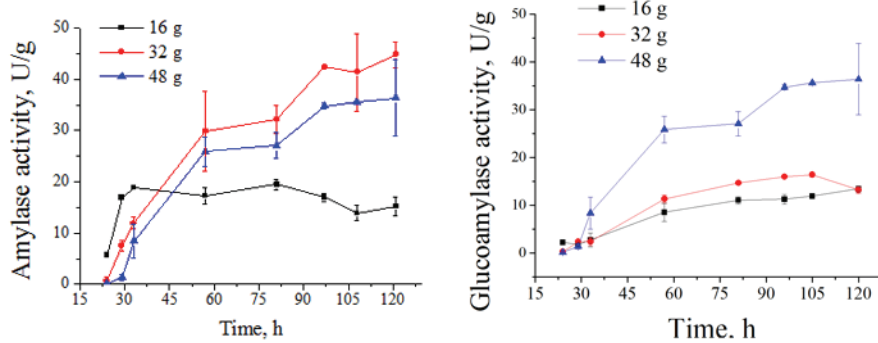

Fig. 5. Impact of substrate height on amylase production in SSF on 16, 32 and 48 g of coarse ground triticale grains in Erlenmeyer flasks with metal cups on *RH* 65 %.

Production of α-amylase and glucoamylase isoforms during SSF under different conditions

Great differences in the production of amylase isoforms after 57 h were observed under different fermentation conditions of SSF. Therefore, the extracts were analyzed by simultaneous zymographic detection of *α*-amylase and glucoamylase, Figs. 6–8, and the obtained results were further quantified using a Gel-Analyzer 2010, Table I.

Using 16 g of coarse ground triticale grains and 16 g of water as substrate, and microbiological cotton wool caps for the Erlenmeyer flasks with *RH* 30 % during SSF ensured the best fermentation conditions for the production of an amylase cocktail according to the zymogram and the relative activity determined
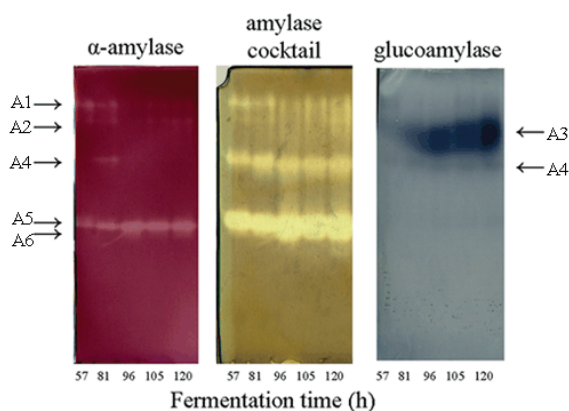

Fig. 6. Zymographic detection of amylase cocktail during different SSF conditions in 57, 81, 96, 105 and 120 h on 16 g of triticale grains, *RH* 30 % and higher aeration. Arrows indicate the positions of α-amylase (left) and glucoamylase (right) isoforms, labeled with A1–A6 (amylase isoform).
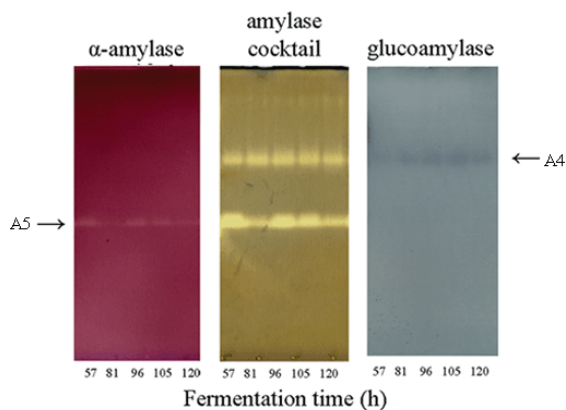
Fig. 7. Zymographic detection of amylase cocktail during different SSF conditions in 57, 81, 96, 105 and 120 h on 16 g of triticale grains, *RH* 65 % and lower aeration. Arrows indicate the positions of α-amylase (left) and glucoamylase (right) isoforms, labeled with A1-A6 (amylase isoform).
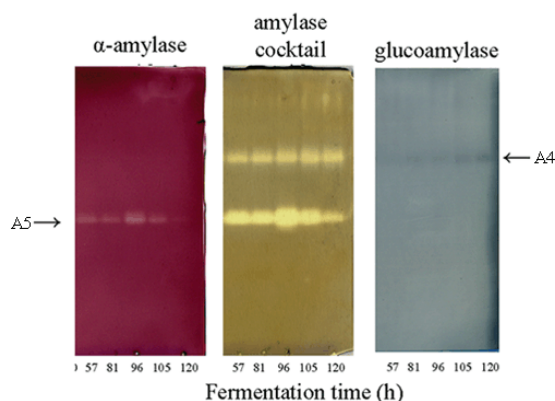

Fig. 8. Zymographic detection of amylase cocktail during different SSF conditions in 57, 81, 96, 105 and 120 h on 48 g of triticale grains, *RH* 65 % and lower oxygen availability. Arrows indicate the positions of α-amylase (left) and glucoamylase (right) isoforms, labeled with A1-A6 (amylase isoform).

by the GelAnalyzer, Figs. 6–8 and Table I. The highest numbers of α-amylase (5) and glucoamylase (2) isoforms were detected when performing SSF under these conditions, as can be seen on the specific zymograms (for α-amylase and gluco-amylase) and on the zymogram for both amylases. A novel glucoamylase isoform A3, detected only under the SSF conditions described above, favors the use of more aerated conditions, Figs. 6–8. In addition, under these conditions *A. niger* produced the highest number of α-amylase isoforms (up to 4) – A1, A2, A5 and A6, while in the other fermentations, only one major isoform A5 could be found. The intensity – relative activity of the individual α-amylase isoforms differed during the fermentation, Fig. 6 and Table I, column a. The maximal activity of α-amyl-ase was detected at 120 h by the A5 isoform on 16 g of triticale, *RH* 30 % and higher oxygen availability (Table I, column a), and also at 96 h by the A5 iso-form under two other fermentation conditions, *i.e.*, with 16 g and with 48 g of triticale, *RH* 65 % and lower oxygen availability (Table I, columns a and b). Under higher oxygen availability (Table I column a), there were no significant differences between the α-amylase production at 96 h (98.81 %) and at 120 h

(100 %), while the production was significantly smaller after 96 h of fermentation under lower oxygen availability (Table I, columns a and b). The maximal activity of glucoamylase was detected at 120 h of fermentation under all compared conditions, but the isoforms were different, Table I.

TABLE I. The relative activity of individual amylases isoforms (%) in amylase cocktails during SSF under different conditions. The most intense isoform per zymogram of the individual enzyme (in each column) is labeled as 100 % activity. Positions of every individual number in table are in correlation with Rf values obtained in the zymogram shown in Figs. 6–8. a) 16 g of triticale grains, RH 30 % and higher oxygen availability. b) 16 g of triticale grains, RH 65 % and lower oxygen availability. c) 48 g of triticale grains, RH 65 % and lower oxygen availability. A1–A6 - amylase isoforms according to the zymographic detection in Figs. 6–8. 1 – α-amylase, 2 – amylase cocktail, 3 – glucoamylase

Fermentation time, h	Amylase isoform	a)			b)			c)		
		1	2	3	1	2	3	1	2	3
57	A1	39.75	11.15	–	–	–	–	–	–	–
	A2	2.62	1.24	–	–	–	–	–	–	–
	A3	–	–	–	–	–	–	–	–	–
	A4	–	28.41	–	–	26.03	18.18	–	50.90	19.85
	A5	42.21	99.83	–	48.14	64.39	–	49.62	96.66	–
	A6	–	–	–	–	–	–	–	–	–
81	A1	10.10	3.56	–	–	–	–	–	–	–
	A2	1.91	0.40	27.89	–	–	–	–	–	–
	A3	–	–	–	–	–	–	–	–	–
	A4	3.66	53.49	0.55	–	38.03	94.32	–	60.23	43.13
	A5	17.17	100.00	–	37.84	64.76	–	21.67	100.00	–
	A6	–	–	–	–	–	–	–	–	–
96	A1	–	–	–	–	–	–	–	–	–
	A2	–	11.58	37.79	–	–	–	–	–	–
	A3	–	–	–	–	–	–	–	–	–
	A4	–	42.11	–	–	44.67	60.23	–	46.51	32.06
	A5	98.81	16.25	–	100.00	100.00	–	100.00	93.41	–
	A6	–	2.89	–	–	–	–	–	–	–
105	A1	–	–	–	–	–	–	–	–	–
	A2	–	–	–	–	–	–	–	–	–
	A3	–	11.11	77.27	–	–	–	–	–	–
	A4	–	43.79	0.75	–	42.49	54.55	–	43.31	61.45
	A5	87.84	7.86	–	48.65	70.52	–	48.33	89.17	–
	A6	–	6.98	–	–	–	–	–	–	–
120	A1	–	4.43	–	–	1.12	–	–	12.08	–
	A2	–	1.98	–	–	–	–	–	–	–
	A3	–	53.19	100.00	–	–	–	–	–	–
	A4	–	5.51	0.17	–	42.42	100.00	–	67.91	100.00
	A5	100.00	10.88	–	7.43	38.36	–	8.33	62.48	–
	A6	–	1.07	–	–	–	–	–	–	–

From the results of specific zymographic techniques, obtained for optimization of amylase cocktail production, it could be concluded that particle size, initial external *RH* and oxygen availability have a highly influential impact on α-amylase and glucoamylase production by *A. niger* in SSF using triticale as the substrate. The intensity of the major α-amylase isoform (A5) was contrary to the intensity of the major glucoamylase isoform (A4) on all zymograms.

Amylase cocktail production during SSF under selected conditions

The level of production of both amylases was analyzed each hour by spectrophotometric assays and by zymography during SSF under selected conditions, *i.e.*, on 16 g of triticale grains, at *RH* 30 % and with high oxygen availability, Figs. 9 and 10. Curves representing the production of the two amylases were parallel, while glucoamylase activity was higher, Fig. 8A. Peak production of glucoamylase was achieved at 96 h of SSF (170 U g^{-1}). After 96 h, the production of glucoamylase started to decrease, while the production of α-amylase continues to rise up at 120 h (158 U g^{-1}).
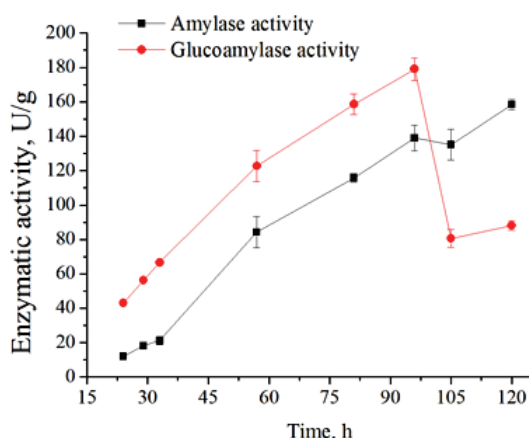

Fig. 9. α-Amylase and glucoamylase activity after SSF with 16 g of triticale grains, at *RH* 30 % and with high aeration.

A. niger started to produce the major α-amylase isoform (A5) during the first days of fermentation (24, 29 and 33 h). The production α-amylase isoforms with lower mobility (A1 and A2) commenced at 57 h at the same time when production of the major α-amylase (A5) started to decrease, Fig. 10. α-Amylase production increased from 96 h to 120 h, as shown by enzymatic assay and zymography, Fig. 10. *A. niger* started to produce glucoamylase (A3 and A4) at 81 h of fermentation, Fig. 10. Selected conditions enable obtaining amylase cocktail with maximal activity of both amylases in 120 h of fermentation, which could test for raw starch hydrolysis.
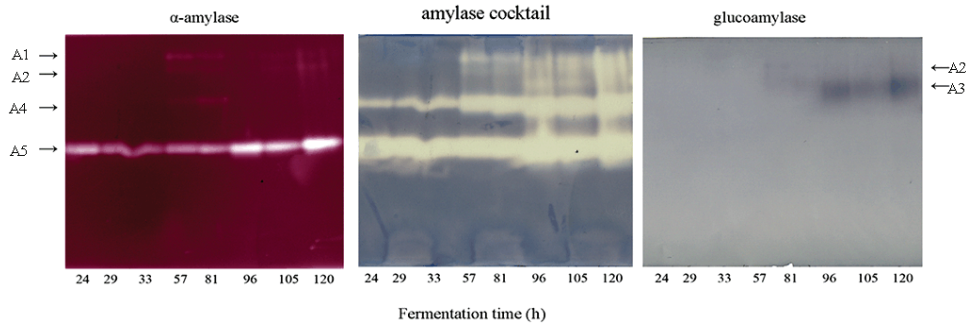
Fig. 10. Zymographic detection of α-amylase and glucoamylase after SSF with 16 g of triticale grains, at *RH* 30 % and with high oxygen availability. Arrows indicate the positions of α-amylase (right) and glucoamylase (left) isoforms labeled with A1-A6 (amylase isoform).

α-Amylase and glucoamylase occur together in many fermentation processes and it has been shown that they have a one-gene precursor,[12,13] but here for the first time, it was demonstrated that they could be co-produced simultaneously in one SSF. This new approach to the fermentation process facilitates peak production of both enzymes or specific *α*-amylase or glucoamylase isoforms, and in addition enables modeling of the process to obtain a specific amylase cocktail, depending on the industry requirements.

Scale-up of amylase cocktail production

When the selected conditions for SSF were employed in large-scale production, very similar levels of *α*-amylase and glucoamylase, calculated on dry substrate, were obtained. The obtained crude extract (769 mL) contained *α*-amylase 31.02 U mL^{-1}, *i.e.*, 149.1 U g^{-1} of dry substrate (coarse ground triticale grains) and glucoamylase 18.14 U mL^{-1}, *i.e.*, 87.2 U g^{-1} of dry substrate (coarse ground triticale grains).

Scaling up of this SSF enabled larger amount of amylase cocktail to be produced. The very similar levels of amylase cocktails (expressed in U g^{-1} of triticale) obtained in the scale-up experiments further confirmed the employment of the selected SSF conditions and justified the usage of triticale as a favorable substrate for the production of amylase cocktails.

Hydrolysis of raw starch by the amylase cocktails

The *A. niger* amylase cocktail was more efficient than a commercial enzyme product in hydrolysis of wheat flour, *i.e.*, it was 29 % more efficient in glucose formation and 10 % more efficient in the formation of total reducing sugars, Table II, a). The hydrolysis of starch isolated from wheat was similar between the *A. niger* amylase cocktail and the commercial cocktail, Table II, b). In terms of the formation of glucose, SAN Super 240L was more efficient by 8 %, while

in the formation of total reducing sugars, the *A. niger* amylase cocktail was more efficient by 5 %.

TABLE II. Comparison of the hydrolysis of raw starch and wheat by *A. niger* amylase cocktail and by a commercial enzyme product. a) Soaked wheat flour as substrate for hydrolysis; b) raw soaked starch from wheat as substrate for hydrolysis

State	Starch mg mL^{-1}	Dextrin mg mL^{-1}	Reducing sugars mM	Glucose mM
a) Wheat flour				
Prior to hydrolysis	1.64	9.22	5.37	11.04
After hydrolysis with SAN Super 240L, Novozyme	0.42	0.00	90.43	146.18
After hydrolysis with the *A. niger* amylase cocktail obtained in this work	0.45	0.00	100.28	206.12
b) Raw soaked wheat starch				
Prior to hydrolysis	1.59	8.51	0.00	11.04
After hydrolysis with SAN Super 240L, Novozyme	0.01	0.00	94.46	231.36
After hydrolysis with the *A. niger* amylase cocktail obtained in this work	0.01	0.00	99.38	213.48

Yields of the hydrolysis considering the amount of starch to be hydrolyzed, when wheat flour was the substrate were 74.39 % with the commercial cocktail and 72.56 % with the *A. niger* amylase cocktail obtained in this work. Hydrolysis of raw starch was more efficient, 99.37 % with both amylase cocktails (commercial and the present cocktail).

Hydrolysis of raw starch by amylase enzymes from *A. niger* was the subject of several papers and it showed high efficiency.[12,33,34] In addition, amylase extracts obtained by SSF from *Aspergillus sp.* were previously used for raw starch hydrolysis with wheat flour as the initial substrate.[23,35] The results mentioned above are not mutually comparable or comparable with the results obtained in this study because of individual calculations of efficiency and of the degrees of hydrolysis. In addition, the efficiencies were not compared to those of commercially available enzymes. Almost complete hydrolysis of raw starch (99.37 %) was attained by the *A. niger* amylase cocktail produced in this work when the yield of the hydrolysis was calculated based on the amount of available starch. It was the same result comparing with the commercial amylase cocktail and was much better compared with other *Aspergillus* amylase cocktails (69 % to 86 % depending on starch origin).[33]

CONCLUSIONS

Triticale grains without the addition of any nutritive components could be used as the sole substrate for fungal production of important enzyme complexes under SSF conditions that could be industrially applicable. Based on triticale

properties as a nutritive rich substrate, competitive and superior amylase cocktails were obtained. The demonstrated higher efficiency of the obtained *A. niger* amylase cocktail in wheat flour hydrolysis indicates a possibility for implementation in starch industries. The use of the obtained amylase cocktail could unify the phases in the starch hydrolysis process, which would significantly reduce the costs of starch hydrolysis.

ИЗВОД

ЕФИКАСНА ПРОИЗВОДЊА АМИЛАЗНОГ КОКТЕЛА ИЗ *Aspergillus niger* МЕТОДОМ ФЕРМЕНТАЦИЈЕ НА ЧВРСТОЈ ПОДЛОЗИ КОРИСТЕЋИ ТРИТИКАЛЕ КАО ДОБРО ИЗБАЛАНСИРАНИ ХРАНЉИВИ СУПСТРАТ

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Тритикале (X *Triticosecale*, Wittmack) је важана индустријска житарица, високог приноса, и садржи велике количине скроба, протеина и макро- и микроелемената, а која још увек није довољно искоришћена. Метода истовремене производње α-амилазних и глукоамилазних изоформи из *Aspergillus niger*, користећи само зрна тритикала као подлогу без додатака других нутритивних елемената, развијена је, оптимизована и подигнута на већу скалу (10 пута) по први пут у овом раду. Специфична комбинација испитиваних фактора ферментације је довела до производње потпуно нове глукоамилазне изоформе. Смањење величине честица, повећање доступности кисеоника и висине стуба супстрата су довели до повећања производње амилазе до 30 %. Смањење релативне влажности са 65 на 30 % је повећало производњу глукоамилазе 2 пута и α-амилазе за 30 %. Максимална производња α-амилазе је била 158 U g^{-1}, а глукоамилазе 170 U g^{-1}, што је потврђено и на мањој и на већој скали. Добијени амилазни коктел из *A. niger* је био ефикаснији у хидролизи сировог скроба из пшеничног брашна од комерцијално доступног амилазног коктела, SAN Super 240L, који се најчешће користи у индустрији, и то 29 % ефикаснији у добијању глукозе и 10 % ефикаснији у добијању укупних редукујућих шећера.

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REFERENCES

1. M. P. Nandakumar, M. S. Thakur, K. S. M. S. Raghavarao, N. P. Ghildyal, *Lett. Appl. Microbiol.* **29** (1999) 380
2. S. R. Couto, M. A. Sanroman, *J. Food Eng.* **76** (2006) 291
3. A. Pandey, C. R. Soccol, C. Larroche, *Current developments in solid state fermentations.* Springer, New Delhi, 2008
4. M. Hoogschagen, Y. Zhu, H. van As, J. Tramper, A. Rinzema, *Biotechnol. Lett.* **23** (2001) 1183

5. R. te Biesebeke, G. Ruijter, Y. S. P. Rahardjo, M. J. Hoogschagen, M. Heerikhuisen, A. Levin, K. G. A. van Driel, M. A. I. Schutyser, J. Dijksterhuis, Y. Zhu, F. J. Weber, W. M. de Vos, K. A. M. J. J. van den Hondel, A. Rinzema, P. J. Punt, *FEMS Yeast Res.* **2** (2002) 245

6. D. F. Salmon, M. Mergoum, H. Gómez Macpherson, in *Triticale improvement and production*, M. Mergoum, H. Gomez-Macpherson, Eds., FAO, Rome, 2004, p. 27

7. A. Pejić, *Agrosvet* **17** (2007) 6 (in Serbian)

8. R. J. Pena, *Food uses of triticale*, in *Triticale improvement and production*, M. Mergoum, H. Gomez-Macpherson, Eds. FAO, Rome, 2004, p. 37

9. K. Lorenz, F. W. Reuter, C. Sizer, *Cereal Chem.* **51** (1974) 376

10. H. Gasdorf, P. Atthasampunna, V. Dan, D. Hensley, K. Smiley, *Carbohydr. Res.* **42** (1975) 147

11. A. Yuhki, T. Watanabe, K. Matsuda, *Starch* **29** (1977) 265

12. A. K. Dubey, C. Suresh, R. Kavitha, N. G. Karanth, S. Umesh-Kumar, *FEBS Lett.* **471** (2000) 251

13. P. Selvakumar, L. Ashakumary, A. Pandey, *Bioresour. Technol.* **65** (1998) 83

14. H. Pedersen, M. Beyer, J. Nielsen, *Appl. Microbiol. Biot.* **53** (2000) 272

15. Q. H. Wang, X. Q. Wang, X. M. Wang, H. Z. Ma, *Process Biochem. (Oxford, U.K.)* **43** (2008) 280

16. M. J. E. C. van der Maarel, B. van der Veen, J. C. M. Uitdehaag, H. Leemhuis, L. Dijkhuizen, *J. Biotechnol.* **94** (2002) 137

17. W. Crabb, D. Mitchinson, *Trends Biotechnol.* **15** (1997) 349

18. D. Pejin, Lj. Mojović, V. Vucurović, J. Pejin, S. Denčić, M. Rakin, *Fuel* **88** (2009) 1625

19. P. Bernfeld, in *Methods in enzymology*, Vol. I, P. De Murray, Ed., Deutcher Acad. Press, San Diego, CA, 1955, p. 149

20. I. Pointe Scientific, *Liquid Glucose (Oxidase) Reagent Set*. Pointe Scientific, Brussels, 2009

21. B. Dojnov, Z. Vujčić, *Anal. Biochem.* **421** (2012) 802

22. D. E. Briggs, *J. Ind. Brew.* **67** (1961) 4

23. R. Wang, L. C. Godoy, S. M. Shaarani, M. Melikoglu, A. Koutinas, C. Webb, *Enzyme Microb. Technol.* **44** (2009) 223

24. H. Anto; U. B. Trivedi; K. C. Patel, *Bioresour. Technol.* **97** (2006), 1161.

25. J. Rodriguez-Leon, C. Soccol, A. Pandey, D. Rodriguez, in *Curent developments in solid state fermentations*, A. Pandey, C. R. Soccol, C. Larroche, Eds., Springer, New Delhi, 2008, p. 26

26. Y. S. P. Rahardjo, J. Tramper, A. Rinzema, *Biotechnol. Adv.* **24** (2006) 161

27. A. Pandey, P. Selvakumar, C. R. Soccol, P. Nigam, *Curr. Sci. India* **77** (1999) 149

28. T. E. Bertolin, W. Schmidell, A. Maiorano, J. Casara, A. V. Z. Costa, *Z. Naturforsch.* **58** (2003) 708

29. R. Gupta, P. Gigras, H. Mohapatra, V. K. Goswami, B. Chauhan, *Process Biochem. (Oxford, U.K.)* **38** (2003) 1599

30. T. Sugimoto, H. Shoji, *Biotechnol. Lett.* **34** (2012) 347

31. A. Sharanappa, K. S. Wani, P. Patil, *Int. J. Adv. Biotechnol. Res.* **2** (2011) 473

32. Z. Hamidi-Esfahani, S. A. Shojaosadati, A. Rinzema, *Biochem. Eng. J.* **21** (2004) 265

33. J. Abe, F. Bergmann, K. Obata, S. Hizukuri, *Appl. Microbiol. Biotechnol.* **27** (1988) 4

34. A. M. Omemu, I. Akpan, M. O. Bankole, O. D. Teniola, *Afr. J. Biotechnol.* **4** (2005) 19

35. M. A. F. Farid, H. M. A. H. Shata, *Iran. J. Biotechnol.* **9** (2011) 267.