Valorization of soybean meal to produce high-protein animal feed and value-added products using a new strain of *Aureobasidium pullulans*

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Abstract

Soybean meal (SBM) is a by-product of soybean oil production. It is a high-quality protein supplement for animal feed. However, it is rich in anti-nutritive factors and indigestible components, among which the special attention is focused on galacto-oligosaccharides, due to the lack of α-galactosidase in monogastric animals. The main goal of this study was to apply fermentation using a selected strain of black yeast-like fungus of the SBM to obtain a high-protein, low-oligosaccharide soy-based product. Screening for an appropriate strain of *Aureobasidium* spp. among natural isolates from grapes has been performed. The highest α-galactosidase activity of 0.89 U cm^{-3} was produced by the strain identified as *A. pullulans* P8. It was applied in SBM submerged (SmF) and solid-state fermentations (SSF). Maximal crude protein yield (~61 % based on dry weight) and the lowest galacto-oligosaccharides content were obtained after 3 days of SmF at 30 °C and 10 % of dry matter. SSF produced ~58 % crude protein after 7 days of incubation at 30 °C with substrate containing 30 % of dry matter. Extracellular enzymatic activities of cellulase, pectinase, amylase, xylanase, and α-galactosidase were detected in the supernatant after SmF, indicating its potential for hydrolysis of various lignocellulosic biomass substrates.

Keywords: Black yeast-like fungus; solid-state fermentation; submerged fermentation; α-galactosidase; extracellular hydrolytic enzymes; grape

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1. INTRODUCTION

Soybean (*Glycine max* (L.) Merril) is an important oilseed crop with a global production of 389 million metric tons estimated in 2026 [1]. Soybean meal (SBM) is a co-product of soybean oil production. It is rich in high-quality proteins thus representing a cost-effective and vastly available food source for livestock, poultry, and fish [2-4]. SBM is also rich in carbohydrates, which comprise about 35 to 40 % of its dry matter [5]. Nonstructural soybean carbohydrates are represented by low molecular weight sugars (among which the most abundant is sucrose), oligosaccharides, and starch. Structural polysaccharides include cellulose, pectin, and hemicellulose, together with mannans, galactans, and xyloglucans.

Although highly wholesome, consumption of soybeans is limited by various anti-nutritional factors, including nondigestible oligosaccharides and polysaccharides. The most prevalent oligosaccharide in soybean is stachyose, followed by raffinose, while only a trace amount of verbascose is found [3]. These are galacto-oligosaccharides (also referred to as raffinose family oligosaccharides (RFOs)) sharing the common feature represented by 1, 2 or 3 galactose residues in raffinose, stachyose and verbascose, respectively, linked to a sucrose unit. Terminal galactose moiety is linked by an α-1-6 glycosidic bond that is cleaved by the enzyme α-galactosidase [6]. Due to the lack of α-galactosidase these oligosaccharides are indigestible for monogastric animals. Although they are degraded by intestinal microbiota, flatulence and diarrhea occur. Young animals are especially susceptible to anti-nutritional factors, as their digestive system is not fully developed [7,8]. The effective removal of indigestible RFOs is achieved by adding exogenous α-galactosidase into animal feed [6]. This treatment showed enhanced nutrient digestibility, improved average daily gain, and reduced diarrhea rate in weaned piglets [9].

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The other approach to improve the nutritional quality of food and food ingredients is fermentation, which could be applied as the only treatment, or together with enzymatic hydrolysis [10-13]. Importantly, fermentation eliminates not only RFOs but also other anti-nutrition factors presented in soybeans [14]. Fermented SBM has lower contents of oligosaccharides and higher crude protein (CP) than the unfermented SBM, so that it has been shown as highly nutritious for weanling pigs [15]. However, not all microorganisms can use complex soybean carbohydrates, thus additional treatments are required before soybean can be used as fermentation feedstock [16]. To overcome this issue different microorganisms were applied to obtain double-fermented SBM [4]. Adding double-fermented SBM in broiler food resulted in better feed efficiency, nutrient digestibility, and ultimately higher quality of breast meat. Additional health-promoting effects could be obtained by choosing probiotic microorganisms for fermentation [8]. Nevertheless, fermentation could also exert negative effects, depending on the microorganism used [17]. Therefore, exploring for new adequate strains is of crucial importance.

Yeast-like black fungi are ubiquitous polymorphic microorganisms primarily recognized as pullulan producers [18-20]. Other biotechnological applications include also single cell protein, melanin source, siderophores, biosurfactants, and different industrially important enzymes [21-23]. We hypothesized that microorganisms with such versatile properties could be suitable candidates for fermented SBM production. To test this hypothesis, we have performed screening for an appropriate strain of yeast-like black fungi among natural isolates from grapes. The highest α-galactosidase producer was applied in solid-state (SSF) and submerged fermentation (SmF) of SBM with the final aim of obtaining a high-protein, low-oligosaccharide product. Moreover, the extracellular hydrolytic enzyme potential of the new isolate *Aureobasidium pullulans* P8 has been explored.

2. EXPERIMENTAL

2. 1. Materials

The working microorganism was isolated from grapes. The grapes of the autochthonous varieties were picked at the moment of full ripeness from the organically certified vineyards of the sample estate "Sremski Karlovci", Faculty of Agriculture in Novi Sad, Serbia. SBM was kindly obtained from Bankom doo., Belgrade, Serbia. Microbiological media (malt extract broth, yeast extract, casein hydrolysate, and agar) were purchased from Torlak, Serbia. Raffinose was purchased from Fluka (Germany), carboxymethyl cellulose (CMC), pectin, and xylan were purchased from Sigma-Aldrich (USA), and soluble starch and microcrystalline cellulose (Avicel) were purchased from Merck (Germany). All other chemicals were of analytical grade and purchased from Merck (Germany) and Lach-Ner (Czech Republic).

2. 2. Isolation and characterization of yeast-like black fungi from grapes

Three different organic grapes (*Vitis vinifera* L.) were used for isolation of black yeast-like fungi, two white varieties (Panonija and Morava) and one black (Dionis). A few grapes of each variety were separately soaked in a sterile physiological solution and vigorously vortexed for 5 min. Serial dilutions were made and plated on sterile malt extract agar (MEA) plates. Seeded plates were incubated at 30 °C for 3 days. Afterward, a few colonies were picked from each group, inoculated in malt extract broth (MEB), and incubated at 30 °C at 130 rpm for two days. To ensure that strains are purified they were re-streaked on MEA plates.

2. 2. 1. Quantitative determination of α-galactosidase activity

Erlenmeyer flasks (100 cm^3) containing 30 cm^3 of malt extract broth supplemented with 20 g dm^{-3} raffinose were inoculated with 1.5 cm^3 of black yeast-like fungi isolates fresh cultures and incubated in an orbital shaker (Ikka, Germany) at 30 °C and 130 rpm. After two days, the fermentation medium was centrifuged at 6000*g* for 15 min and α-galactosidase activity was determined in the supernatant by using raffinose as substrate according to the procedure described by Carević *et al.* [24]. Concentration of released galactose was determined by the 3,5-dinitro-salicylic acid (DNS) method [25]. Absorbance was read using a UV/visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Sweden). One unit (U) of α-galactosidase activity was defined as the amount of enzyme that

released 1 μmol of galactose equivalents per minute. The isolate with maximal determined α-galactosidase activity was selected for further experiments.

2. 2. 2. Identification of the selected strain

DNA extraction was carried out from a 4-day-old yeast culture grown in a liquid ISP1 (5.0 g L^{-1} of casen hydrolysate, 3.0 g L^{-1} of yeast extract) medium. Detailed procedure of DNA extraction and molecular identification of yeast by PCR method using ITS1/ITS4 primer pair (ITS1: 5'-TCC GTA GGTGAA CCT GCG G-3', ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'), is described in our previous study [26]. Macrogen double-sequenced amplified PCR products, quality verified and aligned them. The obtained sequences, which represented a partial sequence of the ITS region were deposited in the NCBI GenBank database.

2. 2. 3. Examination of the enzymatic potential of the selected yeast-like black fungi strain

To explore the enzymatic potential of the selected strain, a qualitative test on a selective agar medium was performed as described earlier [26]. The activity of α-galactosidase was detected on MEA with added raffinose by the same procedure.

2. 2. 4. Determination of optimal growth conditions for the selected strain

To determine the optimal temperature for growth, the selected strain of yeast-like black fungus was incubated in MEB at different temperatures in the range of 4 to 37 °C for 48 h. To determine the optimal acidity for growth, the selected strain of yeast-like black fungus was incubated in MEB, whose pH value was previously adjusted in the range from 3 to 10. The number of viable cells was determined for each tested condition on MEA plates after incubation for 48 h at 30 °C

2. 3. Solid-state fermentation of soybean meal

The SBM was weighed in 300 cm^3 Erlenmeyer flasks and wetted with distilled water or ammonium sulfate solution (2.4 mg g^{-1} of SBM dry matter) to obtain a final moisture content of 70 %. After autoclaving (121 °C, 30 min) and cooling, the flasks were inoculated with a selected strain of yeast-like black fungus fresh culture (approximately 10 % of inoculum). Incubation was carried out at 30 °C for 1 to 7 days. For each sampling day, three Erlenmeyer flasks were prepared and analysed. Additional three Erlenmeyer flasks were prepared for control, which underwent all the steps as samples except for inoculation. For the biomass analysis, 1 g of the sample was aseptically transferred to 9 cm^3 saline and vigorously vortexed for 5 min. Afterward, serial dilutions were made and plated on sterile MEA plates. Colonies were counted after 3 days of incubation at 30 °C. The rest of the fermentation medium was analysed for carbohydrate and protein contents. The same procedure was performed with the control samples.

2. 4. Submerged fermentation of soybean meal

For SmF 10 % SBM in water was prepared in Erlenmeyer flasks of 300 cm^3, autoclaved (121 °C, 20 min), and after cooling seeded with a selected strain of yeast-like black fungus fresh culture and incubated at 30 °C and 130 rpm for 3 days. For each sampling day, three Erlenmeyer flasks were prepared and analysed. An additional three Erlenmeyer flasks were prepared for control, which underwent all the steps as samples except for inoculation. For the biomass analysis, an aliquot of 1 cm^3 was aseptically transferred to 9 cm^3 saline and the rest of the fermentation medium was centrifuged for 15 min at 6000*g*. Pellets were subjected to protein and carbohydrate analyses and supernatants were analysed for hydrolytic enzyme activities.

2. 5. Protein analysis

Fermented soybean samples (and unfermented controls) were dried at 45 °C overnight and CP content was determined by the Kjeldahl method [27].

2. 6. Carbohydrate analysis

To analyse the carbohydrate content the dried SBM samples (fermented and unfermented controls) were extracted with 50 cm^3 of 40 % ethanol per gram of dry mass at 40 °C and 120 rpm for 2 h. The procedure was repeated three times and collected extracts were combined and then evaporated using a rotary evaporator (Büchi, Switzerland). The evaporated residue (from 3 g of dry product) was dissolved in 10 cm^3 of HPLC-grade water, and the resulting solution was analysed by using high-performance liquid chromatography (HPLC). Analyses were performed using a Dionex Ultimate 3000 HPLC device (Thermo Scientific, USA) with a Hi-Plex Ca^{2+} column (Agilent, USA) for carbohydrates (300 × 7.7 mm, 8 µm) at 80 °C. HPLC-grade water at a flow rate of 0.6 cm^3 min^{-1} was used as the mobile phase. Product detection was performed using an RI detector RefractoMax 520 (ERC, Germany) set at 40 °C. Data collection and further processing were performed using Chromeleon 7.2. software [28].

2. 7. Determination of hydrolytic enzyme activities

The supernatant obtained after SmF was used as a source of extracellular hydrolytic enzymes, *i.e.* α-galactosidase, amylase, xylanase, pectinase, and cellulase. The enzymatic activities were determined as previously described [24,26].

2. 8. Statistical analysis

All results are presented as mean values of three replicates ± standard deviation. Statistical analysis was performed by Origin Lab software (OriginPro 9.0) [29]. The significance of difference of experimental data was analysed by analysis of variance (ANOVA), followed by Tukey's test, and a confidence level of 95 % was chosen.

3. RESULTS AND DISCUSSION

3. 1. Isolation of yeast-like black fungi from grapes and screening for a high α-galactosidase producer

In this study over 30 microorganisms, natural inhabitants of grapes, have been isolated and preliminary characterized. The isolates were designated as P, M or D, followed by a number, based on the grape variety from which the strain was isolated, *i.e.* Panonija, Morava and Dionis. After first screening based on the morphology of their cells on MEA and microscopic observation (Fig. 1), ten isolates with an appearance typical for *Aureobasidium* spp. [30], were selected for further work.

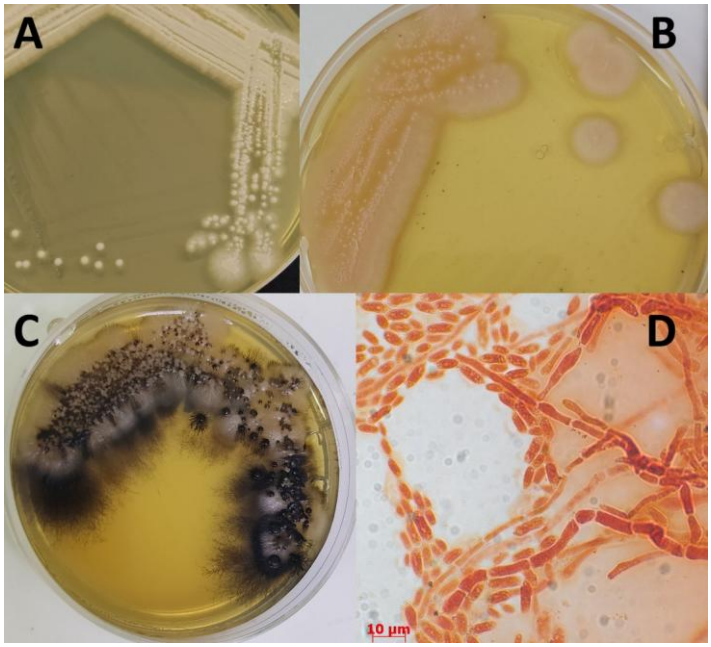

Figure 1. Appearance of black yeast isolates on an MEA plate incubated at 25 °C: strain M12 (A), strain P8 (B), and strain D3 (C), and optical micrograph of P8 cells (D)

The macroscopic morphology of isolates was characterized by pale yellow to pinkish colonies with smooth surfaces and aerial mycelium (Fig. 1a). Individual colonies increased in size over time reaching 5 to 20 mm diameter (Fig. 1b). Over time the colony surface became slimy and turned black due to melanin synthesis (Fig. 1c). Microscopic observation of the cultures from MEA plates revealed typical spherical yeast-like cells as well as conidiogenous cells and blastoconidia (Fig. 1d). These properties indicated that isolated strains belonged to black yeast-like fungi, and they were screened for α-galactosidase activity.

The α-galactosidase activity was detected in all tested strains and was in the range of 0.13 to 0.89 U cm^{-3}, significantly (p <0.05) differing among isolates (data not shown). However, the isolate P8 was selected as the most potent producer for further work.

3. 2. Identification and characterization of the new yeast-like black fungus strain

Based on morphological properties, the strain P8 was classified under the phylum Ascomycota. It was anticipated that the new isolate belongs to the genus *Aureobasidium*. Through the examination of a sequence that contained the internal transcribed spacer region (ITS), the novel strain P8 was specifically identified as *Aureobasidium pullulans* P8. The obtained sequence has been deposited in the NCBI-GenBank database and assigned the following accession number: OR921445.

Utilization of complex polysaccharides prevailing in soybeans is possible only for microorganisms capable of producing enzymes for their degradation. Therefore, the new isolate was cultivated with different polysaccharides as a sole carbon source under qualitative test for enzymatic potential examination. Besides already determined α-galactosidase activity, extracellular activities of amylase, pectinase, cellulase (degradation of CMC and avicel), and xylanase were detected (Fig. 2). Namely, halo zones appeared on selective agar plates around growth regions indicating the area of the substrate hydrolysis [26]. This result was expected since *Aureobasidium* spp. are well recognized for producing versatile enzymes [21]. However, not all strains produced cellulase [31,32], while the others lacked xylanase [33], suggesting that the ability for the desired enzyme synthesis is strain-specific.

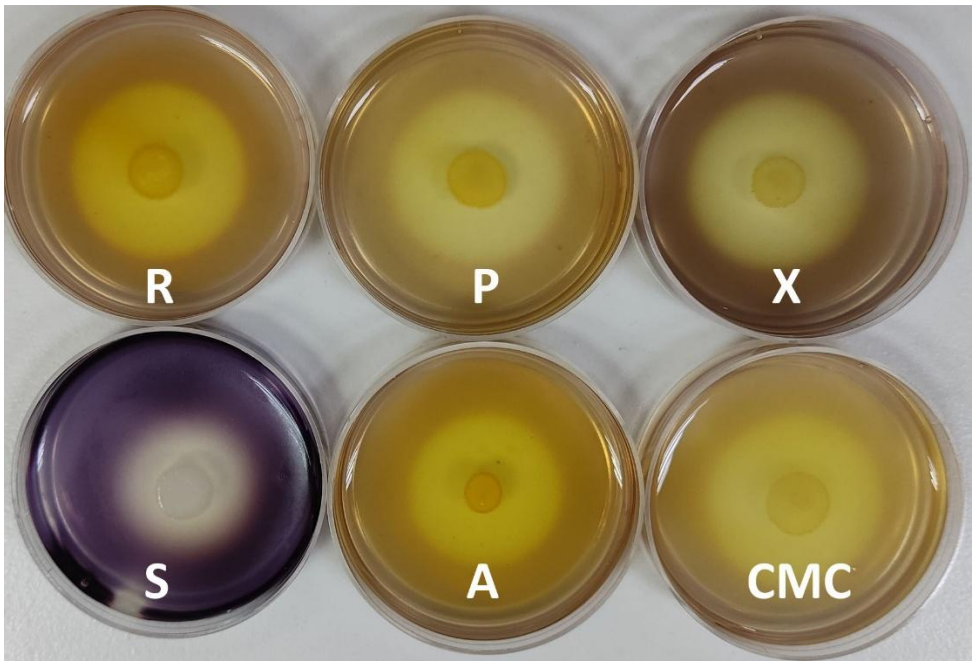

Fig. 2. Qualitative detection of extracellular enzymatic activities (α-galactosidase, pectinase, xylanase, amylase, exoglucanase, and endoglucanase, respectively) of novel strain A. pullulans P8 on selective agar medium plates containing: raffinose (R), pectin (P), xylan (X), starch (S), avicel (A) and carboxymethyl cellulose (CMC), respectively

Testing the growth possibility under different conditions (temperatures in the range of 4 to 37 °C and pH values ranging from 3 to 10) showed that *A. pullulans* P8 can grow under all examined conditions (Fig. 3). However, the

temperature optimum was found to be in the range of 25 to 30 °C. Temperatures lower than the optimal supported growth more than those higher than the optimal ones. These results are in accordance with the literature data reporting mesophilic nature of *Aureobasidium* spp. [23,34]. Considering the medium acidity, neither one of pH values could be chosen as optimal as high growth was observed at all tested pH values. *Aureobasidium* spp. are ubiquitous organisms resistant to various stress conditions [23]. It has been reported that different cell forms are prevalent at specific pH values, enabling its survival. However, growth inhibition was observed for *A. pullulans* Cau 19 at pH 9.5 [32]. From the aspect of technological application, the ability of *A. pullulans* P8 to grow at a wide range of pH values is a very favorable feature, enabling its application for fermentation utilizing versatile substrates.

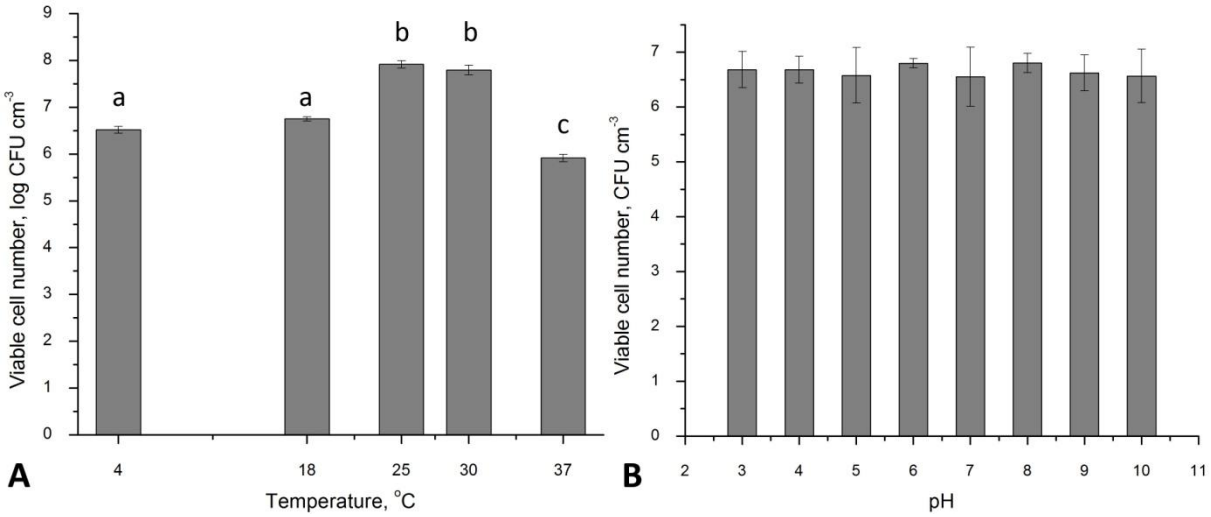

Figure 3. Effects of temperature (A) and pH (B) on A. pullulans P8 growth. The results are presented as mean values of three independent experiments ± standard deviations. Values having the same letters are not significantly different from each other.

3. 3. Fermentation of soybean meal

3. 3. 1 Crude protein content analysis

SBM is an attractive component of animal feed primarily due to its high protein content that can be additionally improved by fermentation. During fermentation microorganisms use carbohydrates and lipids remaining after oil extraction from soybean as carbon sources to enlarge the biomass, thus an increase in the proportion of CP in fermented SBM occurs [4]. In this study, the potential of new isolate *A. pullulans* P8 for high protein SBM production was exploited under two types of fermentation. The CP content had an increasing trend during both SSF and SmF (Tables 1 and 2, respectively). The lowest increment in the CP content was observed after 24 h of SSF when the least viable cells were detected as well. After 72 h of SSF, the microorganism entered the stationary growth phase. However, prolonged fermentation time resulted in higher CP content, finally reaching 57.78±0.49 % based on dry matter (dm) after 168 h. Compared to unfermented control an increase in the CP content by 20.8 % was achieved.

Table 1. Crude protein content and viable cell number in SBM fermented by A. pullulans P8 during SSF

Fermentation time, h	Crude protein content, % dm		Viable cell number, log CFU g^{-1} dm	
	Water	AS	Water	AS
0 (unfermented control)	47.83±0.25		0	0
24	48.94±0.70*	48.48±0.43*	6.92±0.13	6.44±0.31
72	52.42±0.60	48.59±0.48	8.65±0.17	7.58±0.30
120	55.59±0.86	51.39±0.83	8.58±0.32	8.03±0.11
168	57.78±0.49	56.42±0.43	8.69±0.23	7.95±0.27

The results are presented as mean values of three independent experiments ± standard deviations
Water -fermented control; AS - ammonium-sulfate supplemented. *Values within rows that do not differ from each other (p >0.05)

The slow growth rate observed during SSF on SBM may have been caused by low availability of free nitrogen. Namely, nonprotein nitrogen content in SBM is usually less than 1 % [35]. To enhance the metabolic activity of *A. pullulans* P8, SBM was supplemented with ammonium sulfate (AS) as the preferable source of inorganic N among *Aureobasidium* spp. [20,36,37]. However, the addition of AS to SBM significantly slowed down the fungal growth and negatively influenced the CP content (Table 3). This result might have been a consequence of the already low C:N ratio in SBM [16] that was further lowered by the addition of the inorganic N source and ultimately became unfavorable for *A. pullulans* P8 growth. Therefore, the inorganic N source was not added to SBM during SmF.

Table 2. Protein content and viable cell number in SBM fermented by A. pullulans P8 during SmF

Fermentation time, h	Crude protein content, % dm	Viable cell number, log CFU cm^{-3}
0 (unfermented control)	49.02±0.19	0
24	54.98±0.25	6.45±0.20
48	60.03±0.25	8.08±0.71
72	61.11±0.11	8.38±0.53

The results are presented as mean values of three independent experiments ± standard deviations

Contrary to SSF, the increase in the CP content during SmF was accompanied by the fungal growth (Table 2). After 48 h of incubation the CP content reached 60 % (dm basis) and significantly increased up to 61 %, observed after 72 h of incubation. Compared to the unfermented control, an increase in the CP content of 24.7% was achieved.

The crude protein content in fermented SBM obtained in this study was higher than those reported in the literature. For example, SBM with 55.71 % CP (9.8 % enhancement) was obtained by solid-state fermentation using *Bacillus subtilis natto* [38]. Although this value is achieved in a shorter time (62.3 h compared to 168 h in this study) it was obtained after optimization, while optimization has not been performed during our study. In another study the protein content increased by 16.6 % in SBM fermented by *Bacillus subtilis* E20 after 96 h of SSF [2]. When two-stage fermentation of SBM was applied with *Aspergillus oryzae* and *Bacillus subtilis* an increase in the CP content by only 3.7 % was obtained [4], while simultaneous fermentation with *Lactobacillus plantarum*, *B. subtilis*, and *Saccharomyces cerevisiae* raised the CP content by 13.3 % [8].

By comparing the highest protein yields obtained by SSF and SmF using *A. pullulans* P8, it is evident that ~5.8 % more protein is produced in the latter case. Moreover, it was obtained after 3 days of fermentation, while SSF lasted for 7 days. The prolonged time of fermentation required for SSF is probably a consequence of slow aeration and reduced mass transfer that are generally recognized and reported as drawbacks of this type of fermentation [39]. During SmF, water-soluble sugars are readily available, while mixing enables their uniform distribution enhancing microbial growth. However, submerged processes are generally unfavorable for industrial applications since they require more equipment and higher energy consumption than SSF. To make SmF more economically acceptable, but also following circular economy principles, the liquid obtained after centrifugation could be further applied as a source of industrially important enzymes.

3. 3. 2 Carbohydrate content analysis

Besides high protein content, another important goal of the present study was to reduce RFOs content in SBM. Fermented SBM samples with the highest CP contents were analysed for carbohydrate content using HPLC. Comparing chromatograms of the control sample and fermented samples (solid-state), two main features could be observed (Fig. 4a). Peaks corresponding to sucrose, raffinose, and stachyose have decreased, while peaks of galactose and higher oligosaccharides have increased. That indicated the activity of α-galactosidase. Namely, during its growth, *A. pullulans* P8 synthesized α-galactosidase that cleaved terminal galactose residues from galacto-oligosaccharides and galactomananas present in SBM. Raffinose was formed as a product of stachyose hydrolysis. It was further hydrolysed to sucrose and galactose that were consumed by microorganism, supporting its growth. However, more sugars were produced than consumed. On the other hand, released galactose served also as a substrate for α-galactosidase in transglycosylation reaction [6] that increased the content of higher oligosaccharides, compared to the control. Moreover, differences among samples were detected as well. More stachyose and raffinose were noticed in the sample

obtained after 7 days of SSF when AS was added to SBM, than in SBM wetted with water. There was a negligible difference in galactose contents among these two samples, while the content of higher oligosaccharides was lower in AS-supplemented SBM, implying that the addition of AS had a negative effect on α-galactosidase production and/or activity. This result corresponds well with already discussed results obtained in this study *i.e.,* the protein content was significantly lower, and weaker growth was observed in samples with AS. Finally, analysis of the chromatogram of the sample obtained after 3 days of submerged fermentation (Fig. 4b) shows the absence of peaks corresponding to raffinose and stachyose. Also, the galactose content was lower than that in the control, while the content of higher oligosaccharides was the same as in the control. According to results obtained by the HPLC analysis, it could be concluded that SBM obtained after 3 days of submerged fermentation has the lowest content of indigestible carbohydrates, which makes it the most suitable for animal feed.

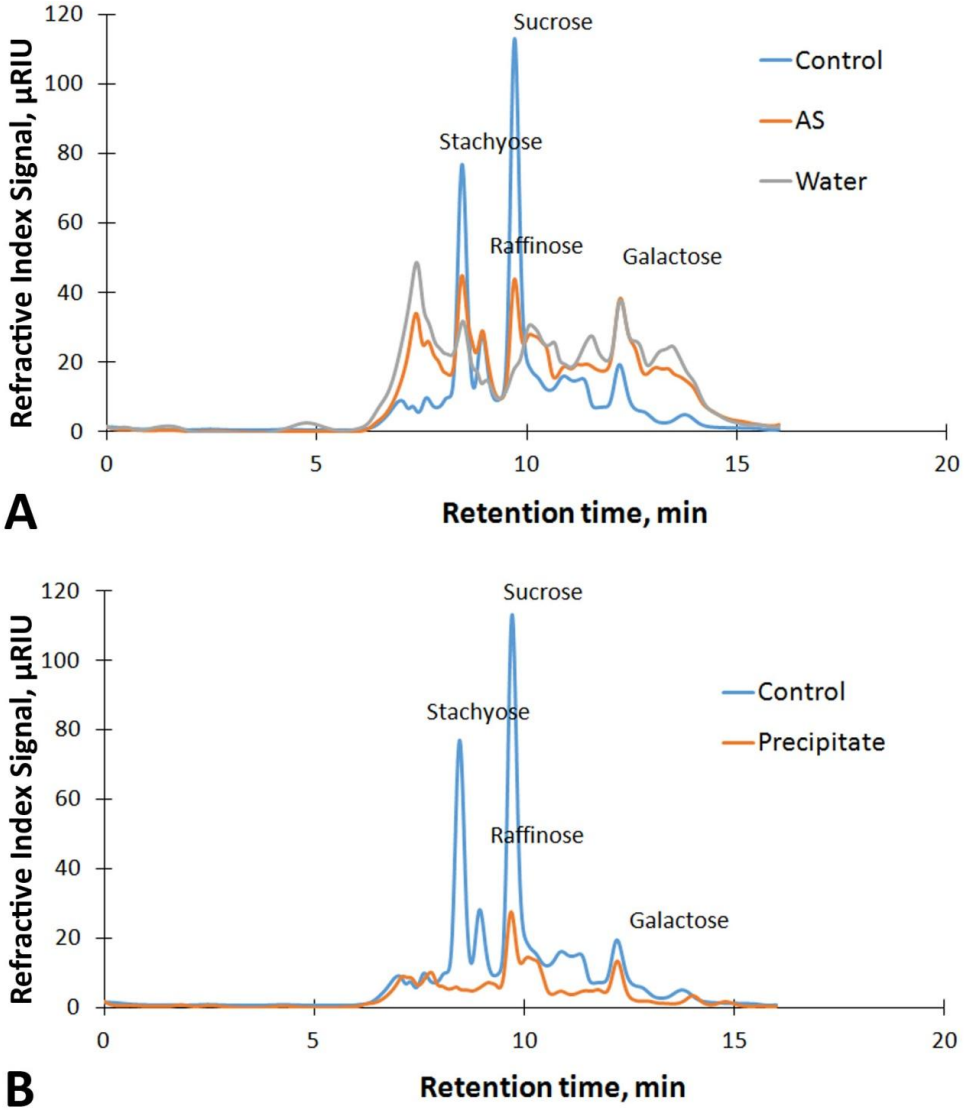

Figure 4. Residual sugars of SBM: (A) after 7 days of SSF with A. pullulans P8 in unfermented control, fermented control (water), and ammonium-sulfate supplemented SBM (AS); (B) after 3 days of SmF with A. pullulans P8 in unfermented control and precipitate obtained after centrifugation

3. 4. Determination of extracellular hydrolytic enzyme activities produced by *A. pullulans* P8 during SmF of SBM

In recent years production of many different enzymes using biomass and by-products from the food industry has been intensively studied [26,40-43]. In the current study, we showed that the new isolate *A. pullulans* P8 could produce amylase, cellulase, xylanase, pectinase, and α-galactosidase during SmF using SBM as a sole nutrient source.

The common feature for all extracellular enzymes examined during the current study is the lowest activity observed in the supernatant obtained after 24 h of SmF (Fig. 5). During the first 24 h of incubation, *A. pullulans* P8 used readily available monosaccharides to increase its biomass. When this source is depleted, extracellular enzymatic activities are required to hydrolyse complex carbohydrates. Significantly higher activities of all examined enzymes were detected after 48 h of incubation, which further increased for cellulase, pectinase, and xylanase, or stayed at the same level to the end of fermentation in the case of amylase and α-galactosidase.

Investigations of genomes of *Aureobasidium* spp. have shown an abundance of genes encoding most of the enzyme families involved in carbohydrate degradation [44]. For a high level of desired enzyme biosynthesis, however, selection of the appropriate substrate is crucial [43,45]. Another approach for obtaining enhanced enzymatic activities is optimization of the substrate concentration, but also of other factors that affect enzyme synthesis by microorganisms, such as the C:N ratio, temperature, aeration, pH, incubation time, *etc.* [40,41,43].

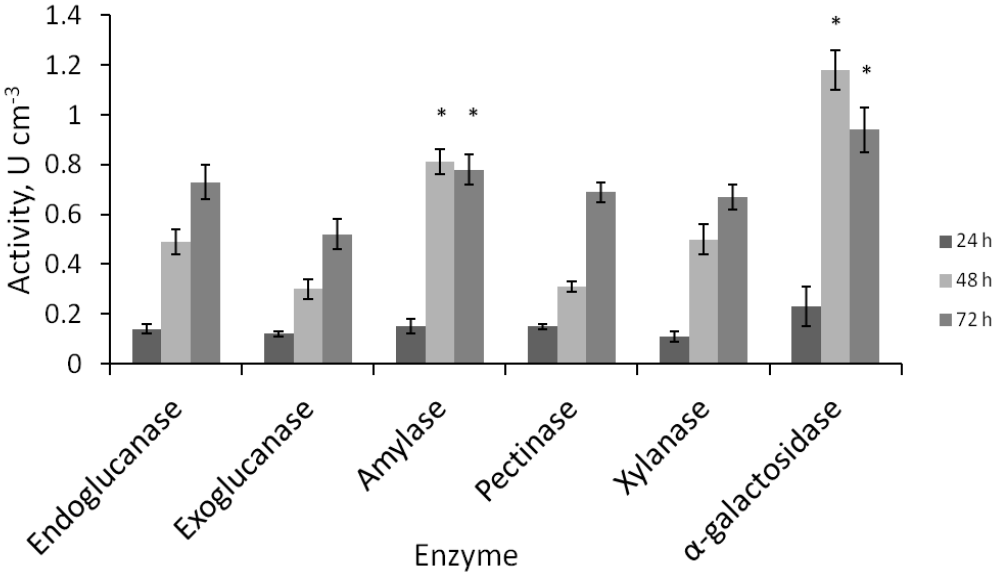

*Figure 5. Extracellular enzymatic activities in supernatant obtained during SmF of SBM using A. pullulans P8. The results are presented as mean values of three independent experiments ± standard deviations. *Values within the same group that do not differ from each other (p >0.05)*

Testing the possibility of different waste substrates to induce the production of xylanase in *A. pullulans* has shown the activity of 0.51 U cm^{-3} on hazelnut skin [45], which was lower than the maximal xylanase activity observed in our study (0.67 U cm^{-3}). However, ten times higher xylanase activity than in our study (67.44 U cm^{-3}) was obtained by the same fungal strain on wheat bran as substrate.

Unexpectedly, high amylase activity of 0.78 U cm^{-3} was determined in the supernatant after 48 h of SmF by *A. pullulans* P8, although the starch content in SBM is very low compared to other polysaccharides [3]. *A. pullulans* Cau 19 produced 0.456 U cm^{-3} after 5 days of growth in medium containing 1 % of starch [32]. After optimization, the amylase activity reached 0.8 U cm^{-3}, a level comparable to the result obtained in our study. This implies that *A. pullulans* P8 is a high amylase producer.

The activity of α-galactosidase obtained after 48 h of SmF using SBM exceeded that obtained in raffinose-supplemented MEB (1.18 *vs.* 0.89 U cm^{-3}), suggesting the potency of SBM to support α-galactosidase biosynthesis by *A. pullulans* P8. This effect could be explained by the ability of different SBM constituents to stimulate α-galactosidase production. Namely, besides RFOs, galactose, glucose, pectin, and galactomannans have been reported as potent carbon sources for the induction of α-galactosidase production by different microorganisms [6].

In our previous study [26], SBM has been shown as the best substrate for inducing pectinase production by *Bjerkandera adusta* TMF1 and *Schizophillum commune* TMF3. In the current study pectinase activity produced by *A. pullulans* P8 was 0.69±0.04 U cm^{-3}. A higher activity (3.8 U cm^{-3}) was reported in the study conducted by Bennamoun *et al.* [46] for a high pectinase-producing strain of *A. pullulans*, using 4 % tomato pomace as substrate.

Both endo and exoglucanase activities were detected in the supernatant after SmF by *A. pullulans* P8. Higher activities, by approximately 3 to 10-fold, for *Aureobasidium* spp. endoglucanase were reported in the literature than those obtained in the present study (0.73±0.07 U cm^{-3}). For example, *A. pullulans* NRRL-Y-2311-1 endoglucanase activity obtained after 120 h of SmF using differently processed canola meal was in the range of 2.27 to 3.17 U cm^{-3} [47]. *A. pullulans* LB83 produced 6.86 U cm^{-3} on SBM during 60 h of SmF [48], while the same strain produced 7.42 U cm^{-3} on sugarcane bagasse. On the other hand, in the study of Leite *et al.* [34] exoglucanase activity was not detected regardless of the substrate used for fermentation. In the current study, exoglucanase activity of 0.52±0.06 U cm^{-3} was obtained, and it was the lowest among all the tested enzyme activities.

Relatively low endoglucanase activities obtained in our study compared to literature data could be a result of activities of proteases produced by *A. pullulans* P8 during fermentation [47]. Although proteases were not the focus of this research, it is reasonable to expect they had been secreted. The effect of protease activity was more pronounced when complex substrates were used as nitrogen sources compared to ammonium sulfate [49]. Likewise, it was observed that different nitrogen sources significantly influenced amylase activity [32]. Since SBM was the only nutrient source in the current study, future experimental work will consider the effect of its supplementation with different inorganic nitrogen sources.

Although the activity of neither enzyme was optimized during the present study, it was shown that *A. pullulans* P8 has the potential to produce carbohydrate-degrading enzymes. Further research will focus on isolating, purifying, and applying these enzymes. Importantly, the crude enzyme mixture obtained in the present study could be used for bioethanol production without the need for time-consuming and expensive purification steps [26]. Additionally, it could be used for SBM hydrolysis for animal food production, as well as other complex lignocellulosic substrates that require the use of various enzymes to be degraded [17,26,43].

4. CONCLUSION

This study has shown the immense potential of a new strain of *A. pullulans* P8 isolated from grapes for producing high protein, low oligosaccharide soybean-based feed. The CP content in fermented SBM was increased by 20.8 and 24.7 % compared to control during SSF and SmF, respectively. The highest CP content of ~~61 % (dry matter basis) was achieved after three days of submerged fermentation using *A. pullulans* P8. This sample also exhibited the lowest galacto-oligosaccharide content, making it the most suitable for animal feed. Furthermore, the study revealed extracellular activities of different carbohydrate degrading enzymes in supernatant obtained after submerged fermentation. It is important to note that this study is only the first screening that elucidated the wide application potential of new strain *A. pullulans* P8. Therefore, subsequent research endeavors will focus on optimization of the fermentation process aiming to further enhance the content of crude protein and hydrolytic enzyme activities and to shorten the incubation time. Scaling-up experiments also need to be conducted. Finally, an animal feeding trial should be performed to verify the real beneficial effect of the new fermented soybean-based product *in vivo*.

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Data availability: The obtained sequences, which represented a partial sequence of the ITS region were deposited in the NCBI GenBank database: https://www.ncbi.nlm.nih.gov/nuccore/OR921445

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Valorizacija sojine sačme za proizvodnju visokoproteinske stočne hrane i proizvoda sa dodatom vrednošću korišćenjem novog soja *Aureobasidium pullulans*

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(Naučni rad)

Izvod

Sojina sačma je nusproizvod koji nastaje nakon ekstrakcije ulja iz zrna soje. S obzirom na to da je bogata visokokvalitetnim proteinima, sojina sačma se koristi kao dodatak za stočnu hranu. Međutim, bogata je takođe i antinutritivnim faktorima i nesvarljivim komponentama, među kojima je posebna pažnja usmerena na galaktooligosaharide, zbog nedostatka α-galaktozidaze kod monogastričnih životinja. Osnovni cilj ovog istraživanja bio je da se odabere pogodan soj crne gljivice nalik kvascu (*Aureobasidium* spp.) među deset prirodnih izolata iz grožđa, koji bi tokom fermentacije sojine sačme dao proizvod sa velikim sadržajem proteina i malim sadržajem oligosaharida. Sa tim ciljem odabran je izolat P8 koji je pokazao najveću aktivnost α-galaktozidaze od 0,89 U cm^{-3}. Odabrani soj je identifikovan kao *A. pullulans* P8. Maksimalni prinos sirovih proteina u fermentisanoj sojinoj sačmi (61 % računato na suvu materiju) i najmanji sadržaj galaktooligosaharida dobijeni su nakon 3 dana inkubacije na 30 °C potopnom fermentacijom pri sadržaju 10 % suve materije sojine sačme. Fermentacijom na čvrstom supstratu dobijeno je ~58 % sirovih proteina nakon 7 dana inkubacije na 30 °C pri sadržaju suve materije od 30 %. U supernatantu dobijenom nakon potopne fermentacije izmerene su aktivnosti ekstracelularnih enzima (celulaze, pektinaze, amilaze, ksilanaze i α-galaktozidaze), što ukazuje na potencijal ovog pomoćnog proizvoda za hidrolizu različitih lignoceluloznih supstrata.

Ključne reči: crna gljivica nalik kvascu; fermentacija na čvrstoj podlozi; potopna fermentacija; α-galaktozidaza; ekstracelularni hidrolitički enzimi; grožđe