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Production of *Aspergillus oryzae* RCAM 01133 biomass with increased protein and polysaccharides content using by-products of food industry

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Abstract. The biomass of filamentous fungi is a promising source of protein and carbohydrate. It can be used as an unconventional substrate in technologies for obtaining food and feed ingredients. The studies show that the fungus *Aspergillus oryzae* synthesizes an insufficient level of protein in the process of solid-state fermentation. The aim of the research was to develop conditions for the production of fungal biomass with a high content of protein and polysaccharides on the basis of solid-state fermentation using by-products of food industry as inexpensive substrate for biomass production. Wheat bran, soybean meal, distilled dry grains with solubles, and brewer's spent grain were used as raw material. Results of fermentation show that the protein content was 73.4% and 82.0%, which is more than 3 times higher than in fungus grown by submerged fermentation. The studies of the biosynthesis properties of the selected strain *A. oryzae* RCAM 01133 confirmed the high biological value of microbial biomass cultivated using food by-products. Fermentation of micromycete on culture media containing soybean meal and DDGS provided an increase of protein content by 1.45 times. Maximum increase of amino acids was observed for isoleucine, leucine, tryptophan, and glutamic acid. The increased content of synthesized polysaccharides related to media containing wheat bran and DDGS. The highest concentrations of polysaccharides were 27.9% and 32.9%, respectively.

Key words: *Aspergillus oryzae*, solid-state fermentation, protein, amino acids, polysaccharides, food by-products.

INTRODUCTION

Active studies of the biochemical and structural-functional properties of fungal biomass are conducted to identify the prospects of its use as a substrate for the production of protein and amino acids additives and functional ingredients (Ward et al., 2006; Feofilova, 2010; Abdel-Gawad et al., 2017).

Filamentous fungi have the ability to synthesize industrially significant biologically active substances. Fungus biomass contains protein, vitamins and valuable polysaccharides,

mainly glucan, chitin, mannan (Polizeli et al., 2005; Kumaresapillai et al., 2011; Gow et al., 2017). The composition of mycelial biomass is not constant. The content of metabolized fungi polysaccharides and protein varies considerably. It's related to the genetic affiliation and productivity of the used strains, the conditions of their cultivation and the media composition (Zhong et al., 2018).

The biomass of the fungi can be used for large scale manufacture of bio-based products and active pharmaceutical ingredients (Meyer et al., 2016). The food industry is constantly under the state and social press to reduce wastes damage to environment and become more eco-friendly. Some by-products are included in recipes of food and feed products as inexpensive sources of protein, dietary fibre, phenolic compound and the other biologically active substances (Laufenberg, et al., 2003). In addition to direct use as an ingredients, food by-products and wastes can be used more effectively as substrates for fermentation to produce biomass protein and polysaccharides (Zhang et al., 2008; Jin et al., 2010; Shin et al., 2018).

Solid state fermentation as a process carried out at a low water content has several advantages over submerged fermentation. Among the main advantages, one can note a higher resistance to contamination, lower costs of energy for sterilization and heat treatment, lesser wastewater production, and a higher concentration of target fermentation products. An important aspect is the possibility of using of solid agro-industrial wastes as substrate in their natural form for the production of fungal biomass (Soccol et al., 2017). *Aspergillus oryzae* is one of most used species for utilisation of food industry wastes such as rice bran (Rudravaram et al., 2006; Shin at al., 2019), brewer's spent grains (Bekatorou et al., 2007; Ogunjobi et al., 2011), soybean meal (Hong et al., 2004; Chean et al., 2013), distiller's dried grains with solubles (Lio & Wang, 2012), pea-processing byproduct (Souza Filho et al., 2018), sweet potato beverage residues and peanut shells (Zuo et al., 2018).

Results of investigations of Serba et al. (2016) show that the protein and polysaccharides content in the fungal biomass of *Aspergillus oryzae* RCAM 01133 produced by submerged fermentation is not high enough and corresponds to 18–25% and 25–33%, respectively. The aim of this study is the development of conditions for the production of fungal *Aspergillus oryzae* RCAM 01133 biomass with a high content of protein and polysaccharides as promising feed additives effective for animal production using solid state fermentation (SSF) of food industry by-products and wastes.

MATERIALS AND METHODS

Microbial Culture, Substrates and Fermentation

The object of study was non-pathogenic strain *Aspergillus oryzae* RCAM 01133 from the microorganisms collection of the Russian research institute of food biotechnology. A distinctives features of the strain are its high growth rate with decreases spore formation.

Substrates for solid-state fermentation of the fungus were by-products of food industry such as wheat bran (WB), soybean meal (SBM), distilled dry grains with solubles (DDGS), brewer's spent grain (BSG) and their combinations in various ratios shown in Table 1.

Fermentation was carried out at 30° within 2 and 3 days. Prepared growth media with moisture content of 55–60% was sterilized at 0.1 MPa for 40 minutes. The culture

efficiency was estimated by the growth rate of biomass, content of polysaccharides and protein, and hydrolytic enzymes activity.

All treatments and analyses were carried out in two sets.

Table 1. Combination of by-products for fermentation medium

No. of medium	Content of food industry by-products in solid phase of the medium, %			
	SBM	DDGS	BSG	WB
1	100	0	0	0
2	0	100	0	0
3	0	0	100	0
4	0	0		100
5	80	20	0	0
6	20	80	0	0
7	20	0	80	0
8	50	0	0	50
9	80	0	20	0
10	0	80	20	0
11	0	50	50	0
12	0	50	0	50

Chemical Analysis

The content of polysaccharides was determined as total reducing sugars (TRS) after acid hydrolysis measured spectrophotometrically by Nelson-Somogyi method (Nelson, 1944). The total protein content was determined by the Kjeldahl method. Approximately 0.5 g of raw material was hydrolyzed with 15 mL) concentrated sulfuric acid ($H_2SO_4$with catalyst tablet containing 5 g Potassium Sulphate (K_2SO_4) and 0.5 g Copper (II) Sulphate ($Cu_2SO_4 \cdot H_2O$) using Turbotherm digestion unit (Gerhard, Germany) at 420 °C for 2 h. Nitrogen was determined using distillation system Vapodest (Gerhardt, Germany). Protein content was arrived by multipliing amount of total nitrogen in the raw materials by the nitrogen-to-protein conversion factor of 6.25. Amino acids profile was estimared by the liquid chromatographic method using the amino acid analyzer Knauer (Germany) with ultraviolet spectrophotometric detector Knauer Smartline 2500 operated at 570 nm. Before analysis, samples were hydrolyzed with 6 N hydrochloric acid (HCl) in a boiling water bath under reflux for 6 hours followed by subsequent autoclaving for 2 hours at 120 °C. Then hydrolyzates were centrifuged at 6,000×gfor 15 minutes. Supernatant was used for quantitative and qualitative total amino acids analysis. Aminograms were calculated by comparing the areas of the standard and the samples curves.

The concentration of amine nitrogen was determined by the copper method of Pope and Stevens in the absence of ammonium salts (Cowan & Steel, 1993).

Iodine Fuwa method (Fuwa, 1954) was used to measure the α-amylase activity (AA). A 300 µL of enzyme was mixed with 600 µL of 1% (w/v) soluble starch dissolved in 100 mM NaAcetate buffer pH 4,7 at 30 °C. One unit of the α-amylase activity was defined as the amount of enzyme that produced 10% reduction in starch-iodine staining after 10 min of incubation under the experimental conditions. Protease Hemoglobin colorimetric Sigma Aldrich assay was used to determine proteolytic activity (PA). One unit of protease activity will hydrolyze Hemoglobin to produce color equivalent to 1.0 µmole of Tyrosine per minute at pH 4,7 at 30 °C (color by Folin & Ciocalteu's Reagent).

Statistical analysis

Statistical processing of the results was carried out using the one-way ANOVA and the post-hoc Tukey's test for multiple comparison. Differences were considered significant for $\alpha < 0.05$.

RESULTS AND DISCUSSION

The process of biosynthesis the mycelial biomass by solid-state fermentation of fungus *A. oryzae* RCAM 01133 in culture media containing by-products of food industry has been studied. During the growth of the culture on by-products media, an intensive formation of characteristic white mycelium and synthesis of hydrolytic enzymes were occurred. Maximim values of the amylolytic enzymatic activity were noted for WB and SBM media. The difference was not statistically significant for both substrates. The levels of amylolytic and proteolytic enzymatic activity during the cultivation of micromycete on wheat bran media were 105 U g^{-1} and 64 U g^{-1}, respectively. The proteolytic activity decreased to 12.0–29.5 U g^{-1} using SBM, DDGS, BSG as nutrient medium (Table 2).

The enzymatic activity confirms the biosynthetic ability of the micromycete *A. oryzae* RCAM 01133 grown on food by-products. Despite the fact that the target cultivation products were protein and polysaccharides, Shi et al. (2015) noted that synthesis of multiple enzymes can be useful to eliminate the antinutritional components and improve the protein quality using the final product as feed additive.

Table 2. Synthesis of α-amylase and protease by the fungus *Aspergillus oryzae* RCAM 01133 on various media during solid-state cultivation

Nutrient medium	Enzymatic activity, U/g	
	Amylolytic (AA)	Proteolytic (PA)
WB	105.0 ± 5.2^{a}	64.0 ± 3.2^{a}
SBM	110.3 ± 5.5^{a}	29.5 ± 1.5^{b}
DDGS	63.1 ± 3.1^{b}	12.0 ± 0.6^{c}
BSG	43.1 ± 2.2^{c}	15.0 ± 0.8^{c}

Values of enzymatic activity with the same letters in each column are not significantly different from each other at $\alpha = 0.05$.

The formation of spores was observed during fermentation only on BSG medium. It was noted the formation of yellow-green spores on the 2nd day of the fermentation.

The content of protein and polysaccharides in the surface culture of a fungus grown within 2 days was studied. It has been established that the highest accumulation of protein has been achieved in SBM and DDGS medium (Fig. 1). The level of total protein content in comparison with the initial medium increased in SBM and DDGS media from 56.6% to 82.0% and from 50.7% to 73.4%, respectively. In the study of Hong et al. (2004), fermented soybeans and fermented soybean meals contained 10% more crude protein than initial substrates as a result of 48 hours fermentation with by *Aspergillus oryzae* GB-107. Chen et al. (2013) showed increase of protein content from 50.47 to 58.93% after 36 h of fermentation of soy meal via *A. oryzae* 12892 from American Type Culture Collection. A lower protein gain in these studies could be associated with a shorter period of fermentation.

Lio & Wang (2012) used DDGS as substrates to evaluate the effect of coculturing three different fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium*, on enzyme production by SSF. These products also had 3.5–15.1% lower fiber and 1.3–4.2% higher protein contents. Authors suggested a potential feed quality improvement. Since the purpose of their experiment was to study the synthesis of enzymes, it is not entirely correct to compare the increase in protein content with this study. Nevertheless, a general tendency toward an increase in protein content can be noted.

The maximum percentage increase in protein content, which amounted to 115.2%, was noted during fermentation using BSG. The study of Bekatorou et al. (2007) revealed

that BSG slurries treated directly using *A. oryzae and A. awamori* at various conditions could increased their protein content by 20–36%. Long-term solid state fermentation of BSG using *Aspergillus oryzae* increased significantly in the percentage protein from 18.22% in the unfermented to 28.33% in the fermented BSG (Ogunjobi et al., 2011).

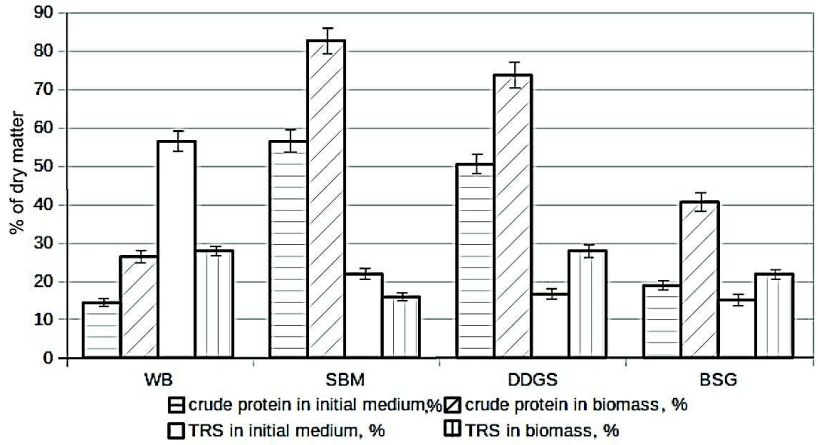

Figure 1. Crude protein and total reducing sugars content in the biomass of fungus *Aspergillus oryzae* RCAM 01133.

Wheat bran as a substrate for cultivation of the micromycete *A. oryzae* RCAM 01133 also showed high protein gain from 14.7% to 26.8%. These results correlated well with the results of Ravinder et al. (2003), who studied the production of single cell proteins from deoiled rice bran by *Aspergillus oryzae* mutants. *A. oryzae* MTCC 1846 and Shan2 mutant increased total nitrogen content from 9.2% to 16.4% and to 28.1% after 3 days fermentation, respectively. Also the protein increase of deoiled rice bran from initial 9.20% to 24.80% was achieved by Rudravaram et al (2006) growing *A. oryzae* MTCC 1846 with optimum growth process parameters such as moisture, pH of the substrate, inoculum size, temperature and nitrogen source. Fermentation of wheat bran with *Candida utilis* and *Rhizopus oligosporus* with optimum growth parameters resulted in a maximum crude protein yield of 41.02% compared with the 4.21% crude protein of the non-fermented wheat bran (Yunus, F.-U et al., 2015).

It should be noted that the protein content in the surface culture of the fungus is more than 3 times higher than in fungus grown by submerged fermentation with *Aspergillus oryzae* RCAM 01133 carried out by Serba et al. (2016).

Analysis of the amino acid composition of the obtained microbial biomass samples with a high protein concentration showed statistically significant increase in the content of all amino acids in comparison with their initial content in medium (Table 3).

Amino acid content in biomass grown on SBM media increased by 1.46 times and amounted to 350.6 mg g^{-1} in comparison with 239.6 mg g^{-1} in initial medium. The maximum increase in mass was observed for isoleucine (from 13.20 to 20.70 mg g^{-1}), leucine (from 21.80 to 36.10 mg g^{-1}), tryptophan (from 3.10 to 18.90 mg g^{-1}), and glutamic acid (from 51.80 to 61.10 mg g^{-1}). In percentage terms, this increase amounted to 56.2%, 65.6%, 509%, and 17.9%, respectively. Histedine showed minimal increase from 7.90 to 10.20 mg g^{-1}. The experimental data correlated with the results of other

researchers, although they have a higher degree of increase. Solid-state fermentation of soy meal via *Aspergillus oryzae* by Chen et al. (2013) showed increase of total amino acid content by 13.13%. The contents of methionine, cysteine, threonine, and tryptophan increased by 11.11, 28.57, 18.65, and 6.76%, respectively. Available lysine and valine content increased by 6.21% and 12%.

Table 3. Amino acid composition of initial media and fungal biomass of *Aspergillus oryzae* RCAM 01133 during SSF

Amino acid	Content, mg g^{-1}			
	on SBM		on DDGS	
	in culture medium	in biomass	in culture medium	in biomass
Aspartic acid	31.20 ± 0.96	38.40 ± 1.02	13.60 ± 0.48	20.60 ± 0.41
Serine	0.70 ± 0.03	5.10 ± 0.26	11.40 ± 0.27	15.70 ± 0.29
Threonine	10.30 ± 0.29	14.50 ± 0.52	8.20 ± 0.21	12.10 ± 0.28
Glutamic acid	51.80 ± 1.27	61.10 ± 2.84	34.00 ± 1.03	44.10 ± 1.21
Proline	13.60 ± 0.35	19.80 ± 0.64	27.10 ± 1.16	33.30 ± 1.18
Glycine	11.30 ± 0.31	14.60 ± 0.38	6.10 ± 0.11	9.40 ± 0.17
Alanine	11.80 ± 0.21	18.40 ± 0.74	16.90 ± 0.35	23.50 ± 1.02
Valine	12.70 ± 0.51	16.50 ± 0.53	7.70 ± 0.19	11.50 ± 0.38
Methionine	3.40 ± 0.05	7.90 ± 0.18	2.70 ± 0.06	6.10 ± 0.11
Isoleucine	13.20 ± 0.32	20.70 ± 0.78	6.20 ± 0.17	13.70 ± 0.29
Leucine	21.80 ± 0.75	36.10 ± 1.21	12.40 ± 0.28	26.70 ± 0.57
Tyrosine	10.30 ± 0.26	14.50±0.38	5.60 ± 0.14	9.80 ± 0.19
Phenylalanine	0.80 ± 0.02	7.30 ± 0.27	7.00 ± 0.14	13.30 ± 0.27
Histidine	7.90 ± 0.21	10.20 ± 0.31	4.50 ± 0.13	6.80 ± 0.14
Lysine	15.30 ± 0.32	21.80 ± 0.78	4.40 ± 0.19	9.50 ± 0.28
Tryptophan	3.10 ± 1.24	18.90 ± 0.45	18.30 ± 0.41	38.60 ± 1.33
Arginine	20.40 ± 0.98	24.80 ± 0.74	7.10 ± 0.14	10.80 ± 0.21
Total number	239.60 ± 6.51	350.6 ± 8.53	193.20 ± 4.05	295.50 ± 6.78
Crude protein, % of *absolute dry substance*	56.60 ± 1.24	82.00 ± 2.10	50.70 ± 1.24	73.40 ± 2.67

All changes in amino acid content in culture medium and in biomass are statistically significant for each amino acid and substrate at $\alpha = 0.05$.

Hong et al. (2004) reported increase of glycine, glutamic acid, and aspartic acid content and the lack of significant changes of any essential amino acids content as result of soybean meals fermentation with *A. oryzae* GB-107.

Ravinder et al (2003) showed significant increase of lysine, threonine, cysteine, and tryptophan content for both *A. oryzae* MTCC 1846 and Shan 2 mutant. after fermentation of deoiled rice bran.

Leucine, glutamic acid, and tryptophan were amino acids with maximim increase in content after fermentation of *A. oryzae* RCAM 01133 on DDGS medium. Minimal rise was observed as well as for SBM medium for histidine.

The highest content of polysaccharides was noted during *Aspergillus oryzae* RCAM 01133 fermentation on WB and DDGS medium, it was 27.9% and 28.0%, respectively. These polysaccharides containing a chitin-glucan-mannan complex can be used can be used in technologies of manufacturing bioproducts and additives with predominant content of dietary fiber.

Further, the ability of fungus strain *A. oryzae* RCAM 01133 to synthesize protein and polysaccharides during solid-state fermentation on combined mediums containing mixtures of SBM, WB, DDGS and BSG according to Table 1 was investigated. A comparison of protein and polysaccharide levels in fungus biomass after 3 days of solid state fermentation on combined mediums is presented in Fig. 2.

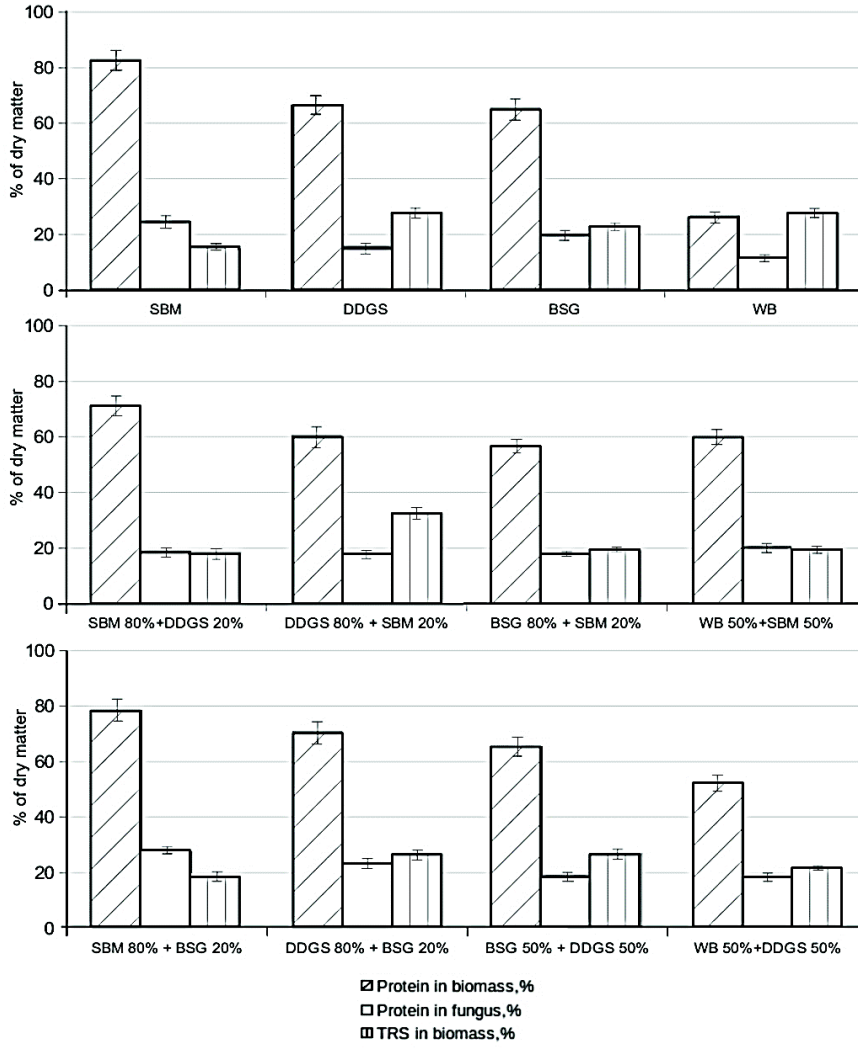

Figure 2. Content of protein and reducing polisaccharides in *A. oryzae* RCAM 01133 fungus biomass after 3 days solid state fermentation.

The results of experimental studies of solid-state fermentation of micromycete made it possible to select culture media that provide the highest level of synthesis of biopolymers in microbial biomass: culture medium No. 4 – 100% wheat bran; culture medium No. 2 – 100% soybean meal; culture medium No. 6 – 20% SBM and 80% DDGS; culture medium No. 5 – 80% SBM and 20% DDGS; culture medium No. 7 – 20% SBM and 80% BSG; culture medium No. 9 – 80% SBM and 20% BSG.

Medium samples No. 4 and No. 6 provided the highest accumulation of polysaccharides (27.9% and 32.9%, respectively) by fungus *A. oryzae* RCAM 01133 (Fig. 3, a) and can be used to produce bio-products with adsorbing properties.

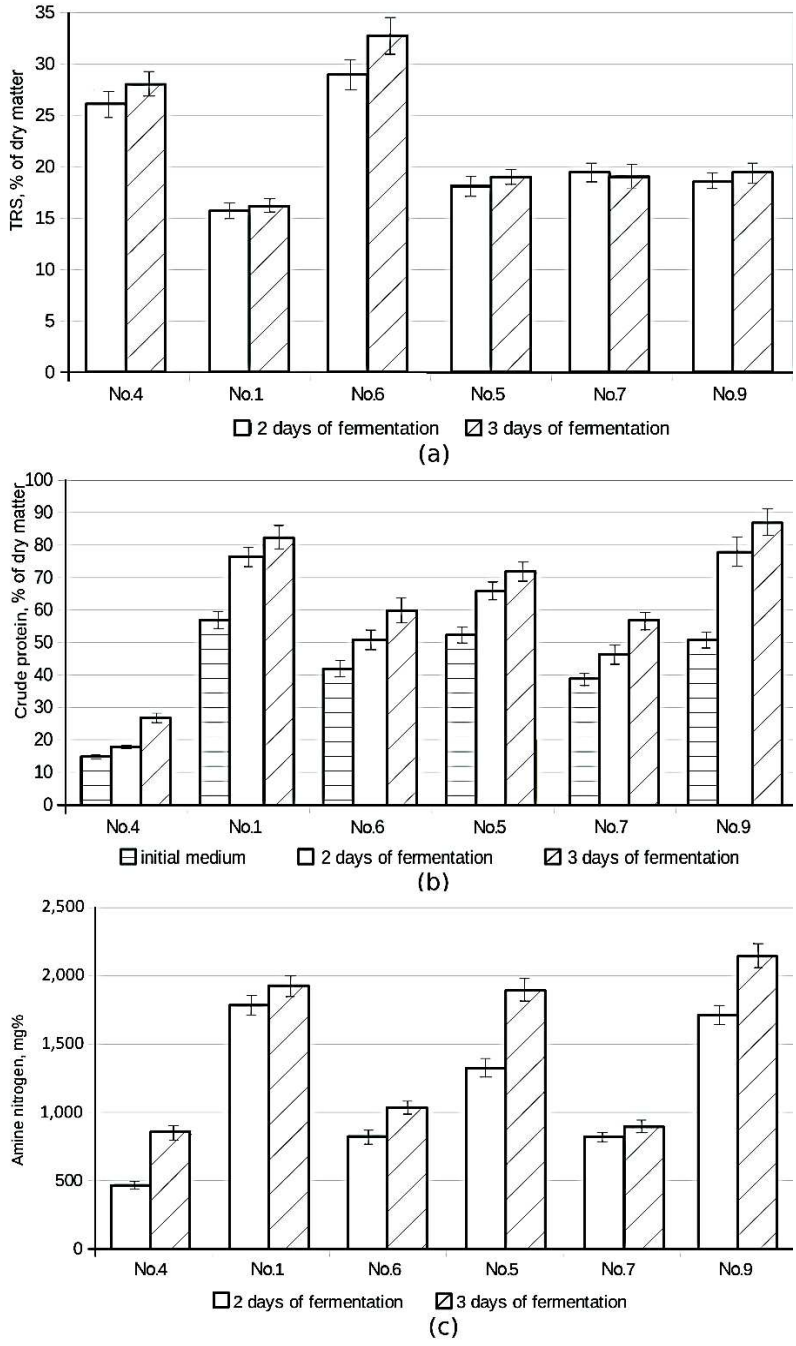

Figure 3. Accumulation of polysaccharides (a), protein (b) and amine nitrogen (c) in the surface culture of the fungus *A. oryzae* RCAM 01133 on selected culture mediums according to Table 1.

Predominant accumulation of protein was observed in culture media No. 1 and No. 9. These medium has a potential to be used in manufacture protein and amino acids food enrichers and feed additives (Figs 3, a and 3, b). A high level of amine nitrogen accumulation indicated an increased synthesis of proteolytic enzymes by fungus *A. oryzae* on these media, especially on the 3rd day of cultivation (Fig. 3, c).

CONCLUSIONS

The obtained experimental data confirmed the possibility of obtaining bio-preparations with the high content of protein substances and polysaccharides based on solid-state fermentation of the fungus *Aspergillus oryzae* on growth medium containing by-products of food industry. Maximum It was found that the protein content in the surface culture of the fungus was 73.4% and 82.0%, which is more than 3 times higher than in fungus grown by submerged fermentation in research of Serba et al. (2016).

The research results of the biosynthetic ability of the *A. oryzae* RCAM 01133 strain confirmed the high biological value of microbial biomass grown on food wastes. Fermentation of micromycete on culture media containing soybean meal and distilled dry grains with solubles provided an increase in the level of protein by 1.45 times. Maximum increase of amino acids for SBM and DDGS media was observed for isoleucine, leucine, tryptophan, and glutamic acid. Higher level of polysaccharides chitin-glucan-mannan complex by *A. oryzae* fungus is achieved on culture media with WB and DDGS (27.9% and 32.9%, respectively), that can be used in technologies for obtaining bio-preparations with predominant content of dietary fiber.

Thus, the results of the study show that fermentation of *Aspergillus oryzae* RCAM 01133 on WB, SBM, DDGS, and BSG culture media increased protein and polisaccharides content. The fungus strain and substrates can be used in technologies for producing protein and amino acid additives and bioproducts as sources of valuable polysaccharides.

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