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STUDY OF THE APPLICATION IN THE BREWING OF ENZYME PREPARATION, CONTAINING LAMINARINASE AND LICHENASE, PRODUCED FROM *TRICHODERMA SP. 405*

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ABSTRACT

The β-glucanase enzymes find wide application in the brewing. It was obtained an enzyme preparation with laminarinase and lichenase activity, produced from Trichoderma sp. 405 and was investigated its application in the technological process of brewing wort preparation. It was found that the studied enzyme preparation has a hydrolytic effect on the β*-glucans and the hemicelluloses of the used ingredients. From economical point of view the obtained enzyme preparation, added at a dose of 0,04 % compared to the used ingredients, leads to receiving of brewing wort, containing* β*-glucans suitable for healthy lifestyle.*

Keywords: laminarinase, lichenase, β*-glucans, enzyme preparation, brewing, brewing wort.*

INTRODUCTION

The β-glucanase enzymes pertain to the group of hemicellulases. They catalyze the degradation of β-D-glucans. Among the microorganisms, synthesizing β-glucanases, are the strains of genus *Trichoderma* - *Trichoderma reesei* [1], *T. harzianum* [2], *T. viride* [3], *T. koningii T 199* [4], *T. sp. GXC* [5], *T. asperellum* [6]. The group of β-glucanase enzymes include also the laminarinase and the lichenase. The laminarinase [E.C. 3.2.1.39] attacks 1,3-β-D-glycosidic bonds into 1,3-β-D-glucans. Its substrate is the polysaccharide laminarin [7]. The lichenase [E.C. 3.2.1.73] hydrolyses β-D-glucans, containing 1,3- and 1,4-bonds. Its substrates are the lichenin and the β-D-glucans in the cereals. The content of 1,3 and 1,4-β-glucans in the endosperm cell walls of the various cereals is different. Linear 1,3-;1,4-β-glucans have been isolated from seed endosperm of barley, oats, rye, wheat, millet, ryegrass. The content of β-glucans of different Bulgarian oats varieties ranges from 2,71 % to 4,26 % [8], in different Bulgarian barley varieties is in the range of 3,15 % - 6,08 % [9], in traditional USA oats varieties is around 4,4 - 4,9 % and in some special selected USA oats varieties reaches to 7,5 - 7,8 % [10]. The content of β-glucans in barley ranges from 1,5 - 2,5 % [11] to 3,0 - 4,5 % [12]. The β-glucans are the main component of barley and malt cell walls [7, 11, 13]. It is considered that they contain approximately 75 % β-1,3-1,4-glucans, 20 % arabinoxylans (noted as pentosans) and 5 % proteins, with traces of ferulic acid. According to Bamforth et al. [14, 15] the internal layer of barley and malt cell walls to the endosperm is composed from dense layer of β-glucans, connected with proteins and the external layer contains xylans, arabinose residues and ester linked acetic and ferulic acid among them (Fig. 1).

Beer is traditionally made from malt, hops, yeasts and water, but the technology is based mainly on the action of the enzymes, activated during the brewing. In the process participate 4 main categories enzymes: α- and β-amylases, (carboxy)peptidases and β-glucanases. All

of them act synergistically.

For brewing is necessary that the brewing wort contains β-glucans as low as possible, i.e. the β-glucans have to be completely degradated. When they are not fully degradated they exert negative influence on the brewing wort and the beer- increased viscosity and deteriorated filtration [16 - 21]. The β-glucans are partially cleaved by the action of β-glucanases and as a result is observed the release of high molecular mass β-glucans from complex compounds with proteins and other substances, as well as a cleavage of the β-glucans with high molecular mass to low molecular mass β-glucans, including glucose [22].

Usually the β-glucanases, which are used in the brewing, are isolated from *Trichoderma reesei, Aspergillus niger, Penicillium emersonii* and *Bacillus subtilis* [18]. It was established better filtration velocity of the brewing wort when enzyme preparations with β-glucanase activity were added in the production process of beer and brewing wort [23]. More effective degradation of glucans and viscosity decreasing of brewing wort and beer were established when enzymes with endo-β-1,3(4)-glucanase and xylanase activities were used in the production process of beer [10].

The aim of this research is to create a technological scheme for receiving an enzyme preparation, containing β-glucanase (laminarinase E.C.3.2.1.39 and lichenase E.C.3.2.1.73), and to study its application in the brewing.

EXPERIMENTAL

It was used *Trichoderma sp. 405*, bought from the National bank for industrial microorganisms and cell cultures - NBIMCC.

A spore inoculation product was obtained from the strain producer cultivated on potato-dextrose agar (PDA- Biokar Diagnostics) slants. Mandel's sowing media was inoculated with the spore sowing material. Mandel's fermentation media was inoculated with the sowing material [24]. The Mandel`s sowing media content is: NH_4Cl 1.00 g l^{-1}; urea 0.30 g l^{-1}; KH_2PO_4 2.00 g l^{-1}; $(NH_4)_2SO_4$ 1.40 g l^{-1}; $MgSO_4.7H_2O$ 0.30 g l^{-1}; $CaCl_2.2H_2O$ 0.40 g l^{-1}; Corn extract 10.00 ml l^{-1}; Glucose 20.00 g l^{-1}. The Mandel's fermentation media content is: NH_4Cl 1.00 g l^{-1}; urea 0.30 g l^{-1}; KH_2PO_4 2.00 g l^{-1}; $(NH_4)_2SO_4$ 1.40 g l^{-1}; $MgSO_4.7H_2O$ 0.30 g l^{-1}; $CaCl_2.2H_2O$ 0.40 g l^{-1}; Corn extract 0.1 %; Wheat bran 1 %. The cultivation of *Trichoderma sp. 405* was carried out by the method of deep fermentation in 500 ml flasks on a shaker "Inkubations - Schüttelschrank BS-4 B.Braun" (220 rpm) for 144 hours at 28°C.

The activity of both enzymes was obtained spectrophotometrically by measuring the reducing groups, which are released when hydrolyzing their substrates - laminarin and lichenin, respectively. An Unicam SP 1800 Ultraviolet spectrophotometer was used. For determination of enzyme activities was applied Somogyi-Nelson's method [25, 26]. One international unit (IU) of laminarinase activity is the amount of enzyme which catalyses the transformation of 1 μmol of reducing sugars in 1 cm^3 per minute at 40°C in a citric phosphate buffer of pH 5.00. One international unit (IU) of lichenase activity is the amount of enzyme which catalyses the transformation of 1 μmol of reducing sugars in 1 cm^3 per minute at 40°C in an acetate buffer of pH 4.00.

The protein contents in the liquid culture in mg ml^{-1} was obtained by the spectrophotometric method (OD_{280}/ OD_{260}) [27].

The lyophilization was carried out in a freeze-drying installation „HOCHVAKUUM" TG-16. The freezing process of the liquid culture was done at -35°C. The lyophilization's duration was 28 hours. The cooling temperature of the desublimator was -75°C and the temperature of the plates was -35°C. The enzyme activities were recovered by adding 10 ml distilled water to the lyophilized liquid culture and then they were analysed by the above mentioned Somogyi-Nelson's method [25, 26]. The residual moisture content of the lyophilized enzyme preparation was determined according to Bulgarian State Standard-1109-89- modified method by using an electronic scale "Sartorius thermo-control" with infrared heating of the samples.

The total mesophilic aerobic microorganisms (CFU g^{-1}) of the lyophilized enzyme preparation is determined according to ISO 4833:2003 [28]; *Escherichia coli* and coliforms (CFU g^{-1}) of the lyophilized enzyme preparation are analyzed according to ISO 21528-2: 2004 and ISO 4832: 2006 [29, 30]. The determination of *Staphylococcus aureus* (CFU g^{-1}) of the lyophilized enzyme preparation is according ISO 6888-1: 1999 [31], and the determination of *Salmonella sp.* (CFU g^{-1}) of the lyophilized enzyme preparation – according to ISO 6579: 2002 [32].

The gamma-irradiation of the samples lyophilized enzyme preparation was performed with the following

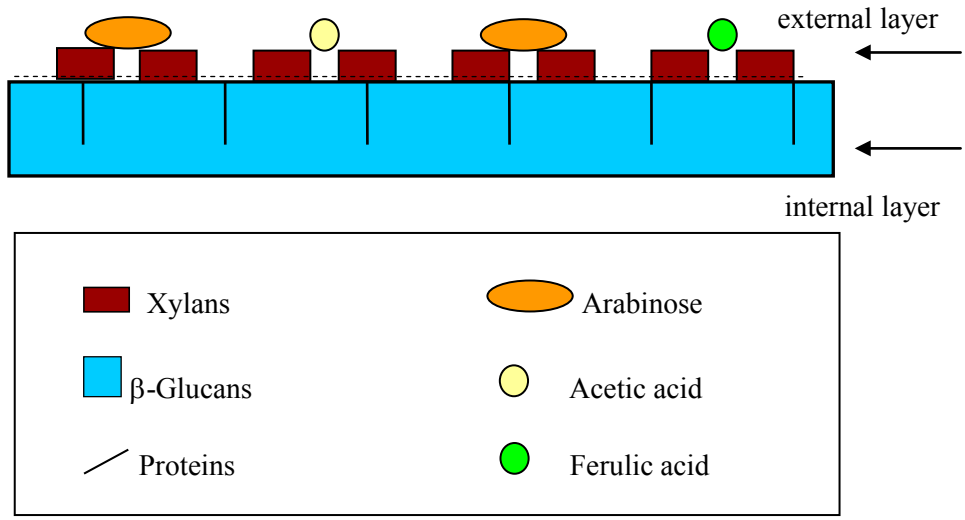

Fig. 1. Structure of the cell walls of barley.

parameters: radionuclide Cs^{137}; type of radiation - gamma rays; activity of Gamma-irradiation installation- „GAMA-1300", dose rate 1,75 Gy min^{-1}; radiation doses: 0, 5 and 10 kGy; operating temperature - 20°C - 22°C.

To study the application of the lyophilized enzyme preparation in obtaining of the brewing wort are conducted laboratory runs according to infusion regime in the production of 10 % bright beer when 30 % of the malt is replaced by 30 % unmalted barley. The enzyme preparation was dosed in the beginning of the process in amounts presented in Table 1 and the doses are conformed with its activity and with the activities of other industrial preparations (from 776 to 12700 IU g^{-1} and recommendatory doses from 150-250 to 350-450 g t^{-1} grist) [23].

The brewing wort is obtained on laboratory automatic apparatus of the German company "Bender & Hobein" by infusion method of the Department of Technology of Beer and Beverages of the Institute of Cryobiology and Food Technology. In this method the malt grist was mixed with water in a ratio 1:4 and after cytolytic, proteolytic and amylolytic degradation is adjusted to a certain volume and then is filtered. The obtained laboratory brewing worts are analysed by the following methods of the EBC Analysis Committee [33]: the time for saccharification is checked with iodine solution [34]; the speed of draining is determined taking into account the past milliliters brewing wort for a certain period of time [34]; the yield of the extract is determined by calculating the amount of the extracted substances from 100 g malt in the result of the mashing process [34]; the brewing wort extract is determined according to the measured relative mass of a certain quantity brewing wort and according to an official table is reported as g extract in 100 g brewing wort [33]; the determination of the brewing worts and beer pH values is made with pH-meter [33]; the brewing wort and beer colour is determined spectrophotometrically (λ = 430 nm) [33]; the brewing wort viscosity is measured with viscometer Höppler [33]; the soluble nitrogen is determined spectrophotometrically (λ = 215 nm and λ = 225 nm) [35]; the β-glucans determination is performed according to the method of Erdal [36].

RESULTS AND DISCUSSION

Technological scheme for receiving an enzyme preparation with laminarinase and lichenase activities

It was developed a scheme for obtaining a lyophilized enzyme preparation with laminarinase and lichenase activities, produced from *Trichoderma sp. 405*, which combines several technological stages, presented schematically in Fig. 2.

Characterization of the received enzyme preparation

The total yield after vacuum-freeze drying of 1 l liquid culture is 7,815 g lyophilized enzyme preparation. The obtained lyophilized enzyme preparation is a powder product with uniform consistency and yellowish-brown colour. The laminarinase activity of the filtered liquid

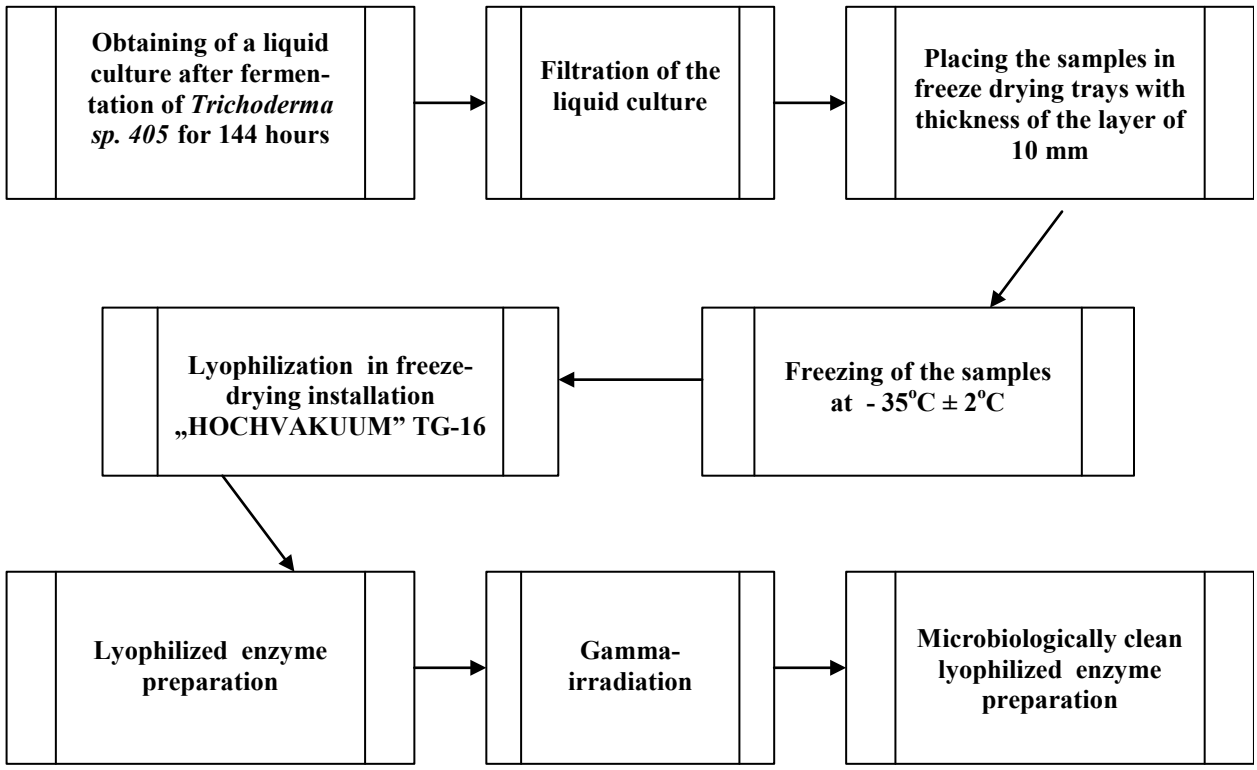

Fig. 2. Technological scheme for receiving an enzyme preparation with laminarinase and lichenase activities, produced from *Trichoderma sp. 405.*

culture, determined immediately after the fermentation is 0,520 IU ml^{-1}, and the lichenase activity - 0,107 IU ml^{-1}. After vacuum-freeze drying the laminarinase and lichenase activities of the lyophilizate, determined immediately after the lyophilization, are respectively 65,26 IU g^{-1} and 13,59 IU g^{-1}. It was found that the obtained lyophilizate is with average residual moisture content of 8,65 %. This ensures the structural and microbiological stability of the product during its storage.

The results from the microbiological analysis of the received lyophilized enzyme preparation show absence of pathogenic microorganisms - *Escherichia coli* and coliforms (CFU g^{-1} enzyme preparation), *Staphylococcus aureus* (CFU g^{-1} enzyme preparation) and *Salmonella sp.* (CFU g^{-1} enzyme preparation) were not isolated. The total mesophilic aerobic microorganisms of the lyophilized enzyme preparation is 10^6 CFU g^{-1}, i.e. this result is above the norm for total mesophilic aerobic microorganisms according to the norms of Bulgarian State Standards and ISO. This can be explained by the fact that the filtering of the liquid culture and the subsequent preparation of the samples before the lyophilization process are performed in non-sterile conditions. In order to reduce the reported high number of total mesophilic aerobic microorganisms the lyophilized enzyme preparation was subjected to a gamma-irradiation with low doses with 5 kGy and with 10 kGy, respectively.

After irradiation with 5 kGy and 10 kGy the lamina-

Table 1. Amounts enzyme preparation used in the receiving of brewing wort.

Content of:	Control sample	Variant 1	Variant 2	Variant 3
Malt, % of raw material	70	70	70	70
Barley, % of raw material	30	30	30	30
Enzyme preparation, % of raw material	-	0,04	0,40	2,0

rinase and lichenase activities remain high. It was found that the irradiation with 5 kGy is more favorable, because the reducing of laminarinase and lichenase activities is less compared to the samples, irradiated with 10 kGy. The percentage of preservation of the enzyme activities is presented in Table 2. The results for total mesophilic aerobic microorganisms are shown in Table 3. From the analysis it can be concluded that the irradiation with 5 kGy is enough to ensure considerable preservation of the enzyme activities of the lyophilized enzyme preparation and its necessary microbiological purity, which makes possible to use it in contact with food products.

Study of the application of the obtained enzyme in the brewing

It was studied the effect of the lyophilized enzyme (subjected to gamma-irradiation with 5 kGy) with laminarinase and lichenase activities 60,13 IU g^{-1} and 12,43 IU g^{-1}, respectively, in the wort preparation. Laboratory runs according to infusion regime in the production of 10 % bright beer, when 30 % of the malt is replaced by 30 % unmalted barley, were conducted.

In Table 4 are presented some physico-chemical parameters of the malt used in the laboratory runs and in Table 5 - some physico-chemical parameters of the barley used in the laboratory runs. With these ingredients it is expected to obtain beer with relatively good quality.

Table 6 shows the parameters of the brewing wort. The used quantities of enzyme influence the filtration speed, the extract values and the viscosity. The time for saccharification for the first three samples is similar. The speed of draining of the brewing wort in the variants is respectively 0,77 %, 2,77 % and 3,41 % higher than that of the control sample. The extract of the obtained brewing worts is in the range of 10,20 - 10,31 % for the control sample and the first two variants. In Variant 3 the increasing of the extract is with 0,44 % relative to the control sample. The pH values meet the requirements for the technology of bright beers. They vary in a narrow range 5,78 - 5,81. The brewing worts viscosity with dosed enzyme is the same 1,57 mPa s and it is 0,04 mPa s lower than that of the control sample.

In Fig. 3 are presented the received results for the yield of the extract. The values are in the range of 72,98 - 76,50 %. The yield in Variant 1 is 0,24 % higher than that of the control sample, in Variant 2 - 0,88 % higher than that of the control sample and in Variant 3 - 3,52 % higher than that of the control sample.

In Fig. 4 are shown the colour changes of the laboratory brewing worts, measured spectrophotometrically. The control sample and Variant 1 have almost the same colour. Probably the enzyme colour and the doses in Variants 2 and 3 affect the colour of the brewing worts. In Variant 2 the increasing is 1,3 times and in Variant 3 - 2,7 times.

Table 2. Preservation of laminarinase and lichenase activities of the lyophilizate, determined immediately after lyophilization and after its gamma-irradiation with 5 kGy and with 10 kGy.

Analysis phase	Laminarinase activity preservation, %	Lichenase activity preservation, %
Immediately after lyophilization of the liquid culture (0 kGy)	98,07	98,15
After gamma-irradiation of the lyophilizate with 5 kGy	90,37	89,81
After gamma-irradiation of the lyophilizate with 10 kGy	86,32	86,11

Table 3. Total mesophilic aerobic microorganisms of the lyophilized enzyme preparation after gamma-irradiation.

Total mesophilic aerobic microorganisms, determined of the lyophilized enzyme preparation	Established result	Norm for Total mesophilic aerobic microorganisms according to Bulgarian State Standards and ISO
Before gamma-irradiation	10^6 CFU g^{-1}	10^3 CFU g^{-1}
After gamma-irradiation with 5 kGy	0 CFU g^{-1}	10^3 CFU g^{-1}
After gamma-irradiation with 10 kGy	0 CFU g^{-1}	10^3 CFU g^{-1}

Table 4. Analysis of the malt used for the laboratory runs.

Parameters	Values
Moisture, %	4,3
Time for saccharification, min.	10
Extract, % ADM., - fine grist - roughly grist	79,4 78,0
Extract difference, %	1,4
Colour, EBC	2,5
Viscosity, mPa.s	1,47
Soluble nitrogen, - mg/100 ml - mg/100 g	76,6 681
Total protein, % ADM	10,4
Kolbach Index, %	40,9

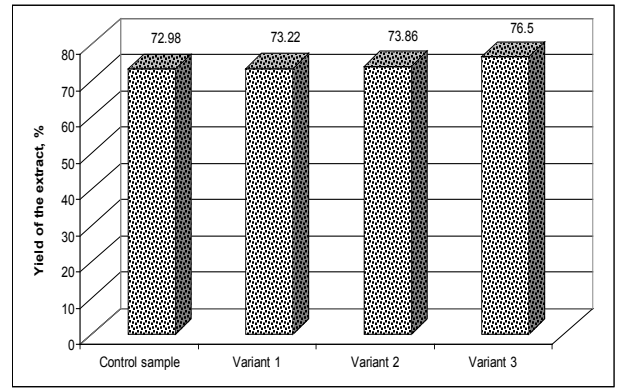

Fig. 3. Yield of the extract in the obtained brewing worts.

Table 5. Analysis of the barley used for the laboratory runs.

Parameters	Values
Moisture, %	12,4
Absolute mass, g	44,8
Uniformity over sieve, %: - 2,8 mm - 2,5 mm (2,5 + 2,8 mm) - 2,2 mm - below 2,2 mm	 70,5 94,9 4,3 0,7
Impurities, %	0,1
Total protein, % ADM	11,3

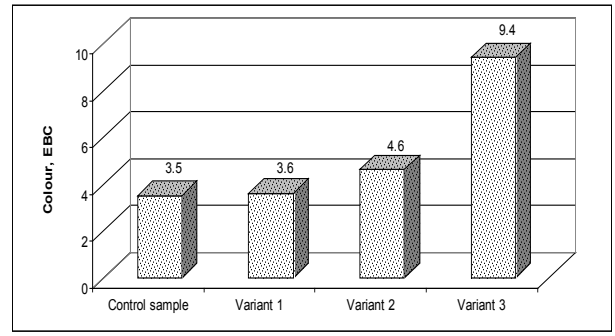

Fig. 4. Colour of the obtained brewing worts.

Table 6. Parameters of the laboratory brewing worts.

Parameters	Control sample	Variant 1	Variant 2	Variant 3
Saccharification, min.	10	10	10	15
Filtration speed, l $h^{-1}.m^{-2}$	16,99	17,12	17,46	17,57
Extract, %	10,20	10,23	10,31	10,64
pH	5,80	5,78	5,81	5,78
Viscosity, mPa s	1,61	1,57	1,57	1,57

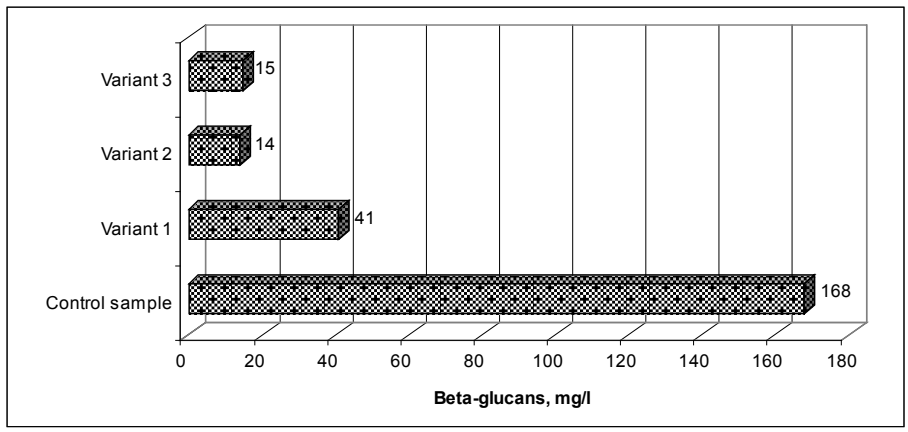

Fig. 5. β-Glucans content in the obtained brewing worts.

Fig. 5 presents the effect of the input enzyme preparation on the contents of β-glucans. In the control brewing wort they are in an amount of 168 mg l^{-1}. The enzyme preparation has clearly underlined effect on them and breaks them down 4,1 times in variant 1, 2-12 times in variant 2 and 3-11 times in variant 3. Independently of the measured low laminarinase and lichenase activities, the obtained enzyme, although it is not purified, can be considered suitable for use for the preparation of brewing wort.

CONCLUSIONS

Based on the results for the brewing wort preparation, it can be concluded that the enzyme prepared with laminarinase and lichenase activities, produced from *Trichoderma sp. 405*, has a hydrolytic effect on the β-glucans and the hemicelluloses of the used ingredients, more pronounced in decreasing of the β-glucans content and less in increasing of the brewing wort filtration speed.

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