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Lupin whey as a perspective substrate for bioethanol production

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Abstract

The study deals with the investigation of processing parameters of acidic water extraction of protein-free compounds from lupin flour in presence of hydrolytic enzymes with different substrate specificity. An important objective of the study was to analyze content of the liquid extract (lupin whey) and its perspectives for biofuel production. Commercial cytolytic enzyme systems (Cellulase-100, Cellolux-A, -F) were used in the extraction process. Multienzyme complex complied of individual enzymes such as cellulase, xylanase and α-amylase was used as an alternative. The optimized multienzyme complex was composed from 1.1 ± 0.2 units g^{-1} of cellulase, 5.2 ± 0.4 units g^{-1} of xylanase and 2.5 ± 0.2 units g^{-1} of α-amylase. The enzymatic treatment resulted in 19 % increase of the total sugar content of lupin whey versus to the control whey obtained without enzyme addition.
The lupin whey was condensed by evaporation to 48−50 % dry matter content. Condensed whey was used as nutrient medium for cultivation of yeasts *Saccharomyces cerevisiae*. After fermentation the yield of bioethanol reached 1.6 g/l.
The proposed technology of complex processing of vegetable raw materials allows to obtain lupin protein concentrates with a crude protein content up to 63.2 ± 1.3 % on dry matter basis and lupin whey with a total sugar content of up to 29 % on dry matter basis. The lupin whey could be used as an organic substrate for biofuel production.

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Keywords: vegetable protein processing; lupin whey; multienzyme composition; rotatable plan; biofuel production; nutrient medium

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1. Introduction

Bioethanol as an alternative motor fuel finds increasing application in the world. Grain raw materials, including wheat, corn, rye, millet are widely used for bioethanol production. However, bioethanol has a high cost when it is obtained from food crops. The use of vegetable protein processing wastes provides a possibility of replacing the existing methods of ethanol production from food crops and allows creating waste-free production technology [1].

By-products and wastes of processing of other legumes, in particular lupin, can be recycled to obtain a nutrient medium for further fermentation similar to soy molasses. Lupin seeds are considered an alternative to soybeans because of high protein content. Carbohydrate fraction of lupin seeds comprises cellulose (11 %), hemicelluloses, pectins (total 10 %), and a small fraction of starch (about 4 %) [2].

Lupin already has many human consumption applications, such as bread making, pasta products, sausage substitutes, egg and milk replacers [3, 4]. Plant protein isolates and concentrates are used in the production of meat products, analogues of dairy products and combined foods. Lupin seeds and lupin protein isolates were used in the manufacture of fermented sausages [5].

One of the known methods of vegetable protein concentrates processing deals with extraction of non-protein compounds from raw materials in acidic water medium with pH close to isoelectrical point of proteins followed by mechanical separation into 2 products: protein concentrate residue and whey waters. It was suggested that the use of hydrolytic enzymes during the extraction process will contribute to a hydrolysis of lupin flour polysaccharides and to their better extraction to liquid extract. The resulting whey is rich in carbohydrates and could be used as a nutrient medium for cultivation of microorganisms.

Microbial hydrolytic enzymes are able to destroy many biopolymers in plant raw material, which perform linking and protective function, such as phytin, cellulose, hemicellulose and lignin, and others [6]. The most widespread, commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *Trichoderma reesei*. The commercial enzyme preparation 'Celluclast' is a non-starch polysaccharide hydrolyzing enzyme for degradation of cellulose, cellobiose and higher polymers of glucose that could be used for improving malt quality [7]. Usage of Celluclast 1.5 L for pectin extraction increases the pectin yield [8]. The degradation of biomaterial by cellulase is accompanied by the release of substrates for the action of other enzymes, particularly for xylanase and mannanase. The glyukuronoksilan and mannan are rapidly decomposed in a system with cellulose [9]. The application of arabinofuranosidase with xylanase leads to complete removal of xylan [10].

The objective of this research was to study parameters extraction of non-protein compounds from lupin flour in acidic water medium in the presence of hydrolytic enzymes with different substrate specificity. The final product will be used as a nutrient medium in bioethanol production.

2. Material and methods

2.1. Materials

Wholegrain lupin flour from *Lupinus angustifolius* seeds was obtained from All-Russian Scientific Research Institute of Lupin, Bryansk, it had the following content: crude protein – 46 %, crude fat – 7.1 %, crude fiber – 4.0 % on dry matter basis.

The following enzymes preparations were used:

- Celluclast BG – carbolytic enzyme preparation made by submerged fermentation of the selected strain of fungus *Trichoderma reesei*, containing 3500 endoglucanase units gram^{-1}. Preparation was provided by Novozymes, Denmark;
- Cellulaza 100 – cytolytic complex enzyme preparation derived from a mixed culture of fungi *Aspergillus foetidus* and *Trichoderma viride*, containing 540 cellulase units gram^{-1}, Sibbiofarm, Russia;
- Pentopan Mono BG – xylanase preparation from fungi *Aspergillus oryzae*, containing 2500 fungal xylanase units gram^{-1}, Novozymes, Denmark;
- Amylosubtilin – amylase preparation containing 950 fungal amylase units gram^{-1}. Preparation was provided by Sibbiofarm, Russia.

- Cellolux-F – cytolytic complex enzyme preparation containing 2000 cellulase units $gram^{-1}$, 8000 fungal xylanase units $gram^{-1}$, 1500 endoglucanase units $gram^{-1}$, Sibbiofarm, Russia.
- Cellolux-A – cytolytic complex enzyme preparation containing 2000 cellulase $gram^{-1}$, Sibbiofarm, Russia.

Saccharomyces cerevisiae yeasts were used for bioethanol production tests.

2.2. Extraction of non-protein compounds of lupin flour

The used method is similar to the one for obtaining the soy protein concentrate in an acidic medium [11]. The wholegrain lupin flour was diluted with water in a ratio of 1:15. The resulting solution was adjusted to pH 4.5 by adding 5 % HCl to achieve protein isoelectric point. The following process was carried out in a thermostatic vessel with a magnetic stirrer. The cellulase enzyme preparations were added at a dosage of 1.08 units g^{-1} after the suspension reached a temperature of 55 °C. The process of extraction lasted for 40 min.

The mechanical separation of phases was performed by centrifugation at 4000 × g for 30 min. The hard residue obtained after centrifugation was the lupin concentrate. Total content of protein, water-soluble carbohydrates in the lupin whey (supernatant) were analyzed.

2.3. Preparation of condensed whey for fermentation and bioethanol production

The lupin whey was pasteurized at a temperature of 72 °C for 15 s and condensed in the rotary evaporator to reach a dry matter content 48−50 %. Then condensed whey was used as a nutrient medium for cultivation of the yeast. Before sterilization, the pH of the medium was adjusted to different pH levels – 4.5; 5.0 and 5.5 with hydrochloric acid. The medium was poured in the Bunsen flask of 250 ml. The flasks were placed in a steam autoclave at a temperature of 120 °C for 30−40 minutes. A suspension of yeast in an amount of 5 % was transferred into a Bunsen flask after cooling. Fermentation was carried out at 26 °C for 74−80 hour. The culture fluid was filtered and then the filtrate was concentrated on a vacuum rotary evaporator 5 times. The distillation was performed to alcohol concentration of 96 %, fuel oils of 1.5 to 2.0 % [12].

2.4. Measurements

Water content in the lupin protein concentrate was determined by the gravimetric method [13]. The content of crude protein was determined by Kjeldahl method on automated analyzer Kjeltec Auto (Tecator, Sweden) according to standard protocol of manufacturer. Crude protein content was estimated using a conversion factor 6.25 from total nitrogen.

The content of crude fat was determined by the Soxhlet method on automated analyzer **SER 148** (VELP Scientifica, Italy) according to the standard protocol of the manufacturer. The ceramic fiber filter method was used to determine the crude fiber [14]. Analysis of the total content of water-soluble carbohydrates was conducted by the Bertrand method [15]. Changes in pH were measured with Orion 920A pH-meter (Russia). For the preparation of the nutrient medium steam sterilizer UT-4735 (ULAB) was used. The filtrate was concentrated using the rotary vacuum evaporator Heidolph Hei-VAP Advantage. Component analysis of mono- and disaccharides was conducted on HPLC analyzer 'Stayer' (Akvilon, Russia) with refractometric detector. The separation was obtained using a mobile phase consisting of acetonitrile and water in ratio of 77 : 23 using the column 'Luna NH_2 5μ', (Phenomenex, USA).

2.5. Statistical evaluation of the data

All experiments were performed with at least three replicates; data was processed by methods of mathematical statistics at theoretical frequency 0.95. Statistical processing of data was carried out using computer programs Microsoft Office Excel 2010 and Mathcad 15.0.

3. Results and discussion

3.1. Preparation of the lupin whey

The resulting lupin whey contains some nitrogen-free extractive compounds of lupin seeds (organic acids, soluble carbohydrates and vitamins, other biologically active substances), low molecular weight nitrogen compounds (amino acids, peptides, albumin fraction of proteins) and lipids, which were released from the initial substrate in the process of hydrolytic destruction of cellular structures. Enzymes with different substrate specificities were tested for the hydrolysis of lupin flour polysaccharides. Bioconversion efficiency was evaluated as the total sugar content in lupin whey (Table 1). The data were compared with the results for the negative control sample (the lupin whey obtained without enzymes).

Table 1. The chemical composition of lupin whey

The name of the enzyme	solids content, %	total nitrogen, %	crude fat, %	nitrogen-free extractives (by difference) , %	total sugar, %	total sugar, % on dry basis
Control sample	1.30 ± 0.05	0.38 ± 0.01	0.17 ± 0.01	0.61	0.13 ± 0.01	10
Cellulaza 100	2.00 ± 0.04	0.22 ± 0.03	0.15 ± 0.02	1.11	0.50 ± 0.01	25
Cellolux-F	2.00 ± 0.05	0.25 ± 0.01	0.11 ± 0.01	1.08	0.54 ± 0.02	27
Cellolux-A	1.90 ± 0.05	0.22 ± 0.02	0.17 ± 0.02	1.08	0.46 ± 0.01	24

Under these conditions, crude protein content of lupin concentrates varied from 50.40 ± 1.30 % on a dry matter basis for the negative control sample to 54.12 ± 1.10 % in the case of Cellolux-F. Some low molecular weight water-soluble proteins were extracted to the whey during the lupin concentrate processing. According to the material balance of the above process, 19 % of total protein of raw materials on average was extracted to lupin whey waters.

The content of total nitrogen in the control whey sample was 0.38 %. The transition of water-soluble proteins to the whey was decreased when using the cytolytic complex enzyme preparations. The total content of all forms of nitrogen was 0.22 % in this case. The content of total sugar was increased on average by 15 % compared with a control sample.

3.2. Selection and optimization of multienzyme complex

Hydrolysis of lupin flour substrate by multienzymatic compositions was used as an alternative way of increasing the efficiency of the process. The degradation of non-starchy polysaccharides was conducted in the presence of Celluclast BG. An attempt was made to improve Celluclast BG hydrolytic efficiency with addition of α-amylase. The synergetic effect is known between xylanase and cellulase. These enzymes act on cellulose, xylan and other hemicelluloses of lupin flour [16]. So it was decided to increase the total sugar content of the lupin whey obtained from extraction process with Celluclast BG additive with addition of another enzyme - xylanase preparation Pentopan Mono. The compositions of the enzymes included cellulases in the optimal dosage of 1.08 u g^{-1} and other enzymes in recommended or higher dosages. The ratio of enzymes in the compositions was calculated according to their declared activity. Hydrolysis was carried out in optimal conditions which were previously found for Celluclast BG [17].The planning matrix of the experiment is shown in Table 2.

Table 2. Plan and results of the experiment

№	Factors in natural scale			Factors in dimensionless scale			
	z_1	z_2	z_3	x_1	x_2	x_3	y
1	1.62	15	2.1	1	1	1	15
2	0.54	5	2.1	−1	−1	1	30
3	0.54	15	2.1	−1	1	1	15
4	1.62	5	2.1	1	−1	1	30
5	1.62	15	0.7	1	1	−1	17
6	0.54	5	0.7	−1	−1	−1	11
7	0.54	15	0.7	−1	1	−1	10
8	1.62	5	0.7	1	−1	−1	25
9	0.172	10	1.4	−1.682	0	0	10
10	1.99	10	1.4	1.682	0	0	30
11	1.08	1.59	1.4	0	−1.682	0	30
12	1.08	18.41	1.4	0	1.682	0	10
13	1.08	10	0.222	0	0	−1.682	17
14	1.08	10	2.577	0	0	1.682	30
15	1.08	10	1.4	0	0	0	30
16	1.08	10	1.4	0	0	0	28
17	1.08	10	1.4	0	0	0	25
18	1.08	10	1.4	0	0	0	32
19	1.08	10	1.4	0	0	0	28
20	1.08	10	1.4	0	0	0	30

Three factors were examined to evaluate their influence on total sugar content of lupin whey (Y, % to dry substance): cellulase dosage (Celluclast) of 0.54–1.62 u g^{-1} (Z_1); xylanas dosage (Pentopan Mono) of 5–15 u g^{-1} (Z_2); α-amylase dosage (Amilosubtilin) of 0.7–2.1 u g^{-1} (Z_3). The mixture was exposed to the hydrolysis for 40 min.

Values of the optimal dosages of enzyme preparations were obtained by means of rotatable plan of the second order and regression equation coefficients were found (1):

$$y = 28.85 + 3.99x_1 - 5.32x_2 + 3.57x_3 - 0.87x_1x_2 - 2.62x_1x_3 - 2.62x_2x_3 - 3.45x_1^2 - 3.45x_2^2 - 2.21x_3^2 \quad (1)$$

The software package Excel 2010, MathCad 15.0 was used to find the optimum point. The resulting response surfaces have the form of an elliptic paraboloid (the contour of the cross section of the response surface is shown in Fig. 1). The significance of the regression equation coefficients was determined by the Student's criterion. The adequacy of the regression equation was estimated by the Fisher test.

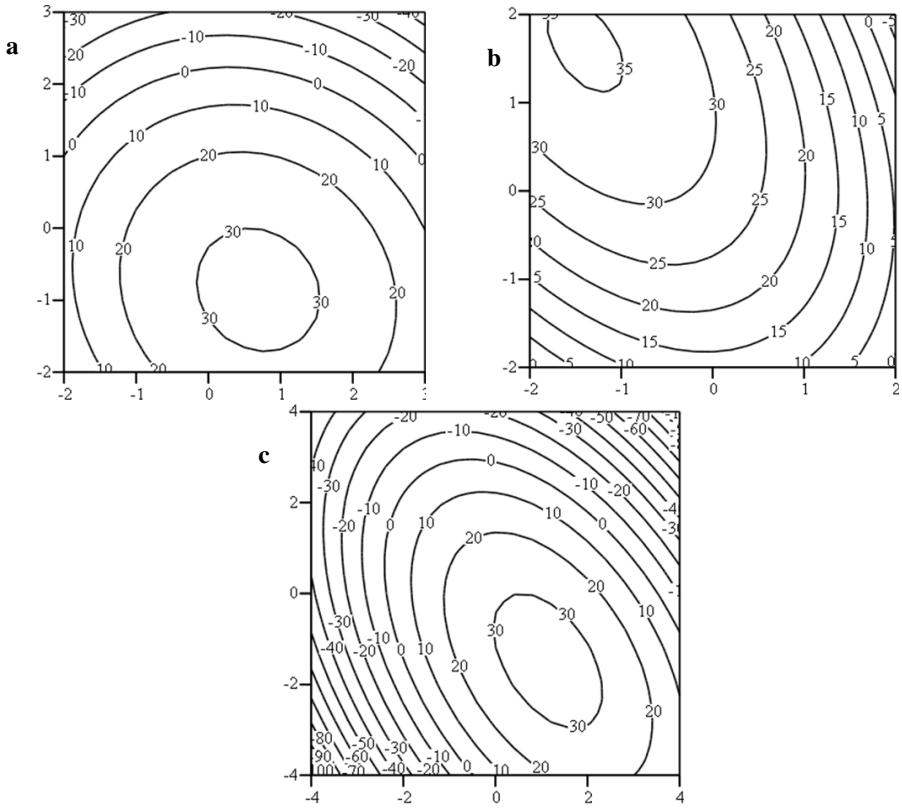

Fig. 1. (a) the contour of the cross section of the response surface of dependence of total sugar content in the lupin whey on Celluclast and Pentopan Mono dosage, respectively; (b) the contour of the cross section of the response surface of dependence of total sugar content in the lupin whey on Pentopan Mono and Amilosubtilin dosage, respectively; (c) the contour of the cross section of the response surface of dependence of total sugar content in the lupin whey on Celluclast and Amilosubtilin dosage, respectively.

Contour sections of response surfaces were used to determine the values of the optimal dosages in coded factors. The targeted value corresponded to the coordinates of the center of the surface. The multienzyme complex in natural values of factors was obtained after statistical processing of the experimental data: 1.1 ± 0.2 units g^{-1} of Celluclast & 5.2 ± 0.4 units g^{-1} of Pentopan Mono & 2.5 ± 0.2 units g^{-1} of Amilosubtilin.

The lupin whey obtained after the usage of the above enzyme complex was characterized by solids content 2.2 %, total nitrogen content of about 0.15 %, crude fat 0.19 %, nitrogen-free extractives of 1.2 %, total sugar content 0.64 % or 29 % on a dry matter basis.

The usage of multienzyme complex with optimized composition for bioconversion of lupin flour polysaccharides was the most effective compared to the data of table 1. The total sugar content of the whey was about 19 % higher than the total sugar content of the control whey and reached 29 % on dry matter basis. The most important that it was achieved alongside with the obtaining of lupin protein concentrate with the highest crude protein level -63.2 ± 1.3 % on dry matter basis.

The proposed technology of processing of lupin flour is waste-free and resource-saving. Bioconversion of carbohydrates results in a maximum concentration of the targeted components in the solid and liquid phases, which leads to obtaining of lupin whey with increased content of sugars. This product has potential for usage as a nutrient substrate component for cultivation of microorganisms inclusive but not limited to bioethanol production.

3.3. Bioethanol production

Carbohydrates of lupin whey were analysed by HPLC [17]. Carbohydrates content of lupin whey presented by sucrose, glucose, fructose with the ratio 10 : 1 : 1 and presence of low molecular nitrogen compounds and extractable bioactive components of whole grain lupin flour showed its potential as a substrate for yeast cultivation.

Prior the cultivation of *Saccharomyces cerevisiae* yeasts pH of condensed lupin whey was adjusted to 4.5; 5.0 and 5.5 with hydrochloric acid to determine the optimum pH levels of cultivation medium. Alcohol yield was the criterion of the fermentation process efficiency (Table 3).

Table 3. Influence of pH of incubation medium on ethanol yield

Initial pH	Final pH	Alcohol yield, g/l
4.5	4.7	1.3
5.0	4.9	1.6
5.5	5.1	1.4

The highest yield of ethanol 1.6 g/l was observed for lupin whey with pH 5.0.

This technology of bioethanol production from condensed lupin whey allows reducing production costs. In the future this technology can be the basis for the creation of ecologically safe production.

4. Conclusions

Enzymatic treatment of the lupin flour is an effective method for increasing the maximum concentration of the components in the solid and liquid phases. Hydrolysis of the lupin flour with optimized multienzyme composition increased crude protein content in the lupin concentrate to 17 % compared with the initial flour and increased total sugar content in the lupin whey to 19 % compared to the final product obtained without enzymes.

The proposed technology allows to save energy costs due to use of water extraction and biotechnological methods and to provide a high degree of substrate bioconversion because of optimized multienzymatic composition.

Implementation of these products can help diversifying production due to the necessity of compensation of technological risks and of using new food sources.

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