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OPTIMIZATION OF HYDROLYSIS CONDITIONS OF WHEAT STRAW BY ENZYME PREPARATION FROM *Fennellia* sp. 2806

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The aim of the work was to optimize the hydrolysis conditions of wheat straw by complex enzyme preparation from *Fennellia* sp. 2806 with endo-, exoglucanase, xylanase and β-glucosidase activities. Bioconversion of wheat straw was carried out by an enzyme preparation obtained from the culture filtrate of *Fennellia* sp. 2806. The two methods of statistical optimization of the experiment — the Plackett-Burman (determination of significant factors) and Box-Behnken (determination of optimal values of defined significant factors) methods were used consequentially to optimize the hydrolysis conditions. Endo-, exoglucanase, xylanase and β-glucosidase activities were assayed in enzyme preparation. Reducing sugars were determined by the modified Bertrand method. As a result of two-stage optimization of the bioconversion process of wheat straw by enzyme preparation from *Fennellia* sp. 2806, it was found that the highest reducing sugars values were formed at temperature 50 °C, pH 5.0, substrate concentration 100 mg/ml, endoglucanase activity — 0.012 u/mg substrate, process duration — 18 h and pre-treatment by 4.5% alkali solution with further exposure to a microwave irradiation 6 W/g WS for 10 min. So it was established that temperature, pH, substrate concentration, pre-treatment of wheat straw by alkali solution and microwave irradiation were the significant factors for the hydrolysis process of substrate by enzyme preparation from *Fennellia* sp. 2806. Reducing sugars concentration was increased 1.5–2.0 times compared with the results obtained for the native wheat straw.

Key words: wheat straw, optimization of hydrolysis conditions, bioconversion, enzyme preparation.

Hydrolysis of agriculture plant residues by fungal enzymes allows receiving sugar mixtures. These enzymes can be used in the process subsequent or simultaneously bioconversion of the substrate with yeast to ferment to ethanol. The amount of reducing sugars (RS) formed in reaction depend on many process parameters — such as type of lignocellulosic substrate, degree of milling, temperature and pH of the medium, the ratio of substrate/buffer, the presence and conditions of pre-treatment of the substrate by physical, chemical or biological methods, the duration of the process, the composition of enzyme preparation, presence of mixing process, etc. As a result, it is possible to obtain sugar mixtures with a concentration of RS up to 70 g/l, in which glucose can make up to 50% of the total amount of sugars [1–5].

In recent years, the chemometric optimization methods have become widespread due to such advantages as a significant reduction in the time and cost of experiments. These methods allow creating mathematical models for assessing compliance, the statistical significance of the impact of the investigated factors and the interaction effects between factors. In addition, in the presence of significant effects of the interaction between factors, the optimal conditions established in classical one-dimensional studies can be differ from the results of multidimensional optimization and be less relevant than the last ones.

The first step of multidimensional optimization is to check the factors being studied by a full or partial factor experiment, for example, by Plackett-Burman design. After determining the relevant factors, the optimal conditions are achieved through the use of more complex three-level designs — the Doehlert matrix, the central composite design and Box-Behnken design [6–9]. Since the comparison of response surface methodology obtained with these designs showed that the Box-Behnken is the most effective of the above mention and was chosen for the second step of optimization of WS bioconversion process.

The aim of this work was to optimize the hydrolysis conditions of wheat straw with enzyme preparation from *Fennellia* sp. 2806 with endo-, exoglucanase, xylanase and β-glucosidase activities.

Materials and Methods

The object of the study was selected strain of the microscopic fungus *Fennellia* sp. 2806, which synthesizes cellulo- and xylanolytic enzyme complex [10]. Strain was grown on potato-glucose agar for 10–14 days at 22–26 °C. The inoculum was cultivated in a potato-glucose medium under submerged conditions at 22–26 °C for 48 h [11].

To obtain the enzyme preparation (EP) microscopic fungus was cultivated under submerged conditions at 22–26 °C for 4 days, the amount of inoculum — 5%. Composition of the nutrient medium (g/l): milled wheat straw (WS) — 50.0; $NaNO_3$ — 1.0; KH_2PO_4 — 4.0; KCl — 3.0; $MgSO_4 \times 7\ H_2O$ — 0.5; yeast extract — 0.5; peptone — 0.1; urea — 0.5; $(NH_4)_2SO_4$ — 0.05; $FeSO_4 \times 7\ H_2O$ — 0.005; $MnSO_4$ — 0.001.

Partially purified enzyme complex was prepared from culture filtrate (CF) *Fennellia* sp. 2806 by precipitation with ammonium sulfate (85% saturation), decanted, centrifuged for 10 min at 3000 rpm. The precipitate was dissolved in 50 mM citrate buffer (pH 5.0) and used for hydrolysis of the WS.

Preliminary processing of milled WS was carried out by a complex physical and chemical method, consisting of treatment with a solution of NaOH with simultaneous microwave irradiation, followed by washing from alkaline with distilled water to neutral pH and drying to constant weight.

Hydrolysis of pre-treated WS was carried out by EP, diluted to appropriate value of endoglucanase activity, 40–60 °C and pH from 4.0 to 6.0 using 50 mM citrate buffer. The amount of RS was determined in filtered from straw liquid for 18–30 h by Bertrand method [11]. The eight factors were used to determine significant for hydrolysis parameters of WS in Plackett-Burman design (Table 1). All variants of the experiment were performed in triplicate.

Table 1. **The experimental variables and their levels in Plackett-Burman design**

№	Code	Parameter of WS hydrolysis	«–»	«+»
1	A	Temperature, °C	40	60
2	B	pH	4.0	6.0
3	C	NaOH,%	1	5
4	D	Power of microwave irradiation, W	300	900
5	E	Duration of microwave irradiation, min	2	10
6	F	Substrate (WS), mg/ml diluted EP	50	100
7	G	Duration of hydrolysis, h	18	30
8	H	CMCase activity, U/mg substrate	0.01	0.05

Table 2. **The influencing variables and their levels in Box-Behnken design**

№	Code	Parameter of WS hydrolysis	«–»	«0»	«+»
1	A	NaOH,%	1	3	5
2	B	Power of microwave irradiation, W	1200	3600	6000
3	C	Substrate (WS), mg/ml diluted EP	50	75	100
4	D	CMCase activity, U/mg substrate	0.005	0.01	0.015

The main influencing variables for RS formation in WS treatment with enzyme preparation were determined and further used in Box-Behnken design (Table 2).

Statistical analysis of data and the creation of experimental designs were carried out using the MiniTab 16 software (Minitab Ltd. UK).

The determination of mono- and disaccharides in hydrolysates was carried out by the method of ascending thin-layer chromatography on Sorbfil PTSC-AF-A-UV (RF) in a solvent system of isopropanol: ethyl acetate: water (7: 2: 1) on silica gel plates. The developer was methanolic solution (83 ml) of 30% orthophosphoric acid (15 ml), diphenylamine (2 mg) and aniline (2 ml) [12].

Endoglucanase activity was determined at 50 °C and pH 5.0 by hydrolysis of 2.0% solution of Na-CMC (Sigma) after 30 min incubation of 0.5 ml of diluted EP with 0.5 ml substrate, exoglucanase — filter paper (1×6 cm) for 60 min [13], xylanase — 1% solution of beech xylan (Sigma) for 5 min [14]. The RS in the reaction mixture after the enzymatic hydrolysis of Na-CMC, filter paper or xylan was determined using a DNS reagent (Sigma) [15].

One unit of endo-, exoglucanase or xylanase activities were defined as the amount of enzyme catalyzing the release of 1 μmol of glucose or xylose equivalent per 1 min under given conditions respectively.

β-Glucosidase activity was determined by incubating 100 μl of diluted EP with 100 μl of 10 mM *p*-nitrophenyl-d-glucopyranoside (pNPG) (Sigma) in 200 μl of 50 mM acetate buffer (pH 5.0) at 50 °C for 10 min [16].

One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per 1 min under given conditions.

Results and Discussion

It is known that the main influencing factors on realize of RS in the hydrolysis process of cellulosic substrates by enzyme preparations are the temperature and pH of the medium, which tend to coincide with the optimum temperature and pH of the action of enzymes that hydrolyse β-glucoside bonds [4, 17]. Therefore, we have previously determined the optimal conditions for the various components of the enzyme preparation from *Fennellia* sp. 2806 [18]. Critical for the process of hydrolysis of lignocellulosic substrates are the ratio between the substrate and the liquid phase, the activity of the preparation, the duration of the hydrolysis, and the method of pre-treatment of the substrate.

According to the literature, preliminary treatment of lignocellulosic substrate by physical and chemical methods contributes to the enhancement of the efficiency of enzymatic hydrolysis [1–5]. Our preliminary results from the study of EP from *Fennellia* sp. 2806 confirmed the significant effect of pre-treatment of WS on its bioconversion [19].

The method of pre-treatment also affects the process of hydrolysis. Thus, the treatment of lignocellulosic substrates with alkali, in contrast to the widespread use of acids, ensures not only a more effective increase in sites for the action of enzymes, but also promotes the removal of lignin, an inhibitor of cellulases and xylanases (while acids generally favor the separation of hemicellulose). In this case, no toxic products which are characterize of acid hydrolysis, such as furfural [2, 4, 20], are formed.

In addition, the combined method of the substrate pre-treatment, used by us reduces the duration and intensity of chemical treatment compared to other methods [21]. Therefore, to determine the optimum conditions for the hydrolysis of pretreated WS by EP from *Fennellia* sp. 2806, the parameters mentioned above were studied (Table 1). The design and the results of the experiment are presented in Table 3.

As a result, it was found that practically all investigated factors were significant for the hydrolysis of wheat straw, except the enzymatic activity of the diluted preparation and the duration of the process (Fig. 1).

To a large extent, the amount of RS affected the temperature and pH of the medium. Pre-treatment conditions were significantly less affect RS formation during the hydrolysis of the substrate in the valid range.

Thus, the model equation to determine the predicted quantities of RS to be formed as a result of enzymatic hydrolysis of WS, had the following form:

$$RS_{pred} = 8.7 + 3.2 \cdot A - 2.8 \cdot B + 1.1 \cdot C - 0.9 \cdot D + 0.8 \cdot E + 1.4 \cdot F$$

Note: the designation of factors in accordance with Table 1.

Thus, the results of experimental and theoretical calculations of RS amount are presented in Table 3. The predicted data obtained from the equation of the mathematical model, diverge from the experimental ones more than the value of the statistical error, for some values the difference

Table 3. **Plackett–Burman design (PBD) of experiments for the study of eight experimental factors in coded values and responses ($n = 3$)**

№	Factors								RS, g/l	
	A	B	C	D	E	F	G	H	Experimental	Predicted
1	+	–	+	–	–	–	+	+	12.8 ± 0.50	14.5*
2	–	–	–	+	+	+	–	+	7.6 ± 0.17	8.5*
3	–	+	+	–	+	–	–	–	6.1 ± 0.23	4.1
4	–	–	–	–	–	–	–	–	4.4 ± 0.28	5.9
5	+	–	–	–	+	+	+	–	18.6 ± 0.49	16.7*
6	–	+	+	+	–	+	+	–	1.9 ± 0.49	3.5
7	–	–	+	+	+	–	+	+	8.7 ± 1.07	7.9*
8	+	–	+	+	–	+	–	–	17.0 ± 0.21	15.5*
9	–	+	–	–	–	+	+	+	3.6 ± 0.99	3.1*
10	+	+	–	+	–	–	–	+	7.1 ± 0.23	4.9
11	+	+	+	–	+	+	–	+	12.1 ± 0.91	13.3*
12	+	+	–	+	+	–	+	–	4.3 ± 0.01	6.5
13	0	0	0	0	0	0	0	0	17.0 ± 0.23	8.7

Note: here and in Table 4 * — the difference between the predicted and experimental values of the RS does not exceed 10%;

A — temperature; B — pH; C — NaOH concentration; D — power of microwave irradiation; E — duration of microwave irradiation; F — WS/EP; G — duration of hydrolysis; H — carboxymethyl cellulase (CMCase) activity;

Endoglucanase activity of EP = 7.6 ± 0.47 U/ml (1.33 ± 0.15 U/mg of protein).

exceeded 10% from the RS_{pred}. A particularly significant difference between the predicted and experimental values of the RS was in the central area of the experimental design. This fact indicates the presence of extremums in the investigated range of factors and the need for describing the surface of the response of higher order functions, in particular, the central composite or the Box–Behnken design (BBD). The BBD has been used to further optimization of reducing sugars formation in wheat straw conversion process with the enzyme preparation from *Fennellia* sp. 2806. For this purpose, four factors from previously studied were selected (Table 2). Since these optimum conditions for hydrolysis, such as temperature and pH of the medium, coincide with the optimum of the action of the individual components of the enzyme complex, it was decided not to investigate them and to concentrate attention on the conditions of WS pre-treatment. The analysis of preliminary data on the influence of power and duration of microwave irradiation indicates that it is advisable to combine them into one — the irradiation power that affect WS during 10 min.

The results obtained for optimization of hydrolysis conditions by BBD (Table 4) confirm the presence of extremums on the response surface in the investigated range of influencing factors. The obtained design equation of the response surface is much better describes the process than the previous one. According to this equation, the optimal parameters are: alkali concentration — 4.5%; total irradiation power — 6 W/g of WS for 10 min; substrate concentration — 100 mg/ml; the concentration of the enzyme preparation is 0.012 U/mg of the substrate.

It is known that in the process of hydrolysis of lignocellulosic agriculture waste by enzyme preparations with cellulolytic and xylanolytic activities produce different amounts of reducing sugars: rice (straw, husk) 25–90 g RS/l, wheat (straw) — 220–320 g glucose/kg, sugar cane (shoots) 12–50 g RS/ l, manioc (husk) 14–15 g glucose/l, rape (straw) 85–90, Miscanthus — 65, corn (stems) 18–55 g/l [1, 2, 5, 20, 22–25]. The main reason for this, in our opinion, may be differences in the ratio of different types of cellulose (crystalline and amorphous) and lignin in the substrate which is related to the species of the plant, different conditions of cultivation, the age of crops, as well as the method of pre-treatment of the substrate [3, 5, 26].

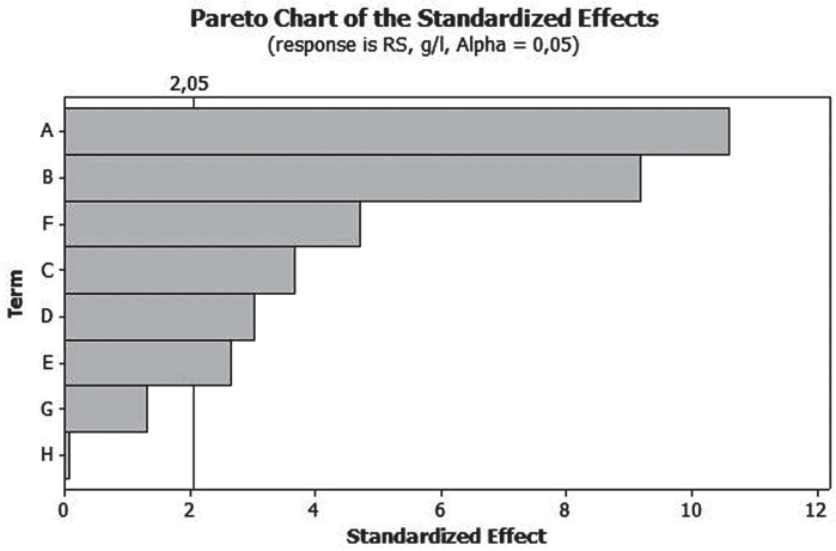

Fig. 1. **Pareto chart of the standardized effects on hydrolysis of WS by enzyme preparation from *Fennellia* sp. 2806**

Table 4. **Experimental matrix by the Box–Behnken design for four factors of WS hydrolysis ($n = 3$)**

№	Factor				RS, g/l	
	A	B	C	D	Experimental	Predicted
1	0	0	-1	1	8.1 ± 0.27	8.3*
2	0	0	0	0	11.2 ± 1.05	11.2*
3	1	0	1	0	14.6 ± 1.16	15.4*
4	0	1	1	0	15.3 ± 0.95	14.8*
5	-1	1	0	0	6.1 ± 0.78	6.2*
6	-1	-1	0	0	5.2 ± 0.51	4.2
7	1	1	0	0	12.1 ± 1.55	12.8*
8	-1	0	-1	0	3.3 ± 0.52	3.6*
9	0	1	0	1	13.4 ± 1.41	11.9
10	1	0	0	1	11.5 ± 0.32	11.5*
11	0	1	0	-1	11.4 ± 0.62	10.9*
12	1	-1	0	0	12.0 ± 0.75	10.8
13	-1	0	0	-1	3.9 ± 0.98	3.9*
14	0	-1	0	-1	8.7 ± 1.11	8.9*
15	0	-1	1	0	13.2 ± 0.40	12.8*
16	0	-1	0	1	9.2 ± 0.92	9.9*
17	0	0	1	1	12.8 ± 0.40	13.5*
18	-1	0	1	0	5.9 ± 0.15	6.8
19	1	0	0	-1	10.6 ± 0.59	10.5*
20	1	0	-1	0	8.0 ± 0.55	8.2*
21	0	0	1	-1	12.1 ± 0.70	12.5*
22	0	0	-1	-1	7.0 ± 1.11	7.3*
23	0	-1	-1	0	6.8 ± 0.31	7.6
24	0	1	-1	0	9.1 ± 0.78	9.6*
25	-1	0	0	1	4.9 ± 1.41	4.9*

Note 1: A — NaOH; *B* — Power of microwave irradiation; *C* — WS/EP; *D* — CMCase activity. Enzyme activity EP: endoglucanase — 5.4 ± 0.11; exoglucanase — 1.0 ± 0.06; xylanase — 1231 ± 49; β-glucosidase — 697 ± 16.1 U/ml.

$$RSpred = 11.2 + 3.3 \cdot A + 1.0 \cdot B + 2.6 \cdot C + 0.5 \cdot D - 2.7 \cdot A2 - 0.8 \cdot D2 + 1.0 \cdot A \cdot C$$

Note 2: the designation of factors is in accordance with Table 2.

Increasing the concentration of substrate from 7.5% to 20% leads to rise in the amount of RS from 28 to 68 g/l for 24 h of cultivation and from 30 to 80 g/l for 72 h [5]. Using of commercial cellulase preparations for hydrolysis of plant residues allows obtaining more than 60 g RS/ l. The amount of EPs used ranges from 5 to 40 U/g substrate for exoglucanase activity (typically 15–20 U/g substrate). The increase in the amount of enzyme preparation from 5 to 40 U exoglucanase activity/g of substrate results in an RS increase by 2–15 times, and allows obtaining of RS values 35–44 g/l, depending on the kind of substrate [3]. In our studies we used partially purified EP from a natural fungal strain with an exoglucanase activity of 1.8–2.8 U/g of WS, which is considerably less (up to 20 times) than mentioned above.

In addition, it should be noted that at a stirring rate of 100–150 rpm and a process time ranging from 48 to 72 h, the concentration of RS reaches 25–90 g/l, while in the case of poor stirring or absence of the mixing, in some cases, the process was extended to 120 h, with the amount of RS, as a rule, was lower than 20–25 g/l. In our experiments, the concentration of RS was 16–20 g/l for up to 30 h and increased to 23–26 g/l for 48 h, which was shown by us earlier [19]. It's known that the most effective enzymatic hydrolysis of lignocellulosic substrates occurs during the first 24 h (75–85%). In this case, glucose is up to 50% of the total amount of RS [1, 2, 4, 19, 20, 22–26].

Since the bioconversion of the lignocellulosic substrate occurs in conditions of high temperature (45–50 °C) and energy-consuming stirring, the expediency of continuing the process up to 48 h more is questionable. Taking into account that in studies of dynamics of bioconversion of pre-treated WS by the EP from *Fennellia* sp. 2806 the value of the RS forming between 16 and 48 h did not exceed 30% [19]. Therefore, we consciously limited the duration of the hydrolysis of 30 h. Conversion of WS was carried out at temperature of 50 °C and pH 5.0, wherein the EP activity decreased for 24 h: cellulolytic — by 45–50%, xylanase — 85–90% [18]. As a result of optimization of the process of wheat straw hydrolysis, the amount of RS increased from 7.2–9.3 g/l [19] to 16.4–18.6 g/l, i.e. 1.5–2.0 times at 18–24 h.

The method of thin layer chromatography was used for determination of the main hydrolysis products of lignocellulosic substrates (pre-treated milled WS and corn cobs) (Fig. 2). The main products of the bioconversion of the

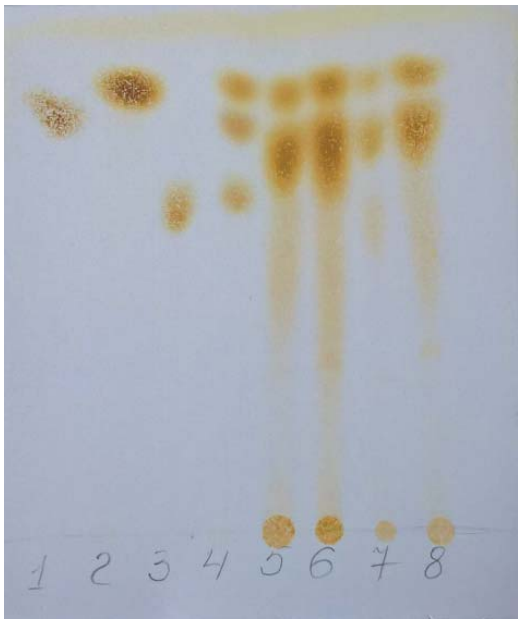

Fig. 2. **Thin-layer chromatogram of RS:** *1* — glucose; *2* — xylose; *3* — cellobiose; *4* — glucose+xylose+cellobiose mixture; *5* and *6* — corn cobs hydrolysates (24 and 48 h respectively); *7* and *8* — WS hydrolysates (24 and 48 h respectively)

WS were, in accordance with TLC, glucose and xylose; while there was almost no formation of cellobiose — the main product of the action of cellobiohydrolases probably due to the high β-glucosidase activity of the EP.

Thus, the optimal conditions for hydrolysis of WS by EP from *Fennellia* sp. 2806 were: temperature 50 °C, pH 5.0, substrate concentration 100 mg/ml, concentration of EP for endoglucanase 0.012 U/mg substrate, duration of process 18 h, and pre-treatment of WS with 4.5% alkaline solution and microwave irradiation with power 6 W/g lignocellulosic substrate for 10 min. As a result, the levels of RS were increased by 1.5–2.0 times compared with the ones for the native WS.

Influencing factors for the process of WS hydrolysis by the EP were established. Optimization of the bioconversion process of the lignocellulosic substrate is an important stage in the study of hydrolysis conditions, with particular attention given to the conditions for substrate pre-treatment, the ratio of its amount and amount of liquid phase, and also the qualitative composition of individual enzymes in EP.

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ОПТИМІЗАЦІЯ УМОВ ГІДРОЛІЗУ ПШЕНИЧНОЇ СОЛОМИ ЕНЗИМНИМ ПРЕПАРАТОМ ІЗ *Fennellia* sp. 2806

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Метою роботи було оптимізувати умови гідролізу пшеничної соломи комплексним ензимним препаратом із *Fennellia* sp. 2806 з ендо- і екзоглюканазною, ксиланазною та β-глюкозидазною активностями. Біоконверсію пшеничної соломи проводили ензимним препаратом, отриманим з культурального фільтрату *Fennellia* sp. 2806. Для оптимізації умов гідролізу пшеничної соломи послідовно застосовували два методи математичного планування експерименту — Плакетта-Бермана (визначення значущих факторів) і Бокса-Бенкена (визначення оптимальних значень встановлених значущих факторів). У ензимному препараті вимірювали ендо- і екзоглюканазну, ксиланазну та β-глюкозидазну активності. Редукувальні цукри визначали модифікованим методом Бертрана. У результаті двоступеневої оптимізації процесу біоконверсії ПС ФП із *Fennellia* sp. 2806 було встановлено, що найвищі величини редукувальних цукрів утворювалися за температури 50 °С, pH 5,0, концентрації субстрату 100 мг/мл, ендоглюканазної активності 0,012 од/мг субстрату, тривалості процесу 18 год за умов попередньої обробки пшеничної соломи 4,5% розчином лугу з подальшим мікрохвильовим опроміненням потужністю 6 Вт/г ПС упродовж 10 хв. Встановлено, що температура, pH, концентрація субстрату, попередня обробка пшеничної соломи розчином лугу і мікрохвильовим опроміненням є значущими факторами для процесу гідролізу субстрату ензимним препаратом із *Fennellia* sp. 2806. Досягнено збільшення кількості редукувальних цукрів у 1,5–2,0 рази порівняно з результатами для нативної пшеничної соломи.

Ключові слова: пшенична солома, оптимізація умов гідролізу, біоконверсія, ензимний препарат.

ОПТИМИЗАЦИЯ УСЛОВИЙ ГИДРОЛИЗА ПШЕНИЧНОЙ СОЛОМЫ ЭНЗИМНЫМ ПРЕПАРАТОМ ИЗ *Fennellia* sp. 2806

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Целью работы было оптимизировать условия гидролиза пшеничной соломы комплексным энзимным репаратом из *Fennellia* sp. 2806 с эндо- и экзоглюканазной, ксиланазной, а также β-глюкозидазной активностями. Биоконверсию пшеничной соломы проводили энзимным препаратом, полученным из культурального фильтрата *Fennellia* sp. 2806. Для оптимизации условий гидролиза пшеничной соломы последовательно использовали два метода математического планирования эксперимента — Плакетта-Бермана (определение значимых факторов) и Бокса-Бенкена (нахождение оптимальных значений установленных значимых факторов). В энзимном препарате измеряли эндо- и экзоглюканазную, ксиланазную, β-глюкозидазную активности. Редуцирующие сахара определяли модифицированным методом Бертрана. В результате двухступенчатой оптимизации процесса биоконверсии гидролиза пшеничной соломы энзимным препаратом из *Fennellia* sp. 2806 установлено, что самые высокие величины редуцирующих сахаров получены при температуре 50 °С, pH 5,0, концентрации субстрата 100 мг/мл, эндоглюканазной активности 0,012 ед/мг субстрата, продолжительности процесса 18 ч при условии предварительной обработки пшеничной соломы 4,5% раствором щелочи с дальнейшими микроволновым облучением мощностью 6 Вт/г в течение 10 мин. Установлено, что температура, pH, концентрация субстрата, предварительная обработка пшеничной соломы раствором щелочи и микроволновым излучением являются значимыми факторами для процесса гидролиза субстрата энзимным препаратом из *Fennellia* sp. 2806. Достигнуто увеличение количества редуцирующих сахаров в 1,5–2,0 раза по сравнению с результатами, полученными для нативной пшеничной соломы.

Ключевые слова: пшеничная солома, оптимизация условий гидролиза, биоконверсия, энзимный препарат.