Features of the Processing of Cellulose-Containing Raw Materials with *Aspergillus Niger* Enzyme Systems to Obtain Bioethanol

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Abstract. In this work, studies were carried out to study the enzymatic activity of the native strain of *Aspergillus niger F-1270* in relation to primary and secondary cellulose-containing raw materials in comparison with the cellulose activity of a commercial enzyme preparation. It was found that the native strain exhibits stable, but low cellulytic activity, lower than when using an enzyme preparation under similar conditions. To enhance the cellulase activity of the native *A. niger* strain, nondirectional physicochemical mutagenesis was used. As a result of mutagenic action on *A. niger F-1270* with UV irradiation or UV irradiation in combination with subsequent treatment with a chemical mutagen, conditionally genetically modified samples were obtained. It was confirmed that as the UV exposure time increases to two hours, the cellulase activity of the surviving part of the culture increases ($r = 0.706; p < 0.05$). Ultraviolet irradiation with an intensity of 253.7 nm for 120 min increases the enzymatic activity of mutant strains by 2-4 times as compared to the native one. The highest enzymatic activity of relative secondary cellulose-containing raw materials is 1.5 U.

1. Introduction

The development of efficient methods for the production of biofuels is becoming increasingly important in light of the depletion of fossil fuels, environmental pollution and the growth of global energy demand [1, 2]. Cellulose-containing raw materials are an attractive substrate for bioethanol production due to their availability and renewability [3, 4, 5].

In order to provide an economically viable process for the production of bioethanol, it is necessary to achieve effective splitting of polymeric lignocellulosic raw materials into monosaccharides, which are subsequently fermented with microorganisms (yeast or bacteria) with the formation of alcohol [6, 7, 8].

The splitting of cellulose requires a synergistic action of three enzymes: endoglucanases (EC 3.2.1.4); exoglucanases, including celloextrinases (EC 3.2.1.74) and celllobiohydrolases (EC 3.2.1.91, EC 3.2.1.176); β-glucosidase (EC 3.2.1.21). Endoglucanase randomly cuts the cellulose chain, celllobiohydrolases attack the end of the cellulose chain, and β-glucosidase hydrolyzes celllobiose and short (soluble) saccharides to glucose.

Cellulolytic enzymes of some bacteria and fungi are capable of hydrolysis of lignocellulosic biomass (pretreated) to monosaccharides. But the cost of such enzyme preparations is high and can reach 50%
of the total cost of the hydrolysis stage and 20% of the total cost of ethanol production. The existing technologies of enzymatic hydrolysis require improvement in order to intensify them. The main disadvantages include the need for a large amount of enzyme preparation and low product yield.

Saccharification of the substrate can be carried out using microorganisms with high cellulolytic activity. To date, more than 14,000 species of fungi that destroy cellulose have been identified, but only a few of them have been studied in more detail. Organisms exhibiting high cellulolytic and hemicellulolytic activity include *Clostridium*, *Cellulomonas*, *Trichoderma*, *Penicillium*, *Neurospora*, *Fusarium*, *Aspergillus*. Research aimed at increasing the enzymatic activity of cellulolytic enzymes in microorganisms is an urgent line of research.

The purpose of this work was to experimentally select the optimal method for increasing the cellulolytic activity of the microscopic fungus *Aspergillus niger*.

2. Materials and methods

Cellulose of Miscanthus genus (Miscanthus Anderss) and cellulose of alder chips, as well as starch and cellulose-containing household waste, were used as a substrate for enzymatic hydrolysis. Cellulose-containing raw materials underwent alkaline and acid delignification [9, 10].

The microorganism *Aspergillus niger* F-1270 was obtained from the collection of VKPM “GosNIIgenetics”.

As a control of cellulolytic activity, the enzyme preparation “Celulase ultra” was used (LLC “Sibbiopharm”, Berdsk, Russia), obtained on the basis of the selection strain *Tr. reeseii*. The activity of the drug is 2500 units / g, the working range is in the range from 30 to 65 °C and pH 2–7 with an optimum of action at 50–60 °C and pH 4.0–5.5.

Physical mutagenesis of Aspergillus niger F-1270 was carried out in BMB-II-“Laminar-S” -1.2 (221.120) Class II (Type A2) using an ultraviolet lamp Philips TUV 30W G13 (LAMSYSTEMS, Mias, Russia) with a maximum radiation at 253.7 nm.

Chemical mutagenesis was carried out using sodium azide and ethidium bromide.

Cellulolytic activity was qualitatively assessed with staining the culture in Petri dishes with 1% Congo red solution (Sigma) for 15 minutes and subsequent washing with 1M sodium chloride solution.

For a quantitative assessment of cellulolytic activity, a wash with 0.05M citrate buffer (pH 4.8) was prepared from a 5-day culture of the microorganism. The resulting spore suspension was centrifuged at 16000g for 15 min. The supernatant containing cellulase was taken into plastic tubes with a volume of 1.5 ml with a piece of filter paper 1 × 6 cm (~ 50 mg). Samples were incubated in 1 ml of enzyme extract at 30, 37, and 50 °C. The concentration of reducing substances (glucose) was measured after 1 and 48 hours.

Enzyme activity is expressed in international units of U (or U) and is equal to the amount of enzyme that catalyzes the conversion of 1 μM substrate in 1 min at a set temperature.

The content of the reducing substance (RS), D-glucose, in the sample was carried out using the reagent 3,5-dinitrosalicylic acid according to the method described in GOST R 54905-2012.

Enzymatic hydrolysis was carried out in 1.0 and 0.25 L conical flasks with cotton-gauze stoppers with a working volume of 200 ml and 50 ml, respectively. The reaction was carried out under static conditions at 37 °C for 6 days. The substrate concentration in all samples was 50 g/L. The inoculum concentration varied from 106 to 108 spores / ml. For the preparation of the inoculum, cultures of microorganisms at pre-exponential or exponential growth stages were used. The concentration of the spore suspension was measured on a SmartSpec Plus spectrophotometer (BioRad Laboratories, Inc., California, USA) at 600 nm. Enzymatic activity was interrupted with heating in an autoclave to 121 °C.

3. Results and discussion

In the course of the studies, the cellulolytic activity of the native *A. niger* F-1270 strain was studied in relation to six types of insoluble substrate, one of which is a model filter paper. Hydrolysis conditions are 37 ° C, pH = 4.7 on inpatient basis. In addition, the cellulase activity of a commercial enzyme preparation was assessed at a concentration of 0.1 mg/ml citrate buffer (table 1).
Table 1. Characteristics of the cellulose activity of the enzyme preparation and *A. niger* in relation to different substrates.

| Substrate                                         | Reducing substance, mg/cm³ |
|---------------------------------------------------|-----------------------------|
|                                                   | “Cellulase ultra”           | A. niger F-1270               |
| Filter paper                                      | 0.39 ± 0.01                 | 0.37 ± 0.03                  |
| Secondary cellulose-containing raw materials      | 0.55 ± 0.01                 | 0.41 ± 0.01                  |
| Secondary starch-containing raw materials         | 1.00 ± 0.01                 | 0.52 ± 0.01                  |
| Miscanthus cellulose after alkaline pretreatment  | 0.54 ± 0.01                 | 0.75 ± 0.01                  |
| Miscanthus cellulose after acid pretreatment      | 0.90 ± 0.01                 | 0.67 ± 0.01                  |
| Wood chip pulp                                    | 0.43 ± 0.01                 | 0.38 ± 0.01                  |

The results obtained make it possible to correlate the level of enzymatic activity in the studied microorganism with a similar enzyme used on a commercial level. When comparing the cellulose activity of *A. niger F-1270* in relation to non-model substrates, it was found that the maximum conversion of cellulose to glucose is observed when using miscanthus cellulose with alkaline pretreatment, and the minimum conversion when using wood chips. The native strain used in this work exhibits stable but low cellitic activity, lower than when using an enzyme preparation (0.1 mg/cm³) under similar conditions.

In further studies, it was decided to enhance the cellulose activity of the native *A. niger* strain with undirected (uncontrolled) physicochemical mutagenic influences.

As a result of mutagenic action on *A. niger F-1270* with UV irradiation or UV irradiation in combination with subsequent treatment with a chemical mutagen, conditionally genetically modified samples of *A. niger F-1270* were obtained, the appearance of which is shown in figure 1.

![Figure 1](image_url)

*Figure 1.* Morphology of *A. niger* conidiophores: native (a); modified UV radiation (b); UV + chemically modified (c); magnification 400x.

A qualitative reaction using a Congo red solution showed an increase in the cellulolytic activity of the modified *A. niger* samples by increasing the intensity of the light zone around the colony (the dye
reacts with glucose obtained from the cleavage of cellulose contained in the medium). For this test, a variant of the medium containing no simple carbohydrates was used.

After mutagenic action, the spore suspension was separated and partly cultivated on a nutrient medium containing cellulose. The other part was cultivated on a medium containing potato waste. The total cellulase activity of the first passage was maximal in cultures that were exposed to UV treatment for two hours. It was confirmed that as the UV exposure time increases to two hours, the cellulose activity of the surviving part of the culture increases \((r = 0.706; p <0.05)\).

The results of studying the cellulolytic activity of the native \(A. \ niger\) strain and strains subjected to mutagenesis are presented in Table 2. Enzymatic hydrolysis in this case was carried out at a temperature of 37 °C. The comparison was carried out between three groups: a native strain (control), a strain treated with ultraviolet light for two hours (UV-120), and a strain that had been exposed to UV radiation and chemical action \(\text{NaN}_3 + \text{I}Br\) for 30 minutes (UV-120 + Chem-30).

An intergroup comparison showed a statistically significant increase in the level of enzymatic activity of mutant strains in comparison with the control (native strain) in relation to all studied substrates. The data obtained are consistent with the literature \([24, 28, 30, 32, 34, 42]\).

In this work, the highest enzymatic activity of relative secondary raw materials was found in the UV strain (1.5 U and 92.3 U / h). Meena et al. (2018) obtained comparatively similar data, where the cellulase activity of \(A. \ niger\) during the hydrolysis of fruit wastes was 64.5 U / h \([31]\). An increase in enzymatic activity was shown with the combined use of recombinant \(A. \ niger\) and \(T. \ reesei\) up to 12 U in the work by Zhao et al. \([41]\). In a study by Abdullah R. et al. cellulase activity of UV-modified Bacillus sp. increased to 11 U / h compared to control (7.5 U / h) \([29]\).

**Table 2.** Comparison of cellulase activity of native and mutant \(A. \ niger\) strains in relation to different substrates.

| Substrate                          | Control                      | UV - 120                    | UV-120+ Chem -30             |
|------------------------------------|------------------------------|-----------------------------|------------------------------|
|                                    | RS \(^a\), mg / h           | EA \(^b\), U/r              | RS, mg / h                   | EA, U/r                     |
| Filter paper                       | 0.37±0.03                   | 0.02±0.01                   | 0.36±0.02                    | 0.01±0.01                   | 1.78±0.14                   | 0.14±0.01                   |
| Secondary cellulose-containing raw materials | 0.41±0.01                   | 0.02±0.01                   | 2.41±0.01                    | 0.18±0.01                   | 2.41±0.01                   | 0.18±0.01                   |
| Secondary starch-containing raw materials | 0.52±0.01                   | 0.01±0.01                   | 16.99±3.86                   | 1.53±0.01                   | 3.76±1.01                   | 0.31±0.03                   |
| Miscanthus cellulose after alkaline pretreatment | 0.75±0.01                   | 0.03±0.01                   | 1.68±0.41                    | 0.12±0.01                   | 1.07±0.01                   | 0.06±0.01                   |
| Miscanthus cellulose after acid pretreatment | 0.67±0.01                   | 0.03±0.01                   | 0.61±0.01                    | 0.03±0.01                   | 4.83±0.01                   | 0.41±0.02                   |
| Wood chip pulp                     | 0.38±0.01                   | 0.02±0.01                   | 0.38±0.01                    | 0.02±0.01                   | 1.31±0.01                   | 0.08±0.01                   |

\(^a\)Reducing substance  
\(^b\)Enzymatic activity expressed in international units of enzyme activity

### 4. Conclusion

As a result of this work, mutant strains of \(Aspergillus \ niger \ F-1270\) with an increased ability to degrade cellulose-containing raw materials were obtained. Ultraviolet irradiation with an intensity of 253.7 nm for 120 min increases the enzymatic activity of mutant strains by 2–4 times compared to the native one.

The enzymatic activity of the strains obtained in this work relative to the primary raw material (miscanthus cellulose, wood chips) and filter paper in some cases is inferior to the commercial enzyme preparation, but there is a possibility of an increase in the total cellulase activity in the case of joint use.
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