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BIOCATALYSIS

Immobilized Fungal Biocatalysts for the Production of Cellulase Complex Hydrolyzing Renewable Plant Feedstock

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Abstract—We discuss characteristics of the directional formation of samples of heterogeneous biocatalysts based on immobilized cells of different microscopic fungi that are characterized by high productivity of cellulases with different substrate specificities (endoglucanases, exoglucanases, and beta-glucosidases). Samples of immobilized cells characterized by maximum productivity of the enzymes of cellulase complex are selected based on our study of the catalytic and operating characteristics of the designed biocatalysts. It is found that a biocatalyst based on *Aspergillus terreus* spores immobilized in polyvinyl alcohol cryogel is the best of the ones available. It is shown for the first time that the developed biocatalyst retains a high level of productivity for the full complex of cellulases when using various substrate inducers of enzyme biosynthesis, such as birch and oak sawdust, and rice and wheat straw. We demonstrate the possibility of efficiently using cellulase complexes obtained as a result of the functioning of immobilized cells in the saccharification of various cellulose-containing agricultural wastes and the conversion of the obtained sugars to organic solvents (ethanol, butanol) considered to be promising alternative fuels. The concentrations of organic solvents in media with immobilized cells are considerably higher than those found for free cells of the same microorganisms.

Keywords: biocatalyst, cellulases, immobilized cells, filamentous fungi, bioethanol, biobutanol

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INTRODUCTION

The production of biofuel from renewable cellulose-containing feedstock is now being widely discussed [1–3]. The process is based on the conversion of sugars formed during the enzymatic treatment of an initial substrate after physicochemical pretreatment. It was found in [4, 5] that using complexes of enzymes with different hydrolytic activities capable of the complex and deep destruction of components of the initial feedstock to ensure the maximum yield of monosaccharides is most efficient.

The most effective cellulase complex synthesized and secreted by microscopic fungi [6, 7] typically contains endoglucanase (EG), exoglucanase (CBH) and β-glucosidase (BG). EG cleaves the internal bonds in cellulose molecules by affecting the amorphous regions of cellulose fibers. CBH attacks cellulose and oligosaccharide molecules from the reducing terminus, successively cleaves cellobiose residues, and acts on crystalline regions of cellulose. BG hydrolyzes cellobiose molecules and soluble oligosaccharides to their final product, i.e., glucose [8].

Using complexes of cellulases consisting of enzymes of the three abovementioned main groups to treat cellulose-containing feedstock (CCF) allows us to attain the maximum degree of degradation of the initial substrate. Obtaining a full complex of cellulases via the cultivation of a producent is more economically attractive than obtaining individual cellulases by cultivating various producents and then preparing a mixture of cellulolytic enzymes, a so-called artificial complex [9].

Various cultures of filamentous fungi are the leading producers of industrial microbial cellulases [10]. Commercially available cellulase preparations are manufactured using representatives of the genus *Trichoderma* [11, 12]. However, a considerable drawback of these cellulase complexes is their relatively low content of the BG responsible for converting the intermediate products of enzymatic cellulose hydrolysis to the final product, glucose. [13]

In contrast to the filamentous fungi of the genus *Trichoderma*, representatives of the genus *Penicillium* synthesize cellulase enzyme complexes of a more balanced composition and efficiently cleave cellulose and cellulose-containing wastes, their individual enzymes having high operational stability [14–16].

Fungi of the genus *Aspergillus* biosynthesize cellulase complexes with a wide spectrum of action [17, 18], but the activity of most of the individual compo-

nents of a complex is not high enough to use these enzyme complexes as universal for all kinds of CCF.

There is thus clear interest in different filamentous fungi that produce cellulase complexes, but there are few universal enzyme preparations.

Enzyme biosynthesis in the traditional approach to solving the problem of their biotechnological production is accompanied by the growth of microorganism cells and biomass accumulation as a result of cultivation. This usually generates the need to solve problems associated with its utilization [19]. In addition, the time required for the biomass accumulation of filamentous fungi cells that produce the necessary enzymes is quite long (7–14 days), due to the low specific growth rates typical of these microorganisms [20].

Using another approach to the production of hydrolytic enzymes is attractive from the viewpoint of creating a base for fundamentally new technological solutions that allow us to simplify the process. This approach is based on the repeated and prolonged use of immobilized cells of filamentous fungi as a biocatalyst for enzyme biosynthesis and secretion with an inductor in the culture medium. In particular, the immobilization of producent cells simplifies and thus lowers the cost of separating the biomass from the enzyme-containing culture liquid. This, in turn, should help solve the economic and environmental problems associated with the regular production and utilization of filamentous fungi biomass.

It should be noted that immobilized filamentous fungi are hardly ever used in industrial processes, and there have been very few studies on the possibility of using immobilized filamentous fungi for synthesizing cellulolytic complex enzymes [21]. The reason for this is the lack of efficient procedures for selecting a carrier that can provide a long metabolically active state of the immobilized producents of cellulases. Adsorption on insoluble carriers [22] or the inclusion of filamentous fungi spores in calcium alginate gel [23] are used for the immobilization of filamentous fungi.

In this work, polyvinyl alcohol (PVA) cryogel was used for the first time as a carrier for immobilizing the filamentous fungi cells that are cellulose producents.

The aim of this work was to determine and cultivate the properties of highly heterogeneous biocatalysts based on immobilized cells of filamentous fungi that ensure the large-scale biosynthesis of cellulolytic enzyme complexes capable of the deep hydrolysis of various CCFs.

MATERIALS AND METHODS

In this work, we used the filamentous fungi *Mucor circinelloides* F-1627, *Rhizopus oryzae* F-873, *Fusarium oxysporum* F-2313, *F. solani* F-819, *Aspergillus terreus* F-728, *A. niger* F-679, *Trichoderma atroviride* F-207, and *T. harzianum* F-214, kept in an agarized medium of the following composition: 20 g/L glucose, 0.2 g/L $MgSO_4$, 0.2 g/L $CaCO_3$, 200 g potatoes, and 20 g/L agar (pH 6.8). To grow spores, the fungal cultures were plated on Petri dishes with an agarized medium. After sporulation, the cultures were stored at +4°C.

The filamentous fungi were grown at 32°C on a minimum medium containing (g/L): 1.4 $(NH_4)_2SO_4$, 0.3 $MgSO_4 \times 7H_2O$, 0.0014 $ZnSO_4 \times 7H_2O$, 2.0 KH_2PO_4, 0.3 $CaCl_2 \times 2H_2O$, 0.005 $FeSO_4 \times 7H_2O$, 0.0016 $MnSO_4 \times H_2O$, 0.02 $CoCl_2$, 1.0 peptone, 2.0 Tween-80, and 2% microcrystalline cellulose (MCC) as a carbon source.

The filamentous fungi cells were immobilized in PVA cryogel following the method developed in [24] for the cells of filamentous fungi responsible for pectinase biosynthesis.

To obtain highly active samples of immobilized biocatalysts containing the filamentous fungi spores and PVA, however, up to 1% of delignified corn stalks (humidity 68 ± 2%) relative to the total mass was introduced into the pellets during their formation.

In this work, we used the thermotolerant cells of *Saccharomyces cerevisiae* T2 yeast to obtain ethanol from CCF hydrolysates prepared under the action of the cellulases of immobilized fungi cells.

To accumulate yeast biomass, we used a cultivation medium of the following composition (g/L): 20 glucose, 5.0 yeast extract, and 10 tryptone.

The yeast cells were cultivated at 26°C under aerobic conditions right to the end of the logarithmic growth phase under constant stirring (150 rpm) using an IRC-1-U thermostated shaker (Adolf Kunner G Apparaebau, Switzerland). The resulting yeast biomass was separated at 10000 rpm for 10 min on a Beckman 2-21 centrifuge (United States) and then used for immobilization in PVA cryogel following the procedure developed in [25] for the yeast cells responsible for alcoholic fermentation.

To obtain organic solvents (ethanol, acetone, and butanol) from CCF hydrolysates prepared under the action of cellulases of immobilized fungi cells, we used immobilized cells of *Clostridium acetobutylicum* B-1787 bacteria. The biomass of *C. acetobutylicum* cells required for their immobilization in PVA cryogel was accumulated under anaerobic conditions at 37°C in a medium of the following composition (g/L): 10 peptone (tryptone), 5 yeast extract, and 25 glucose. The composition of the biocatalyst was the same as described in [26]. Prior to fermentation, the pH in the CCF hydrolysates was adjusted to 7 and the total glucose content to 45 g/L. Nutrition components were introduced, and anaerobic fermentation was performed at 37°C for 96 h.

In our experiments, pH was monitored potentiometrically using a PBL model pH meter (Switzerland).

The CCFs were pretreated by grinding in an activator-type planetary ball mill for 5 min (centrifugal acceleration, 300 m/s^2; rotation frequency, 1290 rpm). To monitor the effectiveness of the pretreatment of various CCF samples, the particles were examined using an optical microscope. The particle sizes that were attained lay in the range of 5–80 μm.

The CCFs were delignified with 10 M NaOH for 3 h at 80°C.

The glucose concentration in the medium was determined from glucosidase levels using a standard reagent (Impakt, Russia).

To quantitatively assess the cellulase activity of endo- and exoglucanases, we determined the amount of reducing sugars formed under the action of cellulase on carboxymethylcellulose (CMC) and microcrystalline cellulose (MCC). The reducing sugars were determined using a modified Somogyi–Nelson test [27].

The β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as a substrate. Our method was based on determining the rate of formation of *p*-nitrophenol released under the catalytic action of β-glucosidase on the substrate [28].

The mass of dry pellets with immobilized cells was determined following the procedure in [29].

The concentration of glucose, which in theory can be obtained through the enzymatic hydrolysis of CCFs, was calculated from the CCF chemical composition, i.e., the cellulose content as determined in [3, 30].

The concentrations of ethanol and butanol were determined via gas chromatography using a Shimadzu chromatograph (Japan) with a flame ionization detector. Nitrogen was used as the carrier gas. The temperature of the thermostated columns was 190°C, while that of the detector and evaporator was 200°C.

RESULTS AND DISCUSSION

Biocatalysts based on filamentous fungi cells immobilized in PVA cryogel. At the initial stage of developing samples of immobilized biocatalysts capable of biosynthesis and the secretion of cellulases, we selected three cultures of filamentous fungi belonging to different genera: *Aspergillus terreus*, *Fusarium solani*, and *Mucor circinelloides* (Fig. 1).

Three strains selected from our seven investigated natural strains of filamentous fungi were characterized by having the three main types of cellulases (EG, CBH, and BG) in the synthesized complexes (Fig. 1). Further use of the cells of the three genera of filamentous fungi for biocatalyst production was required to determine the possibility of the same procedure for immobilization in PVA cryogel affecting the cells' ability to perform biosynthesis and the secretion of cellulases differently. To do this, prepared samples of biocatalysts were placed in a medium containing 1% delignified corn stalks or 2% birch sawdust as a carbon source.

The immobilized filamentous fungus *A. terreus* exhibited the highest cellulolytic activity at different stages of cultivation (Fig. 2). It should be noted that the different levels of the detected cellulase activities and the kinetics of the accumulation of these activities in the culture medium pointed to differences in the composition of enzyme complexes synthesized by immobilized and free cells of the same filamentous fungi (see Figs. 1 and 2).

In addition, differences were revealed in the kinetics of cellulase activity accumulation of in the free and immobilized fungi cells. The accumulation of cellulases in the culture medium thus began much earlier in the immobilized cells. The higher and more rapid productivity of immobilized cells could be due to the immobilized cells of filamentous fungi being better adapted to the synthesis of enzymes. It is known that filamentous fungi functioning in solid-phase cultivation are characterized by high metabolic activity [31, 32].

However, solid-phase fermentation is known to have a number of drawbacks when compared to the submerged cultivation method; the most important of these is the need for longer cultivation of the cells [33, 34].

Moreover, the subsequent extraction of cellulase from the substrate mass is required in the solid-phase cultivation of fungi. The submerged cultivation of free cells allows us to avoid this step, since the cells secrete cellulase directly into the liquid culture medium. Immobilizing the cells and cultivating them in liquid media combines the advantages of submerged cultivation and solid-phase fermentation, since PVA cryogel acts as a solid support for the mycelium and stimulates the continued high-level biosynthesis of cellulases and their secretion into the liquid medium. The use of immobilized forms of the fungus can intensify the submerged cultivation of filamentous fungi. The most productive of these is the strain of *A. terreus* filamentous fungus that can perform biosynthesis and the accumulation of extracellular enzymes of cellulase complex in both the free and the immobilized form.

In order to raise the productivity of immobilized biocatalyst, inductors of the biosynthesis of cellulolytic enzymes were introduced directly into its composition; delignified corn stalks (1% of the total mass of a pellet) were used as the inductors.

It was shown that *A. terreus* cells, spores of which were immobilized in PVA cryogel with the addition of cellulose material, ensured the biosynthesis and secretion of the full range of cellulases containing EG, CBH, and BG, but the level of cellulase activities for such immobilized biocatalyst was 60% higher than the level of the same biocatalyst when it initially did not contain the cellulose material (Fig. 3).

We thus established the expediency of simultaneously incorporating a carbon source that induces cellulase synthesis into pellets and immobilized cells of filamentous fungus. We used such cellulose-con-

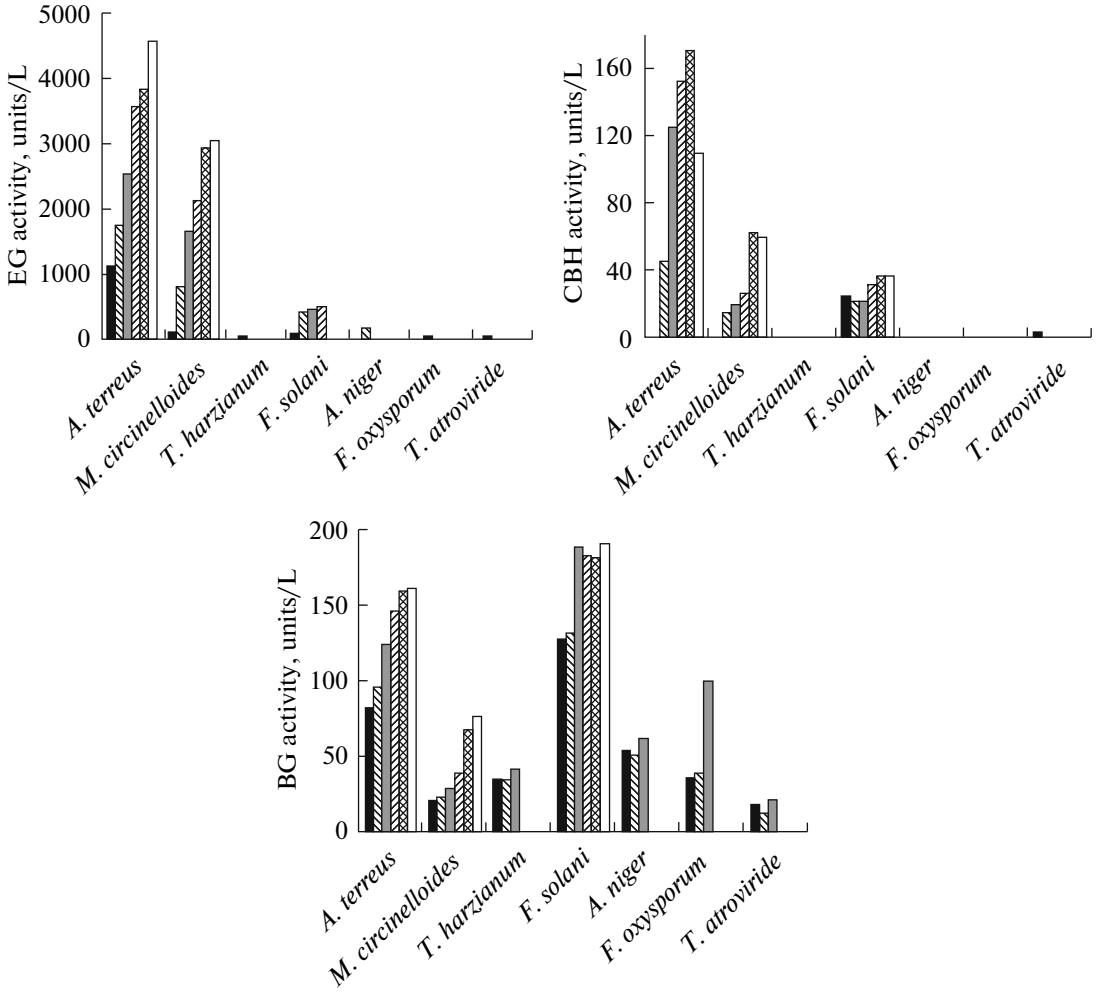

Fig. 1. Cellulase activities detected in media with filamentous fungi after (■) 2, (▧) 5, (▩) 7, (▨) 9, (▩) 11, and (□) 13 days of cultivation (with a medium of 1% delignified corn stalks used as a carbon source).

taining immobilized biocatalysts in subsequent experiments.

Biosynthesis of cellulase enzymes by immobilized cells of *A. terreus* filamentous fungus in media with different CCF samples. Analysis of the effect of a culture medium's pH and the process temperature allowed us to determine the following optimum conditions necessary for accumulation of the maximum activity of cellulase complex enzymes in a medium containing MCC as a carbon source (pH = 5.0–5.5; process temperature 28°C). The maximum values of EG-, CBH-, and BG-activities obtained under these conditions were 9250, 720, and 400 units/L, respectively (Table 1). Further experiments were performed under these conditions.

Since samples of various industrial and agricultural wastes with complex chemical composition were planned to be used as substrates for the enzymes of cellulase complexes secreted by our biocatalyst, further studies were conducted using just such substrates. In cultivating immobilized cells of filamentous fungus in a medium containing 2% crushed corn stalks, the maximum values of endoglucanase, exoglucanase, and β-glucosidase activities fell slightly, relative to the activities observed in cultivating the same samples of immobilized cells in a medium containing purified MCC as the inductor of cellulase synthesis (Table 1).

This tendency was obviously due to the presence of natural inhibitors of cellular metabolism and cellulases in the natural substrate that we used, in contrast to commercially available purified preparations. Similar tendencies in the changing activity levels of cellulases produced by free cells were reported in [10].

Analysis of our results showed that raising the concentration of substrate (sawdust) in the culture medium from 2 to 5% (on dry substances) had a positive effect on the level of EG activity accumulation, resulting in a twofold increase.

During cultivation of immobilized cells in a medium containing the crushed rice straw as a source of carbon needed for the cellulase biosynthesis, the maximum activities of EG, CBH, and BG accumu-

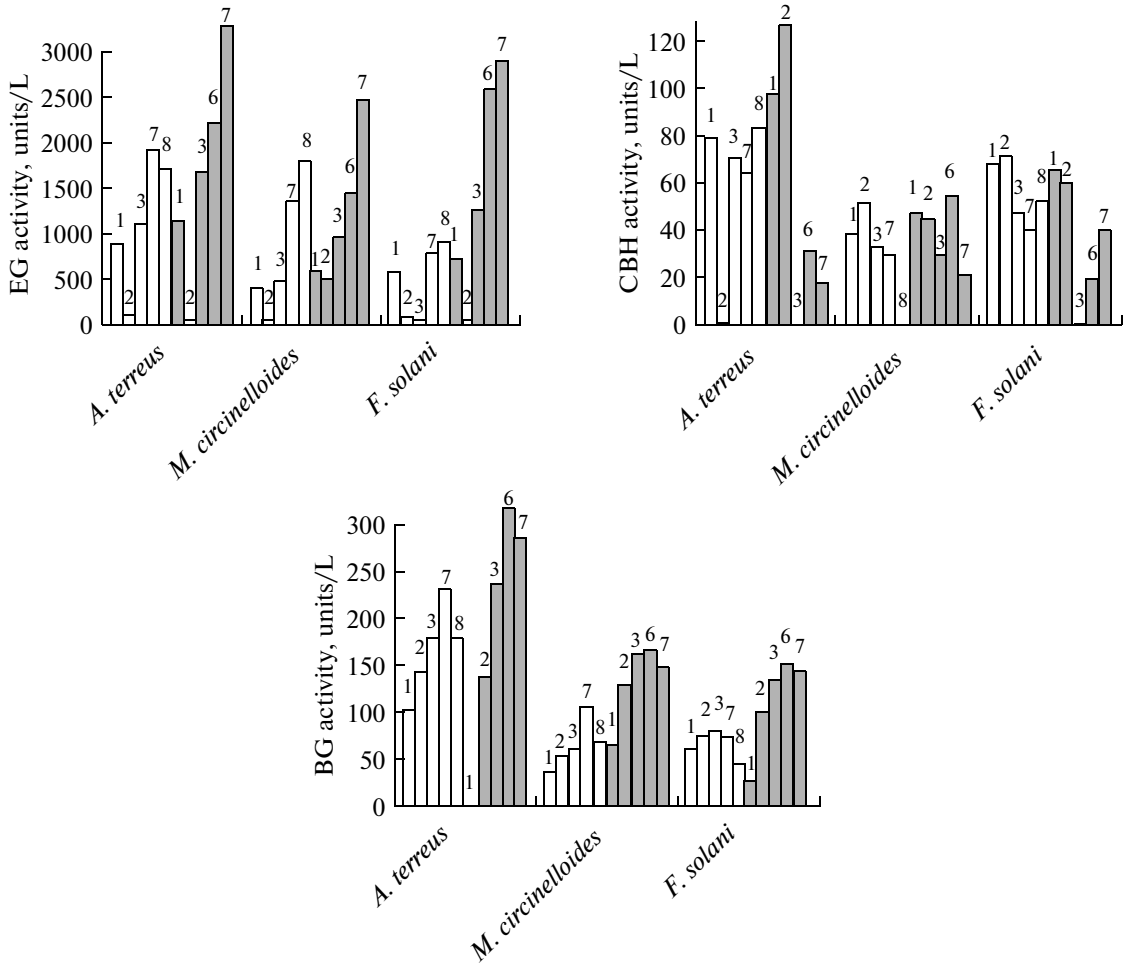

Fig. 2. Cellulase activities detected in media with immobilized biocatalysts: (□) medium with 1% delignified corn stalks used as a carbon source; (■) medium with 2% birch sawdust. Numbers above the columns indicate the number of days from the beginning of cultivation with respect to the time of analyzing the enzymatic activity.

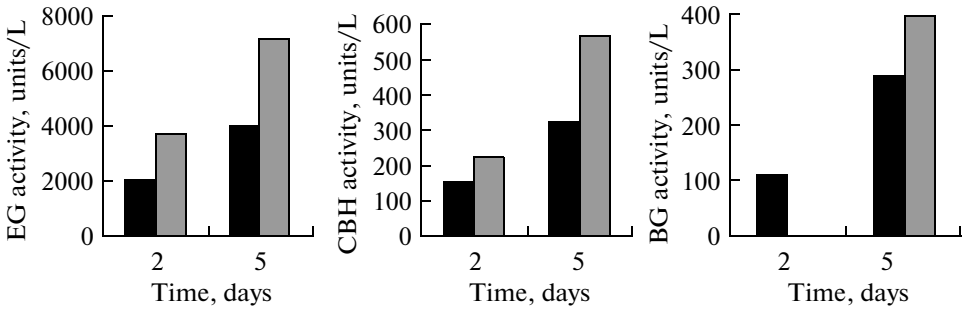

Fig. 3. Accumulation of cellulase activity in a medium for the cultivation of samples of immobilized biocatalyst based on *A. terreus* cells (■) with and (■) without an inductor of cellulase synthesis (1% delignified corn stalks).

lated in the culture fluid exceeded the levels observed on our other investigated substrates. It should be also noted that the greatest changes in the level of detected activity were observed for EG upon varying the substrates introduced into a medium to produce cellulase complex.

Repeated use of immobilized cells for producing enzymes of cellulase complex. It is known that immobilized cells are worthy of attention due to the possibility of their repeated use in implementing various biocatalytic processes [35, 36]. In this regard, we investigated the possibility of the prolonged use of

Table 1. Maximum values of the activity of enzymes of cellulase complex, detected after cultivating immobilized cells for 6 days in media containing different carbon sources

Concentration of CCFs in medium (dry substances), %	Activity, units/L		
	EG	CBH	BG
MCC, 2	9250	720	400
Corn stalks, 2	8540	550	365
Delignified oak sawdust, 2	530	90	65
Birch sawdust, 2	3680	550	390
Birch sawdust, 5	7100	550	330
Rice straw, 5	13390	840	400

Table 2. Concentrations and yields of glucose in fermentolysates of different CCFs obtained using a culture medium containing cellulases of *A. terreus* immobilized cells

CCF	Glucose	
	concentration, g/L	yield relative to the one theoretically possible, %
Corn stalks	16.8 ± 0.2	81.9 ± 0.9
Wheat straw	17.1 ± 0.3	96.0 ± 1.7
Rice straw	18.0 ± 0.1	95.2 ± 0.5
Birch sawdust	14.5 ± 0.3	59.4 ± 1.2
Oak sawdust	13.9 ± 0.3	51.1 ± 1.1

immobilized cells of filamentous fungi to produce cellulase complex in media containing rice straw as a carbon source (Fig. 4).

It was found that the biocatalyst can continuously perform biosynthesis and the secretion of enzymes of cellulase complex in batch cultivation over at least five operating cycles with complete replacement of the culture medium at the end of each operating cycle. In terms of EG, CBH, and BG activities, the average productivity of the process using immobilized cells was 1200, 70, and 30 units/(L/day), respectively. The half-inactivation period of our immobilized biocatalyst was 50 days.

Culture fluids containing a complex of cellulases secreted into the culture medium for immobilized cell-based biocatalysts were used for the targeted hydrolytic treatment of various CCF samples.

It should be noted that as an inducer of enzyme synthesis, the culture medium for the production of cellulases contained a CCF source that was later treated with the obtained complex.

The CCF concentration in the medium was 50 g/L dry substances. The dosage of culture fluid containing cellulases and used for CCF treatment was normalized to the total protein concentration at 15 mg of total protein per 1 g of dry CCF substance. Enzymatic hydrolysis was performed at 45°C and pH 5 for 40 h. After the process was complete, the concentration of accumulated glucose was analyzed for all of our samples (Table 2).

According to our data, the maximum glucose concentration (18 g/L) was attained during the enzymatic treatment of rice straw with cellulase complex produced by immobilized cells of filamentous fungi. These data are consistent with the results presented in Table 1, suggesting that this substrate ensures the highest level of accumulation of the three major cellulase activities. The yield of glucose in this case was 95% of the theoretically possible level. When using sawdust as a degradable substrate, the yield of glucose was more than 50% of the theoretical level, due possibly to insufficient initial pretreatment of this feedstock prior to enzymatic hydrolysis.

Producing organic solvents from enzymatic hydrolysates of CCFs under the action of immobilized yeast and bacteria cells. Enzymatic hydrolysates produced as a result of treating various types of CCFs with cellulases secreted by immobilized cells of fungi can be used to obtain various commercially important products, e.g., organic solvents (ethanol, acetone, and butanol).

Experiments in which the immobilized yeast or bacteria cells were used as catalysts in the transformation of the obtained sugars have confirmed this [37].

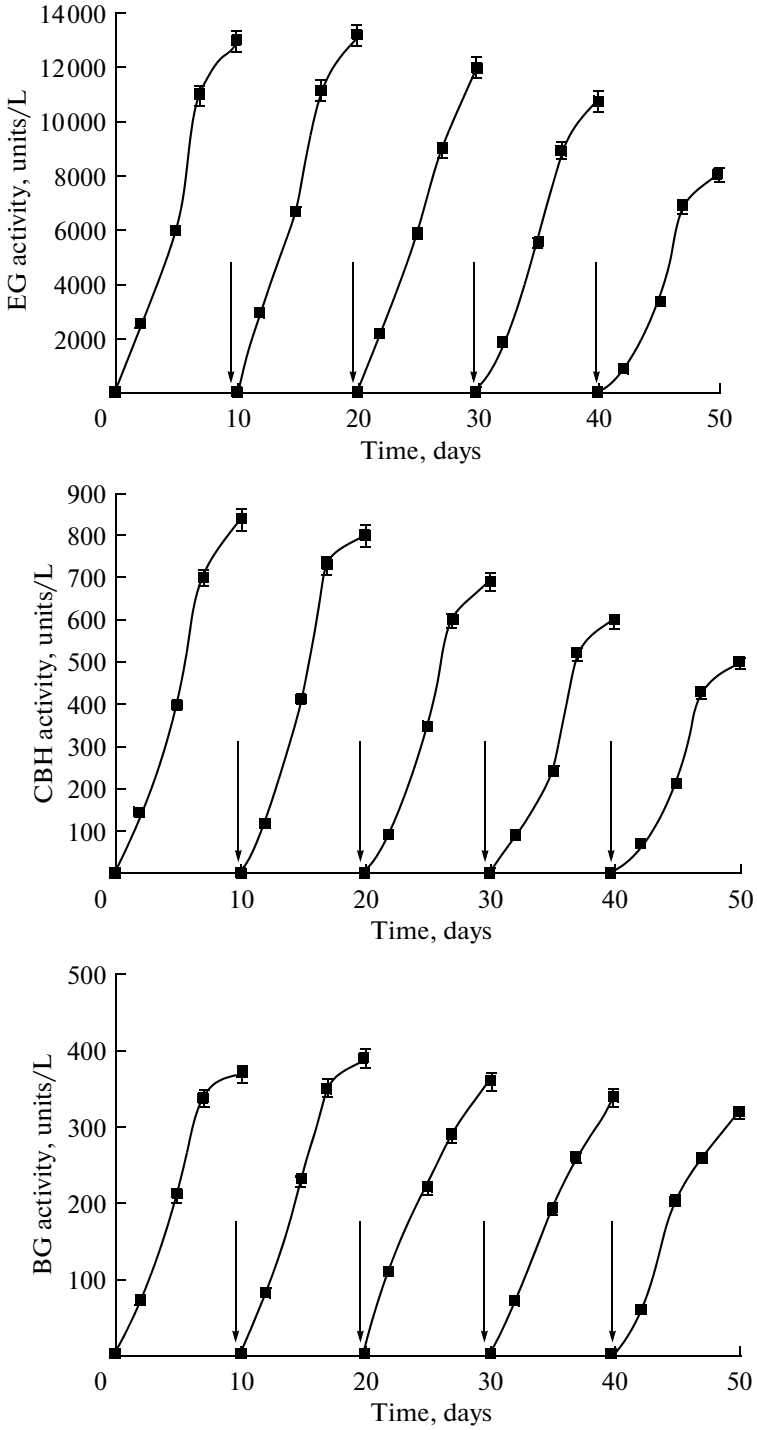

Fig. 4. Dynamics of the accumulation of cellulase activities in a medium of batch cultivation with repeated use of immobilized biocatalyst. Arrows indicate replacement of the medium in a reactor with immobilized cells producing cellulases.

Immobilized cells of thermotolerant yeast were used to ferment hydrolysates of various CCFs (Table 3). The reaction mixture was prepared on the basis of 0.05 M citrate buffer (pH 5); CCF (5% relative to dry substance) and cellulase complex in a concentration of 15 mg of the total protein per 1 g of CCF dry substance were added to it. The process was conducted at 45°C for 48 h. After it was complete, the concentration of accumulated ethanol was determined for all of the samples (Table 3).

According to our data, the yield of the target product was 86–91%, depending on the initial CCF source.

We thus demonstrated the possibility of the highly efficient conversion of CCF hydrolysates, obtained

Table 3. Concentration and yield of ethanol during the conversion of glucose contained in the CCF hydrolysates to ethanol under the action of immobilized yeast cells

CCF	Ethanol	
	concentration, g/L	yield relative to the one theoretically possible, %
Corn stalks	7.7 ± 0.2	89.8 ± 2.3
Wheat straw	7.9 ± 0.1	90.6 ± 1.1
Rice straw	8.4 ± 0.1	91.5 ± 1.1
Birch sawdust	6.4 ± 0.3	86.5 ± 4.0
Oak sawdust	6.1 ± 0.2	86.0 ± 2.2

Table 4. Concentration of organic solvents accumulated during the acetone–butanol–ethanol fermentation of enzymatic CCF hydrolysates under the action of immobilized bacterial cells

CCF	Concentration, g/L		
	acetone	butanol	ethanol
Corn stalks	3.0 ± 0.2	9.4 ± 0.3	0.5 ± 0.1
Wheat straw	3.6 ± 0.3	10.2 ± 0.2	0.9 ± 0.2
Rice straw	3.8 ± 0.3	11.6 ± 0.2	1.2 ± 0.3

under the action of cellulases of immobilized cells of filamentous fungi, into bioethanol.

To investigate the possibility of transforming various CCFs into organic solvents, we used immobilized biocatalyst based on *Clostridium acetobutylicum* cells incorporated into PVA cryogel (Table 4).

The maximum concentration of solvents (11.6 g/L) was achieved in an enzymatic hydrolyzate of rice straw. It should be noted that the correlation between the concentrations of solvents remained virtually the same regardless of the type of treated CCF.

CONCLUSIONS

A unique heterogeneous biocatalyst has been developed in the form of cells of *Aspergillus terreus* filamentous fungus immobilized in PVA cryogel with the addition of a cellulase complex synthesis inductor containing endoglucanase, eoglucanase, and β-glucosidase. This biocatalyst has a long period of half-inactivation (up to 40 days) when used four consecutive times for producing cellulase complex in the batch mode.

It was demonstrated for the first time that our biocatalyst can continuously maintain a high level of productivity for the full complex of cellulases when using different substrate inducers of enzyme biosynthesis, e.g., birch and oak sawdust, and rice and delignified corn stalks.

Culture fluids containing cellulase complexes obtained as a result of the action of our immobilized biocatalyst in a medium with a specific CCF were used for the targeted hydrolytic treatment of the same feedstock.

The possibility of the highly efficient conversion of CCF hydrolysates into organic solvents under the action of immobilized yeast and bacteria cells has been demonstrated.

ACKNOWLEDGMENTS

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