Immobilized *Saccharomyces bayanus* yeast cells in ethanol production from hydrolyzates of agricultural wastes

N. Stepanov[1,2], A. Skomarovsky[2], A. Sinitsin[2], E. Efremenko[2,3*]
[1] Moscow State University of food production, Moscow, Russia
[2] The M.V. Lomonosov Moscow State University, Moscow, Russia
[3] The N.M. Emanuel Institute for Biochemical Physics, Russian Academy of Sciences, Moscow, Russia
E-mail: efremenko@enzyme.chem.msu.ru

Introduction

The rapid development of bioethanol production in the world is observed due to sharp growth of petroleum cost and intensive economic advancement of Asia countries (Chine and India) provoking further increase in demand for oil and oil products. Bioethanol can be added into petrol up to 10% without modification of motors. The toxicity of motor exhaust can be reduced by 40-50% (Gray et al., 2006).
Ethanol as a efficient part of petrol is already used in Brazil, Germany and USA. Around 5 million cars used fuel E-85, containing 15% ethanol in USA. In nature, ethanol can be degraded more promptly as compared as compared to petrol and many other known fuel additives (Fulton, 2004). Today the general volume of ethanol produced in the world is 40 milliard liters actually, whereas the total demand for ethanol is 50-times higher than that volume (Zervos et al., 2004).
To obtain ethanol various agricultural crops are used (Pimentel et al., 2005), whereas the cellulosecontaining agricultural wastes are considered to be effective sources for ethanol production (Olsson et al, 1996; Sun et al, 2002; Kim et al., 2004). The later process consists of two main steps:

- hydrolysis of cellulose and hemicellulose
- fermentation of glucose containing hydrolyzates

The principal problem appeared in the ethanol production from cellulose hydrolyzates is the pronounced inhibiting effect of various substances (furfural, tannins, acetic acid, laevulinic acid, formic acid, humic substances, etc.) on fermentation catalyzed by microorganisms (Clark et al., 1984; Olsson et al, 1996).
Immobilized microorganisms possessing increased resistance to the action of toxic substances, can be used for fermentation of the hydrolyzates. The yeast cells *S.cerevisiae ph.r bayanus* immobilized into cryogel of poly(vinyl alcohol), characterized by high mechanical strength, chemical resistance and high operational stability of developed systems (Lozinsky et al., 1998), were tried in the work, when various enzymatic hydrolyzates of wastes of agriculture and pulp-and paper industry were used.

Materials and methods

The *Saccharomyces cerevisiae ph.r bayanus* cells (AEB group, Switzerland) were used in the work. The cells were kept at 4^{o}C in refrigerator. To accumulate cell biomass for biocatalyst production, the cells were cultivated in the following medium (g/L): glucose - 2.5; yeast extract - 2.0; NaCl - 1.0; $(NH_4)_2SO_4$- 2.0; $MgSO_4x7H_2O$ - 1.0; KH_2PO_4- 13.5. Cultivation was done under aerobic conditions at 20^{o}C for 24 h on a shaker (Lab-therm, Adolf Kühner, Switzerland) with constant agitation (220 rpm). Yeast biomass was separated from the cultivation broth by centrifugation (10,000 g, 10 min, Beckman J2-21 centrifuge, USA), and immobilized into polyvinyl alcohol cryogel (PVA CG) according to previously patented procedure (Efremenko, et al., 2006).
Fermentation was carried out under anaerobic conditions at 30^{o}C for 48 h. The tubes with fermented media were periodically opened and samples were analyzed for ethanol content. All

analyses were carried out in triplicate and the average value of each controlled parameter was calculated.
Ethanol was estimated by HPGC (Hewlett Packard HP 4890D, Poropak Q) using nitrogen as a carrier gas and hydrogen gas in the flame detectors, both at a flow rate 32 ml/min.
Intracellular ATP concentration in the free and immobilized yeast cells was determined using bioluminescent method and luciferine-luciferase reagent (Lumtek, Russia). To extract ATP from yeast cells, an aliquot (0.1 ml) of cell suspension in fermentation medium was treated with 0.9 ml of dimethylsulfoxide (DMSO). The determination of ATP concentration in immobilized cells included the treatment of weighted granules with 1 ml of DMSO. Cell extracts (50 μl) were added to the cuvette with an aliquot of luciferase reagent (50 μl) and the intensity of bioluminescence was measured on a microluminometer 3560 (New Horizons Diagnostic, USA). ATP concentration in the samples was calculated using the calibration graphs plotted with ATP standards.

Results and discussion

The fermentation of cellulose-containing substrates, treated with various enzymatic preparation, by immobilized cells was investigated herein. The following materials were used in the work as main substrates: bagasse, beet pulp fibers, parchment and wheat straw (Table 1).

Main substrate	Enzyme prepara-tion	Yeast cells	Ethanol yield, %
Bagasse pH 5.75	B 221-151	Free	64.7
		Immobilized	79.2
	BIOACE	Free	75.9
		Immobilized	89.7
Beet pulp fibers pH 5.0	B 221-151	Free	76.6
		Immobilized	81.9
	BIOACE	Free	86.2
		Immobilized	83.6
Parch-ment pH 5.3	B 221-151	Free	80.4
		Immobilized	82.6
	BIOACE	Free	82.4
		Immobilized	88.0
Wheat straw pH 5.0	B 221-151	Free	81.1
		Immobilized	90.9
	BIOACE	Free	82.4
		Immobilized	91.6
Bagasse pH 5.5	B 221-151	Free	85.2
		Immobilized	72.6
	BIOACE	Free	54.1
		Immobilized	72.7
Bagasse pH 6.5	B 221-151	Free	67.2
		Immobilized	96.0
	BIOACE	Free	65.9
		Immobilized	78.9

Table 1. Ethanol yield after 48 h fermentation of enzymatic hydrolyzates of agriculture and pulp-and paper industry wastes

The treatment of substrates was carried out at 50°C for 40 h using metal ball (diameter 0.5 cm) with constant agitation at 250 rpm. Enzymatic preparation were applied: BIOACE complex preparation, containing cellulases, isolated from *Trihoderma reesei* cells and B-221-151 complex preparation, containing cellulases, isolated from *Penicillium variable* cells. All enzymatically obtained hydrolyzates were centrifugated before use at 10000 rpm for 10 min. The initial concentration of free and immobilized cells was actually the same and equal to $(2.0\pm0.1)\times10^6$ cell/ml.

It was established that yield of ethanol from theoretical level was more than 70% in all samples with immobilized cells (Table 1). That fact testified to very active metabolic state of immobilized yeast cells. The maximum yields of ethanol was obtained in the case of Bagasse (pH 6.5), treated preparation B-221-151, and wheat straw, treated by preparation BIOACE (96 and 91,6 % respectively). It should be noted that ethanol yields reached by other investigators, worked with various hydrolyzates of bagasse, wheat straw were 50-80% from theoretical level, when free cells were applied for fermentation process (Martín et al., 2002). So, results obtained in the works for immobilized cells were notably better as compared to previously known data for free cells under similar conditions.

The fermentation of enzymatic hydrolyzates of corn-cob, treated by various complexes of cellulases, hemycellulases and pectinases (Table 2) also was investigated in the work. Enzymatic preparation were applied: B1 complex preparation, containing cellulases and hemicellulases isolated from *Penicillium verruenlosum* cells, PCA complex preparation, containing hemycellulases and pectinases isolated from *Penicillium canescens* cells, Genecor (USA) complex preparation, containing cellulases and Novozyme (Denmark) complex preparation, containing amylases and β-glucosidase.

Enzymes used for corn-cob pretreated	Yeast cells	Ethanol yield, %
B1	Free	39.0
	Immobilized	45.4
B1+ PCA	Free	48.7
	Immobilized	74.7
Genencor	Free	58.5
	Immobilized	94.2
B1 + Novozyme	Free	87.4
	Immobilized	98.3

Table 2. Ethanol yield after fermentation of enzymatically pretreated corn-cob solution by free and immobilized yeast cells for 48 h

The experiments were carried out with concurrent application of free and immobilized yeast cells. The solutions of hydrolyzates were centrifugated (10000 rpm, 20 min) before their use. The concentration of both free and immobilized cells was the same and equal to $(2.0\pm0.1)\times10^6$ cell/ml.

The yield of ethanol was higher by 6.4-37.7% in the samples with immobilized yeast cells as compared to probes with free yeast. The most effective fermentation was observed in hydrolyzates obtained with mixture of B1 and Novozyme.

The data of analysis of intracellular ATP concentration in free and immobilized cells (Table 3) confirmed the active state of immobilized cells. That allowed multiple use of the cells in ethanol production.

Yeast cells	[ATP], mole/mg of cells
Before fermentation	
Free	$(4.5\pm0.5)\times10^{-10}$
Immobilized	$(3.9\pm0.2)\times10^{-10}$
After fermentation	
Free	$(1.5\pm0.2)\times10^{-11}$
Immobilized	$(1.9\pm0.5)\times10^{-10}$

Table 3. Intracellular ATP concentration in yeast cells before and after their use in fermentation process for 48 h

Conclusions

Thus, it was shown, that immobilized yeast cells could be successfully used for ethanol production from various cellulose-containing materials treated by preparation with various active complexes of cellulases.

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