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Immobilised cells of *Pachysolen tannophilus* yeast for ethanol production from crude glycerol

Nikolay Stepanov, Elena Efremenko*

Department of Chemistry, Lomonosov Moscow State University, 1/3, Lenin Hills, 119991, Moscow, Russia

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ABSTRACT

Screening among naturally occurring yeast strains of Pachysolen spp. that are capable of producing ethanol from glycerol under aerobic conditions identified the most active culture, *P. tannophilus* Y −475. Conversion of glycerol by this producer immobilised in poly(vinyl alcohol) cryogel resulted in a 90% yield of ethanol relative to the theoretical limit. The maximum rate of alcohol accumulation was $0.64 \pm 0.01\ g\,L^{-1}\,h^{-1}$ at a $25\ g\,L^{-1}$ concentration of glycerol in the culture medium. We demonstrated the efficacy of reusing immobilised cells (for a minimum of 16 working cycles for batch mode of crude glycerol conversion to ethanol) and the possibility of long-term (for a minimum of 140 h) use of the cells in continuous mode with a maximum process productivity of $0.63 \pm 0.02\ g\,L^{-1}\,h^{-1}$, while the medium dilution rate in the reactor was $0.062 \pm 0.001\ h^{-1}$. Reduction of metabolic activity did not exceed 5–7% relative to baseline. Immobilised cells were demonstrated to withstand long-term storage in frozen form for at least 2 years while retaining high metabolic activity.

1. Introduction

The growing global demand for biodiesel fuels has resulted in the sustained growth of production volumes year after year. However, this growth is accompanied by the generation of large volumes of crude glycerol as a by-product (100 kg per each tonne of biodiesel). Such a source of glycerol and the appearance of the latter on the global market exert strong downward pressure on its price [1], making glycerol a readily accessible and competitive substrate for manufacturing commercially important products, such as bioethanol, which is increasingly used as a fuel additive [2].

Citrobacter spp., *Klebsiella* spp., *Enterobacter* spp. and *Escherichia* spp. are capable of anaerobic conversion of glycerol to ethanol; nevertheless, with these bacteria, the rate of the process and yield of the finished product is extremely low. This low yield is compounded by the need to maintain strictly anaerobic cultivation conditions by purging culture media with nitrogen [3].

As *Saccharomyces cerevisiae* yeast, which are traditionally used in ethanol fermentation technology, cannot directly convert glycerol to ethanol, studies were undertaken to produce recombinant *Saccharomyces cerevisiae* YPH499, which convert glycerol to produce ethanol in the culture medium at a concentration of $2.4\ g\,L^{-1}$ over 96 h, process productivity being $0.025\ g\,L^{-1}\,h^{-1}$ [4]. When other recombinant cells of *Hansenula polymorpha* yeast were used, ethanol was produced from glycerol over 8 h with a process productivity of $0.39\ g\,L^{-1}\,h^{-1}$ [5].

However, industrial-scale application of genetically modified microorganisms is always associated with a range of significant limitations which are predominantly connected with environmental issues [6]. Owing to this, there is a huge interest in natural strains producing ethanol from glycerol. Another natural yeast strain, *Pachysolen tannophilus* CBS4044, is known to convert glycerol to ethanol ($17.5\ g\,L^{-1}$) under aerobic conditions with a yield of 56% relative to theoretical limit. However, this conversion is characterised by a relatively low process productivity of $0.16\ g\,L^{-1}\,h^{-1}$ [7].

Accordingly areas of interest include screening for new ethanol producing strains and searching for new ways to boost productivity of known producers. To increase the efficiency of the productivity of this process it seems appropriate to use immobilized cells forms. The advantages of using these forms compared with free cells are shown in the ethanol fermentation [8,9]. Additionally, glycerol obtained as a waste product of biodiesel production contains high levels of contaminants, in particular, methanol (up to 50%), which is a cytotoxic substance [10,11].

Production of effective and stable biocatalysts is important to make a right choice of support for cell immobilization. Poly(vinyl alcohol) (PVA) cryogel is a promising cell carrier that can be

* Corresponding author.
E-mail address: elena_efremenko@list.ru (E. Efremenko).

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successfully applied for cell immobilisation of different types of microorganisms [12]. Unique features of this support include its macroporous structure and high strength of PVA cryogel produced by freezing and subsequent thawing of a polymer solution. The carrier pore size can be tailored to the size of immobilised cells by adjusting the concentration of polymer solutions [13].

In view of the above, the purpose of this work was to develop and use a biocatalyst in the form of PVA cryogel-immobilised *Pachysolen tannophilus* yeast that can efficiently transform glycerol to ethanol in the batch mode and continuous mode of cultivation.

Material and methods

Pachysolen tannophilus yeast strains (Y-2246, Y-475, Y-476, Y-3269) were used in this work, which were obtained from the Russian National Collection of Industrial microorganisms [http://eng.genetika.ru/service-offer/vkpm/].

The batch cultivation was carried out under aerobic conditions using Adolf Kuhner AG (Switzerland) and BioSan ES-20 (Lithuania) temperature controlled shakers-incubators that allow for continuous agitation of the culture medium (200 rpm).

Culture medium of the following composition (g L^{-1}) was used to accumulate yeast biomass at 28 °C: glucose(20); yeast extract (5); tryptone (10), pH 5.6.

Cells were immobilised in PVA cryogel according to a technique previously developed for yeast cells (Efremenko et al., 2011). In accordance with this procedure, yeast biomass (10%, w w^{-1}) was thoroughly mixed with (11%, w w^{-1}) aqueous PVA solution and pipetted into 96-well microplates at 0.2 mL per well. After filling, these microplates were placed in a freezer at −80 °C for 24 h and then thawed. This procedure yielded biocatalyst granules with the shape of microplate wells.

Crude glycerol was obtained in our laboratory as a by-product of biodiesel production from the lipids of filamentous fungi used for treatment of complex food wastewater [14]. The crude glycerol before use was pre-treated and purified by solvent extraction according to the procedure [15]. The final glycerol feedstock contained 6.5% ash, 0.5% methanol, 10% water and 83% glycerol, and it was used as a sole carbon source.

Conversion of glycerol to ethanol in batch mode was carried out in Erlenmeyer flasks (750 mL) under continuous agitation (200 rpm) for 24 h at 28 °C in a medium (100 mL) of the following composition: glycerol feedstock was added to the medium to a final concentration of 10–60 g L^{-1}; yeast extract (5 g L^{-1}), tryptone (10 g L^{-1}), pH 5.6.

Continuous-mode process utilised a continuous flow reactor (F1/1) with a working volume of 0.8 L (LLC «Teksa», Russia). The flow was established using a Masterflex L/S peristaltic pump (Corepalmer, USA) maintaining a minimum flow rate of 0.36 mL h^{-1}. The medium dilution rate in the reactor was calculated as a ratio of the reactor volume to medium residence time (10–20 h) and varied in the range from 0.04 to 0.08 h^{-1}. Reactor aeration was performed using a regulator valve of compressor with a flow rate of 0.05 L min^{-1}.

The viability of immobilised cells was estimated by the concentration of intracellular adenosine triphosphate (ATP), which was assayed using the luciferin–luciferase technique and a reagent by Lumtek (Russia) [16,17]. Bioluminescence intensity in samples was recorded using a 3560 microluminometer (New Horizons Diagnostics Co, USA).

The concentration of intracellular ATP in immobilised cells was determined by the bioluminescent luciferin–luciferase method using cell-containing granules immediately after thawing. For this purpose, granules were weighed (0.15 ± 0.05 g), transferred to dimethyl sulfoxide (1 mL) and allowed to stand at 25 °C for 2 h to extract intracellular ATP.

The growth of free yeast biomass was monitored using spectrophotometry. The absorbance of culture fluid samples was measured at 540 nm (Agilent UV-853 spectrophotometer, Germany), and the precise concentration of free cells was determined from calibration curves of optical absorbance vs. accurately measured concentration of cells in sample.

Cells dry weight (CDW) was determined by allowing a sample of cell biomass separated from the culture fluid by centrifugation (10 000 rpm, 5 min) on an Avanti J25 centrifuge (Beckman Coulter) to dry to constant weight.

Ethanol concentration was determined by gas chromatography as described previously [18]. Glycerol concentration was determined using the Megazyme glycerol assay kit (Ireland). The yield of ethanol was calculated based on glycerol as the sole carbon source and not total carbon in the medium.

All experiments were carried out in triplicate. The microbiological purity of cultures used in this work was monitored by microscopy using a BIOMED Lum microscope (Russia).

Results and discussion

Screening among naturally occurring strains of *P. tannophilus* yeast was carried out in order to prepare a sample of immobilised cells capable of transforming glycerol to ethanol at a maximum

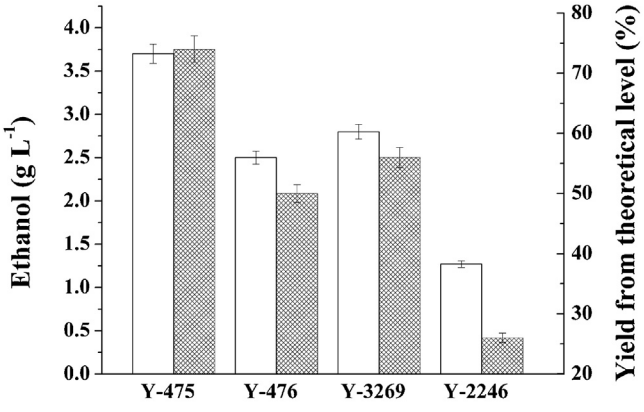

Fig. 1. Concentration of ethanol produced (▯) and its yield relative to the theoretical limit (▯) for the action of free cells of various natural strains of *P. tannophilus* in a medium (0.06 ± 0.02 g CDW L^{-1}) containing glycerol (10 g L^{-1}) at 28 °C for 24 h.

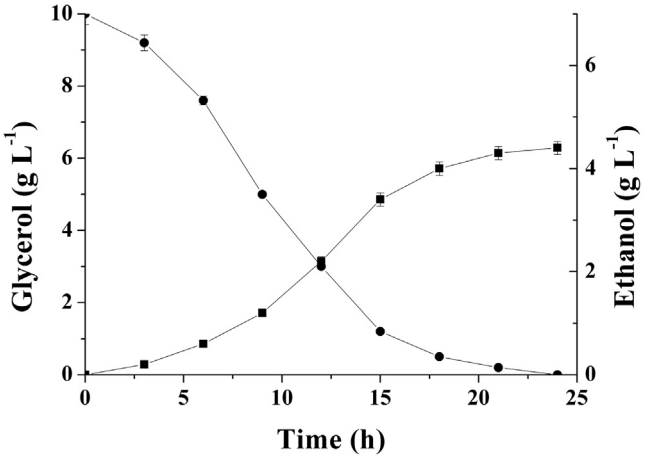

Fig. 2. Kinetics of glycerol (●) to ethanol (■) conversion by *P. tannophilus* strain Y-475 immobilised in PVA cryogel at a concentration of 0.6 g CDW L^{-1}.

Table 1
Parameters of glycerol conversion to ethanol by immobilised P. tannophilus yeast strain Y- 475 (0.6 g DCW L^{-1}) over 21 h.

Glycerol concentration in medium ($g\,L^{-1}$)	Residual concentration of glycerol ($g\,L^{-1}$)	Ethanol (gL^{-1})	Y_s[a] (%)	Process productivity in terms of ethanol over 21 h ($g\,L^{-1}\,h^{-1}$)
15	0.01 ± 0.001	6.2 ± 0.12	83 ± 1	0.30 ± 0.015
20	0.05 ± 0.002	7.6 ± 0.14	76 ± 1	0.36 ± 0.018
25	0.10 ± 0.003	8.3 ± 0.21	66 ± 2	0.40 ± 0.02
30	1.50 ± 0.045	8.2 ± 0.21	58 ± 2	0.39 ± 0.019
35	5.50 ± 0.28	7.9 ± 0.18	54 ± 2	0.38 ± 0.019
40	9.80 ± 0.50	7.5 ± 0.17	50 ± 2	0.36 ± 0.018
45	14.30 ± 0.70	7.2 ± 0.16	47 ± 1	0.34 ± 0.017
50	20.00 ± 1.00	6.8 ± 0.13	45 ± 1	0.32 ± 0.016

[a] Where Y_s is ethanol yield relative to the theoretical limit taking into account the concentration of glycerol consumed by cells.

rate. The efficacy of glycerol conversion to ethanol by the yeast strains assessed was estimated by performing the process in a culture medium containing 10 $g\,L^{-1}$ of crude glycerol for 24 h at 28 °C. To this end, cells were grown on a glucose-containing medium (20 $g\,L^{-1}$) to a stationary growth phase (19 h), separated from the medium by centrifugation (10 000 rpm, 10 min) and transferred to a glycerol-containing medium (10 $g\,L^{-1}$) at a concentration of 0.6 ± 0.02 g CDW L^{-1}. The tests demonstrated that the greatest concentration of ethanol (3.7 ± 0.1 $g\,L^{-1}$) could be achieved when the yeast strain Y-475 (Fig. 1) was used as the producer. The resulting yield of ethanol was 74% of the theoretical limit, while the process productivity was 0.15 $g\,L^{-1}\,h^{-1}$. Based on the results obtained, the cells of this strain were then immobilised and used in the subsequent stages of this work.

The analysis of intracellular ATP concentration before and after the incorporation of cells in PVA cryogel demonstrated that after immobilisation, intracellular ATP concentration in yeast cells dropped by a factor of just 1.5 relative to baseline, to (2.3 ± 0.1) × 10^{-10} mol g^{-1} dry weight of granules with immobilised cells. This suggests that the cells were rather successful in surviving the immobilisation procedure consisting of freezing a cell suspension in a PVA solution with simultaneous formation of a cryogel matrix around the cells, which is driven by the formation of multiple hydrogen bonds between polymer molecules and the subsequent thawing of cells surrounded by the carrier matrix. Retention of a sufficiently high cellular energy state indicated that one could reasonably expect effective functioning of the cells in the process of ethanol production.

Metabolic activity of the immobilised cells was assessed by carrying out the process of ethanol production for 24 h in a glycerol-containing (10 $g\,L^{-1}$) culture medium. The initial concentration of immobilised cells in the medium was 0.6 g CDW L^{-1} (Fig. 2). The test demonstrated that at the initial glycerol concentration in the medium equal to 10 $g\,L^{-1}$, immobilised cells taken at the same concentration as free cells (Fig. 1) produce 16% more ethanol over the same time period (4.3 ± 0.1 $g\,L^{-1}$), while the yield of ethanol relative to the theoretical limit grows to 86%. Almost all the glycerol initially added to the medium was consumed by cells in 21 h. The productivity of this process in terms of ethanol was 0.2 $g\,L^{-1}\,h^{-1}$, or 1.3 times greater than with free cells. This can be associated with a greater oxygen limitation of PVA cryogel-immobilised cells relative to free cells, which reduces the accumulation of free biomass in the medium and drives the yield of ethanol up. High oxygen transfer rate is known to have a negative effect on ethanol yield and production and regulate carbon flow distribution between cell growth and ethanol production [7].

To study the effect of initial substrate concentration in the medium on the effectiveness of immobilised cells, they were cultivated in culture media containing different concentrations of glycerol in the range from 20 to 50 $g\,L^{-1}$ (Table 1). The initial concentration of immobilised cells in the medium was 0.6 g CDW L^{-1}. It was demonstrated that as the initial concentration of glycerol in the medium increased from 15 to 25 $g\,L^{-1}$, so did the accumulated concentration of ethanol, while the process productivity in terms of ethanol rose by a factor of 1.5–1.9. As the initial concentration of glycerol was increased above 30 $g\,L^{-1}$, the process productivity dropped, as did the yield of the target product considering the substrate consumed. This was due to the accumulation of lower biomass concentrations, reaching

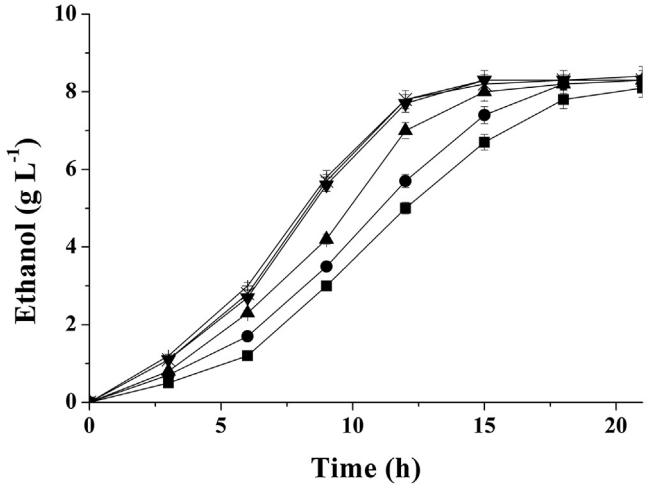

Fig. 3. Kinetics of glycerol to ethanol conversion by *P. tannophilus* yeast strain Y-475 immobilised in PVA cryogel at concentrations between 0.9 and 3 g CDW L^{-1}. Symbols correspond to cell concentration (g CDW L^{-1}): ■—0.9; ●—1.2; ▲—1.5; ▼—2.0; ×—2.5; *—3.0.

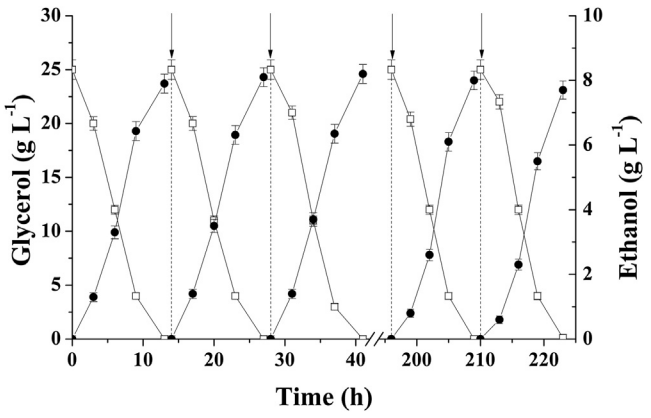

Fig. 4. Glycerol consumption (□) and ethanol accumulation (●) during re-use of immobilised *P. tannophilus* Y–475 cells (2 CDW g L^{-1}) in batch mode (dashed lines and arrows indicate the points of culture fluid replacement with fresh nutrient medium).

Table 2
Results of continuous-mode glycerol conversion to ethanol by immobilised cells.

Rate of medium dilution in reactor (h^{-1})	Ethanol ($g\,L^{-1}$)	Productivity ($g\,L^{-1}\,h^{-1}$)
0.040	8.5 ± 0.26	0.43 ± 0.010
0.044	8.5 ± 0.26	0.47 ± 0.010
0.050	8.5 ± 0.25	0.53 ± 0.015
0.058	8.4 ± 0.25	0.60 ± 0.015
0.062	8.2 ± 0.24	0.63 ± 0.020
0.066	7.4 ± 0.21	0.62 ± 0.010
0.072	6.8 ± 0.20	0.62 ± 0.015
0.076	6.2 ± 0.18	0.59 ± 0.015
0.080	5.8 ± 0.15	0.58 ± 0.015

0.05–0.35 g CDW L^{-1} depending on the initial glycerol concentration. Similar results were also observed with free cells as the glycerol concentration increased from 2% to 10 (%, v/v) [7].

This study also involved investigation into the possibility of augmenting the conversion of glycerol (25 g L^{-1}) to ethanol by increasing the concentration of immobilised cells introduced into the medium (Fig. 3). It was demonstrated that the rate of ethanol accumulation in the medium increases as the concentration of immobilised cells rises from 0.9 to 2 g CDW L^{-1}. Glycerol is completely consumed by immobilised cells at the initial concentration of 2–3 CDW L^{-1} as early as in 13 h of cultivation. In the remaining cases a similar result was observed in 15–18 h. The greatest process productivity (0.64 g L^{-1} h^{-1}) was observed at the initial concentration of cells of 2 CDW g L^{-1}. Increasing the concentration of immobilised cells above 2 g CDW L^{-1} failed to boost the process productivity, which was evidently associated with substrate limitation of the immobilised cells. The maximum yield of ethanol relative to the theoretical limit was 67%. Remarkably, the ethanol productivity of immobilised *P. tannophilus* Y-475 cells investigated in this work was significantly greater than the value reported for free cells of *P. tannophilus* CBS4044 (0.16 g L^{-1} h^{-1}) [7],*P. tannophilus* ATCC 32691 immobilised on Celite 545 (0.07 g L^{-1} h^{-1}) [19,20] and recombinant cells of *Hansenula polymorpha* (0.39 g L^{-1} h^{-1}) [5].

One of the known advantages of immobilised yeast cells lies in the fact that they can be re-used many times in biotechnological procedures [21]. For this reason, *P. tannophilus* Y–475 cells immobilised in PVA cryogel were used to produce ethanol from crude glycerol in a batch process (Fig. 4). To this end, the culture fluid was replaced with a fresh nutrient medium with a glycerol concentration of 25 g L^{-1} after each cycle in a reactor with immobilised cells. Immobilised cells were demonstrated to retain 95–100% of metabolic activity after 17 working cycles under batch conditions.

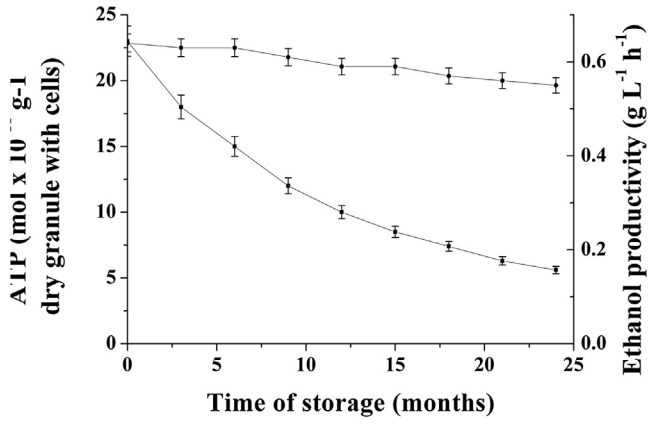

Fig. 5. Intracellular ATP concentration (■) and process productivity in terms of ethanol (●) vs. storage time of frozen immobilised cells at −20 °C.

The continuous process of ethanol production from glycerol using immobilised cells is a matter of particular interest as it allows the maintenance of optimal conditions for yeast metabolism in a flow-through system, including continuous addition of substrate, removal of metabolites and maintaining constant cell functioning conditions. The use of immobilised yeast cells in the continuous mode of ethanol production allows carrying out the process while maintaining high concentrations of immobilised cells in the reactor. In addition, 1 h-delays (Fig. 4) caused by replacing a spent culture medium in the reactor with a fresh medium are eliminated [22]. In connection with this, we tested a continuous process of ethanol production from glycerol using immobilised *P. tannophilus* Y–475 cells. The process of glycerol (25 g L^{-1}) conversion was performed continuously in a flow-through column reactor with a volume of 0.8 L at 28 °C and initial concentration of immobilised cells of 2.0 CDW g L^{-1}. The maximum process productivity of 0.60–0.63 g L^{-1} h^{-1}can be achieved by maintaining the medium dilution rate in the reactor at 0.062–0.072 h^{-1}, with a maximum at a dilution rate of 0.062 g L^{-1} h^{-1} (Table 2). It should be noted that we found no previous reports on the possibility of a continuous-mode ethanol production from glycerol by immobilised *P. tannophilus* yeast cells. Parameters of the continuous process of ethanol production were highly constant for at least 5 days. Meanwhile, the ethanol concentration was constant for the entire time of the study, which confirms the efficiency of immobilised cells in this process mode (0.062 h^{-1}).

In addition, the possibility of long-term storage of *P. tannophilus* yeast immobilised in PVA cryogel was investigated. To this end, the biocatalyst was stored at −20 °C for 24 months (Fig. 5). In order to analyse the viability of immobilised cells, the concentration of intracellular ATP was determined immediately before introducing cells into the process. In 24 months the concentration of intracellular ATP decreased just four-fold relative to baseline. Similar observations were previously made for another yeast species, *S. cerevisiae*, immobilised in PVA cryogel [10] at the same concentration of cells in granules (10% w/w). The analysis of metabolic activity of immobilised cells (conversion of glycerol 25 g L^{-1}) that were stored frozen confirmed that they retain productivity in terms of the target product in the range from 0.57 to 0.64 g L^{-1} h^{-1}. A small drop in productivity is due to the fact that cells withdrawn from long-term storage exhibited a short lag period at the initial stage of glycerol conversion to ethanol. Thus, we demonstrated in principle the feasibility of long-term storage of frozen immobilised biocatalyst for at least 2 years, which can be of great practical importance for the industrial-scale application of such biocatalysts.

Conclusions

Today the technology for producing bioethanol from cellulosic raw material is actively developing [23,24]. Major costs (up to 60%) are associated with physicochemical treatment of cellulose-containing feedstock and its enzymatic hydrolysis with subsequent

conversion of the sugars obtained to ethanol. The total process duration can be up to 200 h [25]. All these drawbacks are eliminated in the process presented in this work, which is based on using a liquid substrate and ready-for-use immobilised biocatalysts. Owing to this, glycerol conversion to bioethanol can become a more attractive process for practical implementation relative to the production of bioethanol from cellulose-containing feedstock. These results can be used for development of bases of innovative technology for producing fuel-grade bioethanol from glycerol, which is a major waste product of biodiesel production.

Acknowledgements

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