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The application of safe for humans and the environment Polyversum antifungal agent containing living cells of *Pythium oligandrum* for biotransformation of prochiral ketones

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ABSTRACT

This report presents the whole-cell biotransformation of benzofuranyl-methyl ketone derivatives with the application of Polyversum antifungal agent containing *Pythium oligandrum* microorganism. Stereochemistry of the reduction of prochiral substrates was modified by the bioconversion conditions (concentration of reagents, a source of the carbon atom, biotransformation medium). In optimized conditions enantioselective process was noted. Secondary alcohols with excellent enantiomeric purity and high yields were obtained. The enantiomeric excess and conversion degree of 1-(benzofuran-2-yl)ethanol, 1-(7-ethylbenzofuran-2-yl)ethanol and 1-(3,7-dimethylbenzofuran-2-yl)ethanol were 99%/98.1%, 94%/94.4% and 99%/72.6%, respectively. In the presence of *P. oligandrum*, one of the enantiotopic hydrides of the dihydropyridine ring coenzyme is selectively transferred to a *re* side of the prochiral carbonyl group to give products with *S* configuration. This study demonstrates an inexpensive, eco-friendly approach in synthesis of optically pure benzofuran derivatives and can be an interesting alternative to organocatalysis. Furthermore, this method can be used in biotechnology processes due to its good chemical performance and a high degree of product isolation.

1. Introduction

Asymmetric reduction of ketones is one of the most important and widely used methods of the synthesis of chiral secondary alcohols, which are valuable building blocks in the synthesis of many pharmaceuticals and agrochemicals. In the enantioselective transformation of prochiral carbonyl compounds, both chemical and biological processes are successfully used. The strategy based on biocatalysis is increasingly used in industry mainly because, in accordance with modern expectations, it is characterized by a high efficiency and a low toxicity. However, the implementation of biotransformation in industrial processes is not so straightforward due to the lack of appropriate biocatalysts for specific reactions and difficulties in scaling-up the processes.

Among the protein catalysts commonly used in technological processes, the most important role is played by hydrolases, especially lipases [1–3]. Proteases, transferases, isomerases, and nitrilases are also used [1]. Biotechnological processes employing oxidoreductases are less common due to the need to use and regenerate expensive cofactors. In contrast, the preparation of whole cells of microorganisms containing enzymes of this class generally does not pose serious problems thanks to the low cost of cell cultures and the simple equipment needed to maintain them. The addition of enzyme cofactors (NADH, NADPH) is not necessary because they are generated by nutrient metabolism.

Among the various biotechnologically important organisms, fungi play a particular role due to the diversity of their applications. Biotransformation of compounds by fungi occurs *in situ*, does not require expensive and time-consuming procedures to obtain and purify the enzyme, and involves additional enzymatic systems that improve the yield of the transformation process [1,4]. *Saccharomyces cerevisiae* is the main fungal species used in biotechnological processes. Among

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compounds obtained in reactions catalyzed by whole yeast cells are chiral hydroxybutanoate [5,6] (an intermediate in the synthesis of carbapenem, daunosamine, benzothiazepine—diltiazem), (*R*)-3-hydroxy-7-methyloct-6-enoic acid [7] (an intermediate for (*S*)-citronellol), and 4-phenyl-2-hydroxybutanoic acid [8] (used as an intermediate in the synthesis of the side chain of the antitumor agent paclitaxel).

For asymmetric bioreduction of ketones, in addition to popular Baker's yeast, most often the cells of bacterial, fungal and plant tissue are used. Plants which are explored as bioreagents are mainly apple (*Malus pumila*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), onion (*Allium cepa*), potato (*Solanum tuberosum*), radish (*Raphanus sativus*), tomato (*Lypersicum esculentum*) and sweet potato (*Ipomoea batatas*) [9]. In particular, the employment of the whole cells of carrot root (*Daucus carota*) for the desymmetrization of prochiral ketones is an effective method of chiral alcohols synthesis that can be successfully used as intermediates in the synthesis of biologically and pharmacologically important compounds. *D. carota* selectively catalyzes the bioreduction of aliphatic, aromatic, cyclic and other various functionalized ketones yielding the corresponding products in high ee [10–13]. Apart from the Baker's yeast, other various microorganisms are used in asymmetric synthesis, e.g. fungi: *Rhodotorula rubra*, *Geotrichum candidum*, *Penicillium citrinum*, *Rhodotorula mucilaginosa*, *Aspergillus sclerotiorum*, *Aspergillus sydowii*, *Aspergillus sclerotiorum*, *Saccharomyces ontanus*, *Rhodotorula glutinis*, *Zygosaccharomyces bailii*; and the bacteria: *Lactobacillus kefir*, *Lactobacillus fermentum*, *Lactobacillus brevis* and *Corynebacterium*. *Geotrichum candidum* is one of the microorganisms that allows selective reduction of the simplest aliphatic ketones as well as ketoestres [14,15]. *Rhodotorula rubra* selectively reduces bromoketones and, for example, *Zygosaccharomyces bailii* and *Saccharomyces ontanus* reduce carbonyl nitrogen compounds [16–18]. The several marine-derived fungi were applied in the bio-reduction of acetophenones, azidoketones, 1*H*-indole-2,3-dione as well as α,β- and α,β,γ,δ-unsaturated ketones, and ene-reduction of Knoevenagel compounds [19–24] whereas dehydrogenase of bacterial origin, for example from *Lactobacillus brevis*, is used to reduce propargyl ketones [25].

Previously, we described an effective biotransformation of prochiral sp^2-hybridized compounds employing *Aureobasidium pullulans* in the Boni Protect® preparation as a catalyst. Chiral secondary alcohols and α- and β-hydroxy esters were obtained with a high optical purity [26–28].

Herein we report a selective desymmetrization of unsymmetrical carbonyl aryl methyl compounds to the corresponding secondary alcohols with a defined absolute configuration, using whole cells of the *Pythium oligandrum* microorganism contained in the antifungal agent Polyversum®. The influence of reaction conditions on the biotransformation selectivity and yield, as well as the potential use of a microbiological method in biotechnological processes, were determined. To the best of our knowledge, this is the first report of a successful use of the Polyversum® preparation in the synthesis of chiral reagents.

Polyversum® is an antifungal agent containing a living organism, intended for the protection of roots and above-ground parts of plants. This agent directly affects phytopathogens, inhibiting the growth of mycelium and the formation of the morphological units of these fungi. Polyversum® is as effective as commonly used chemical preparations [29]. The oospores of *Pythium oligandrum* included in the Polyversum® biopreparation inhabit the soil, colonizing the ecosystems of many crop species. Among the fungi of the *Pythium* species, only *P. oligandrum* is non-pathogenic and is completely safe for humans and the environment. *P. oligandrum* strains display the ability to spread into the root tissues without inducing symptoms. This "benignity" is very beneficial for plants. It provides better protection against various factors causing biotic stress due to the induction of resistance, as well as growth promotion via the production of tryptamine an auxin precursor. *P. oligandrum* strains provide enhanced protection of crops against fungal and bacterial diseases by a direct attack on soil-borne pathogenic fungi including ascomycetes, basidiomycetes, and oomycetes. The *P. oligandrum* species antagonistically affects pathogens mainly through mycoparasitism, competition for nutrients, and antibiosis [29–32].

2. Material and methods

2.1. General information

Nuclear magnetic resonance (NMR) spectra were performed with Bruker spectrometers (400 MHz/700 MHz). Chemical shifts are reported in δ ppm from tetramethylsilane (TMS) as internal standard.

The enantiomeric excess of the chiral products (**1′–3′**) was determined by a chiral stationary phase of high-performance liquid chromatography (HPLC). HPLC analyses were performed on a Shimadzu SCL-10A *VP*.

Compound **1′** was separated on a column Lux® 5μ Cellulose-1, LC Column 250 × 4.6 mm, Phenomenex (Poland). The mobile phase was *n*-hexane and propan-2-ol (95:05 v/v), at the flow rate of 0.5 mL per min.

Compound **2′** and **3′** were separated on a column Lux® 5μ Cellulose-3, LC Column 250 × 4.6 mm, Phenomenex (Poland). The mobile phase was *n*-hexane and propan-2-ol (98:02 v/v), at the flow rate of 0.5 mL per min. The retention times of **2**, **3**, (*S*)-**2′**, (*R*)-**2′**, (*S*)-**3′**, (*R*)-**3′** were 14.1 min, 11.4 min, 20.4 min, 30.0 min, 27.2 min, 31.5 min respectively.

The samples were incubated in an orbital shaker (VORTEMP 1550 S2050; Equimed, Poland).

The absolute configuration of the **1′** was determined in [8]. The absolute configurations of **2′** and **3′** were assigned on the basis of a comparison of the specific rotations to the respective unsubstituted analogue **1′**.

2.2. Reagents and solvents

The chemical substances of analytical grade were commercially available and they included: sucrose, glucose, fructose, ethyl acetate, ethanol, $MgSO_4$, Na_2HPO_4, NaH_2PO_4, *n*-hexane for HPLC, propan-2-ol for HPLC from POCH (Poland), Potato dextrose broth (PDB) from Merck (Germany), Polyversum® WP from Target Natural (Poland) containing 10^6 oospore *Pythium oligandrum* fungus in 1 g of the preparation.

2.3. General procedure

2.3.1. Asymmetric reduction by Pythium oligandrum. Method I

For a typical experiment, to a suspension of Polyversum® (0.5 g) in 7.5 mL of potassium phosphate buffer (pH 7.0/pH 6.5) was added 1×10^{-4} mol glucose/fructose/sucrose and the resulting suspension was incubated in an orbital shaker (350 rpm) at specific temperature (27 °C, 30 °C, 33 °C) for 30 min. After pre-incubation, ketone **1** was added in the following amounts: 5×10^{-5}, 3×10^{-5}, 2.5×10^{-5}, 2×10^{-5}, 1.25×10^{-5}, and ketones **2–3** in the amount of 1.25×10^{-5}. Substrates were dissolved in 0.5 mL of ethanol and added to the suspension of biocatalyst in phosphate buffer. Then the stirring was continued at the same temperature. The reaction progress was monitored by TLC (the solvent system used was *n*-hexane:ethyl acetate 4:1 v/v). After the reaction, hyflo-super cell and ethyl acetate were added and the mixture was filtered. The solid residue was washed with ethyl acetate and the supernatant was extracted with ethyl acetate (3 × 20 mL). The collected organic layer was dried over anhydrous $MgSO_4$ and the solvent was evaporated in a vacuum. The conversion degrees of the substrates and enantiomeric ratios of the products were determined on HPLC system using a chiral column.

2.3.2. Asymmetric reduction by Pythium oligandrum. Method II.

Pythium oligandrum microorganism was cultivated in 50 mL Falcon

Table 1
Conditions for cultivating *Pythium oligandrum*.

T [°C]	pH	PDB[1] [g/L]	Fructose [mol]	Glucose [mol]	Sucrose [mol]
30	7/6.5	50	5×10^{-3}	–	–
30	7/6.5	50	–	5×10^{-3}	–
30	7/6.5	50			5×10^{-3}
30	7/6.5	50	–	–	–
30/27	7	100	–	–	–
30/27	7	200	–	–	–
30/27	7	300	–	–	–
30/27	7	400	–	–	–
27	7	50	–	5×10^{-3}	–
27	7	50	–	–	–

[1] PDB - potato dextrose broth.

tubes under the following conditions (Table 1):

P. oligandrum was grown for 3 days at 27 °C or 30 °C with shaking (170 rpm) on PDB medium in phosphate buffer (pH = 7/6.5). Then the biomass was separated by filtration and rinsed with distilled water to remove medium residual.

After 3 days pre-cultivation, 1.25×10^{-5} mol of **1** was added to 0.5 g of the culture of *Pythium oligandrum*. The bioconversion was carried out for 3 days, at 27 °C in phosphate buffer (pH = 7/6.5), under shaking condition (350 rpm). After the reaction, hyflo-super cell and ethyl acetate were added and the mixture was filtered. The solid residue was washed with ethyl acetate, and combined filtrates were extracted with ethyl acetate (3 × 20 mL). The organic phase was dried over anhydrous $MgSO_4$, and the solvent was evaporated in a vacuum. The conversion degrees of the substrates and enantiomeric ratios of the products were determined on HPLC system using a chiral column.

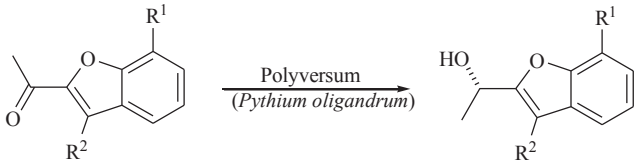
2.3.3. Asymmetric reduction by Pythium oligandrum on a semi-preparative scale.

Polyversum® or biomass received after cultivation (5 g) was suspended in 75 mL of potassium phosphate buffer (pH 7.0/pH 6.5) containing 1×10^{-3} mol fructose/sucrose. The obtained suspensions were incubated in an orbital shaker (350 rpm) at specific temperature (27 °C, 30 °C) for 30 min. After pre-incubation, ketone **1** was added in the amount of 1.25×10^{-4}. After 3 days biomass was removed by filtration. The bio-reduction product (**1′**) was eluted from the aqueous phase and the recovered biomass with ethyl acetate. The organic phase was dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure using rotary evaporator. Then, the product was separated from the unreacted substrate on the preparative plate (PLC). On this basis, the chemical yield was calculated and the degrees of isolation of compounds **1** and **1′** from the post-reaction mixture were assessed. The enantiomeric excess was determined on HPLC system using the Cellulose-1.

2.3.3.1. 1-(benzofuran-2-yl)ethanol (1′). ^{1}H NMR (700 MHz, $CDCl_3$): δ (ppm) = 1.68 (d, 3H, J = 2.7 Hz, CH_3), 2.70 (br s, 1H, OH), 5.05–5.07 (dq, 1H, J = 6.3 Hz, 0.7 Hz, H), 6.65 (s, 1H, CH), 7.23–7.24 (m, 1H, CH), 7.30–7.31 (m, 1H, CH), 7.48–7.49 (d, 1H, J = 3.3 Hz, CH), 7.57–7.58 (d, 1H, J = 3.4 Hz, CH); $[\alpha]_D^{20}$ – 6.3 (*S*; 60% ee; c = 1.4).

2.3.3.2. 1-(7-ethylbenzofuran-2-yl)ethanol (2′). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.38 (t, 3H, J = 7.6 Hz, $-CH_2\underline{CH_3}$), 1.67 (d, 3H, J = 6.4 Hz, CH−($\underline{CH_3}$)−OH), 2.18 (br s, 1H, OH), 2.96 (q, 2H, J = 7.6 Hz, $-\underline{CH_2}CH_3$), 5.05 (dq, 1H, J = 6.4 Hz, 1.2 Hz, $\underline{CH}$−(CH_3)−OH), 6.63 (d, 1H, J = 1.2 Hz, 3-$\underline{CH}$), 7.11–7.13 (m, 1H, $\underline{CH}$), 7.16–7.19 (m, 1H, $\underline{CH}$), 7.39–7.41 (m, 1H, $\underline{CH}$); $[\alpha]_D^{20}$ – 38.0 (*S*, 78% ee, c = 0.99).

2.3.3.3. 1-(3,7-dimethylbenzofuran-2-yl)ethanol (3′). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.68 (d, 3H, J = 6.8 Hz, CH−($\underline{CH_3}$)−OH), 1.95 (br s, 1H, OH), 2.28 (s, 3H, 3-$\underline{CH_3}$), 2.54 (s, 3H, 7-$\underline{CH_3}$),



1 R^1- H; R^2 - H — **1'**
2 R^1- CH_2CH_3; R^2 - H — **2'**
3 R^1- CH_3; R^2 - CH_3 — **3'**

Scheme 1. Desymmetrization of benzofuran derivatives by Polyversum antifungal agent.

5.12 (q, 1H, J = 6.8 Hz, $\underline{CH}$−(CH_3)−OH), 7.08–7.11 (m, 1H, $\underline{CH}$), 7.13–7.17 (m, 1H, $\underline{CH}$), 7.32–7.34 (m, 1H, $\underline{CH}$); $[\alpha]_D^{20}$ – 18.2 (*S*, 99% ee, c = 0.78).

3. Results and discussion

An important factor determining the enzymatic activity of fungi is the selection of appropriate reaction conditions. All modifications of the biotransformation procedure should be carried out in such a way as to find the most efficient, selective, and economical method of substrate bioconversion. The most common modifications of biotransformation conditions concern temperature, the bioprocess duration, and the reaction environment.

Pythium oligandrum included in the Polyversum® antifungal agent was tested for its use in the bio-reduction of unsymmetrical ketones of benzofuran derivatives (Scheme 1).

Experiments were started with the evaluation of the ability of *P. oligandrum* to conduct the conversion of 1-(benzofuran-2-yl)ethanone (**1**). In order to perform an efficient bio-reduction with a high catalytic activity of *P. oligandrum* cells correlated with a high enantioselectivity, the reaction of compound **1** was carried out using different sources of carbon in different environments and with different substrate concentrations. As the source of the carbon atom, commonly used sugars: glucose, sucrose, and fructose, were selected. The bio-reduction reaction was performed in a phosphate buffer solution at pH 7 and pH 6.5 for the following amounts of compound **1**: 5×10^{-5}, 3×10^{-5}, 2.5×10^{-5}, 2×10^{-5}, and 1.25×10^{-5} mol. The reaction mix was incubated at 30 °C for 3 days. The duration of the bioprocess was determined on the basis of preliminary reactions.

The effectiveness of bioconversion is usually strongly affected by substrate concentrations. Bio-reduction was initially carried out, as in the reaction catalyzed by *A. pullulans*, in a 6.5×10^{-4} mol solution of compound **1** [27]. Under these conditions, the highest degrees of conversion were obtained in the solution at pH 6.5 with the addition of sucrose (21.7%), while the highest enantiomeric excess (99%) was obtained in the phosphate buffer system at pH 6.5 with fructose as the source of the carbon atom. The reduction reaction proceeds selectively to generate the enantiomer with the *S* configuration. A major problem encountered while using the enzymatic catalytic system in the biotransformation of a non-physiological reagent is the possibility of toxicity of substrate for the cells of the bioreagent and intracellular enzymes. It appears that a low degree of conversion may be caused to some extent by the toxicity of the reagent, which acts as an inhibitor of the native form of the enzyme. Therefore, the efficiency of the compound **1** bioconversion was strongly dependent on its concentration. It was observed that, while the reagent concentration decreases in most cases, the catalytic activity of the microorganism increases (Table 2). Another reason for the decrease in bioconversion with increasing reagent concentration may be a competitive reaction - deracemization (stereoinversion). The bio-reduction reaction product can be reoxidized to the appropriate ketone, which may consequently reduce the conversion degree and the optical purity of the obtained alcohol [33–35].

Table 2
The efficiency and enantioselectivity of the bioconversion of compound **1** at 30 °C by *P. oligandrum*.

Conditions	The amount of substrate [mol]	Degree of conversion [%][1]	ee [%][1]
Phosphate buffer; pH 7; glucose	5×10^{-5}	10.2	87
	3×10^{-5}	30.5	96
	2.5×10^{-5}	**48.1**	**99**
	2×10^{-5}	95.3	90
	1.25×10^{-5}	68.5	85
Phosphate buffer; pH 6.5; glucose	5×10^{-5}	12.9	93
	3×10^{-5}	17.8	93
	2.5×10^{-5}	**35.7**	**99**
	2×10^{-5}	38.8	91
	1.25×10^{-5}	45.3	76
Phosphate buffer; pH 7; sucrose	5×10^{-5}	12.9	85
	3×10^{-5}	78.7	97
	2.5×10^{-5}	93.0	93
	2×10^{-5}	93.2	97
	1.25×10^{-5}	**96.5**	**99**
Phosphate buffer; pH 6.5; sucrose	5×10^{-5}	21.7	93
	3×10^{-5}	79.9	96
	2.5×10^{-5}	90.4	89
	2×10^{-5}	90.4	97
	1.25×10^{-5}	**96.5**	**99**
Phosphate buffer; pH 7; fructose	5×10^{-5}	7.7	94
	3×10^{-5}	77.1	97
	2.5×10^{-5}	87.3	96
	2×10^{-5}	97.5	90
	1.25×10^{-5}	**96.6**	**99**
Phosphate buffer; pH 6.5; fructose	5×10^{-5}	14.2	99
	3×10^{-5}	83.2	98
	2.5×10^{-5}	88.2	99
	2×10^{-5}	96.6	98
	1.25×10^{-5}	**98.1**	**99**

[1] The ee and degree of conversion were determined by HPLC.

During the reaction with the addition of glucose in the solution at pH 7, a decrease in the amount of reagent to 1.25×10^{-5} mol resulted in a decrease in the degree of conversion. For such an amount of substrate at 30 °C, a high chemical yield correlated with a high optical efficiency was observed in the sucrose and fructose system. A slightly higher catalytic activity of dehydrogenases contained in the *P. oligandrum* microorganism was shown in the solution at pH 6.5 with the addition of fructose and sucrose. A much higher degree of substrate conversion was observed using glucose at the pre-incubation stage in the solution at pH 7 compared with the solution at pH 6.5.

Based on the obtained results, it can be concluded that the activity of the enzymatic system involved in the bio-reduction of ketone **1** depends on the energy source and the biotransformation medium. The Polyversum® antifungal agent containing *P. oligandrum* strains demonstrates an unusual catalytic ability and enantioselectivity to reduce the unsymmetrical ketone in phosphate buffer at pH 6.5 with fructose added before the biotransformation stage.

Transformation of benzofuranyl methyl ketone by free-living cells of *P. oligandrum* is more effective compared with the process using the Boni Protect® antifungal agent containing free-living cells of *A. pullulans*.

Our previous study demonstrated that Boni Protect® exhibited a lower reductive activity toward a benzofuran derivative (61-% ee) and, in order to improve enantioselectivity, it was necessary to add appropriate inhibitors, i.e. additives which inhibit oxidoreductases with a specific stereopreference [27]. Compound **1′** has also been reduced by means of other biocatalysts such as *Rhizopus arrhizus*, Baker's yeast and *Daucus carota* [36–39]. In the presence of *Rhizopus arrhizus* 1-(benzofuran-2-yl)ethanol was obtained with 99.3% yield and around 92% ee [36]. Baker's yeast gave, depending on the reaction conditions, compound **1′** with 60% yield and 55% ee and 93% yield and also with 99% optical purity [37,38]. Utilizing the catalytic properties of *D. carota* makes it possible to obtain a chiral carbinol (**1′**) with > 95% ee and 47% isolated yield [39].

An important factor influencing the selectivity of biotransformation is temperature. For a concentration of 1.5×10^{-4} mol/L, transformation of compound **1** at 27 and 33 °C was conducted (Table 3). As temperature decreases, the selectivity of the bioprocess increases. At 27 °C, irrespective of the pH of the reaction medium as well as the source of the carbon atom, an optically pure compound **1′** with the *S* configuration was obtained.

By increasing the temperature to 33 °C in a biological system using sucrose and fructose, a slight decrease in the conversion degree of the substrate was observed. However, in a reaction conducted with a glucose solution at pH 6.5, the temperature increase caused an increase in the catalytic activity of *P. oligandrum*. The presented study demonstrated that considering process enantioselectivity, a temperature increase had a generally decreasing effect on the enantiomeric purity of the product, and only in the solution at pH 6.5 with sucrose, no such a relationship was observed. Under these conditions, an optically pure compound **1′** was obtained.

Reduction of compound **1** by fermentation using Polyversum® was also conducted. The culture conditions are a very important factor which determines the activity of the fungal enzymatic system under specific reaction conditions. Modification of the bioconversion procedure can yield a more effective and economical biotransformation method. In the procedure used, the appropriate biotransformation was preceded by the bioreagent culture stage in a liquid medium using potato dextrose broth (PDB). Initially, the optimal fermentation conditions were selected. In order to investigate the influence of selected culture factors on the growth of *P. oligandrum*, the pH of the medium, the amount of medium, and the source of the carbon atom were changed. The culture was maintained for 3 days in an incubator at 27 or 30 °C with shaking (150 rpm). Once the culture was ceased, dry mass was determined by weighing. The results are shown in Fig. 1.

Table 3
The efficiency and enantioselectivity of the bioconversion of compound **1** at 27 °C and 33 °C by *P. oligandrum*.

Conditions	Temperature [°C]	The amount of substrate [mol]	Degree of conversion [%][1]	ee [%][1]
Phosphate buffer; pH 7; glucose	**27**	**1.25×10^{-5}**	**88.5**	**99**
	33	1.25×10^{-5}	63.9	85
Phosphate buffer; pH 6.5; glucose	**27**	**1.25×10^{-5}**	**76.9**	**99**
	33	1.25×10^{-5}	90.0	95
Phosphate buffer; pH 7; sucrose	**27**	**1.25×10^{-5}**	**91.7**	**99**
	33	1.25×10^{-5}	93.9	55
Phosphate buffer; pH 6.5; sucrose	27	1.25×10^{-5}	96.5	99
	33	**1.25×10^{-5}**	**97.5**	**99**
Phosphate buffer; pH 7; fructose	**27**	**1.25×10^{-5}**	**88.6**	**99**
	33	1.25×10^{-5}	93.2	93
Phosphate buffer; pH 6.5; fructose	**27**	**1.25×10^{-5}**	**94.4**	**99**
	33	1.25×10^{-5}	89.1	88

[1] The ee and degree of conversion were determined by HPLC.

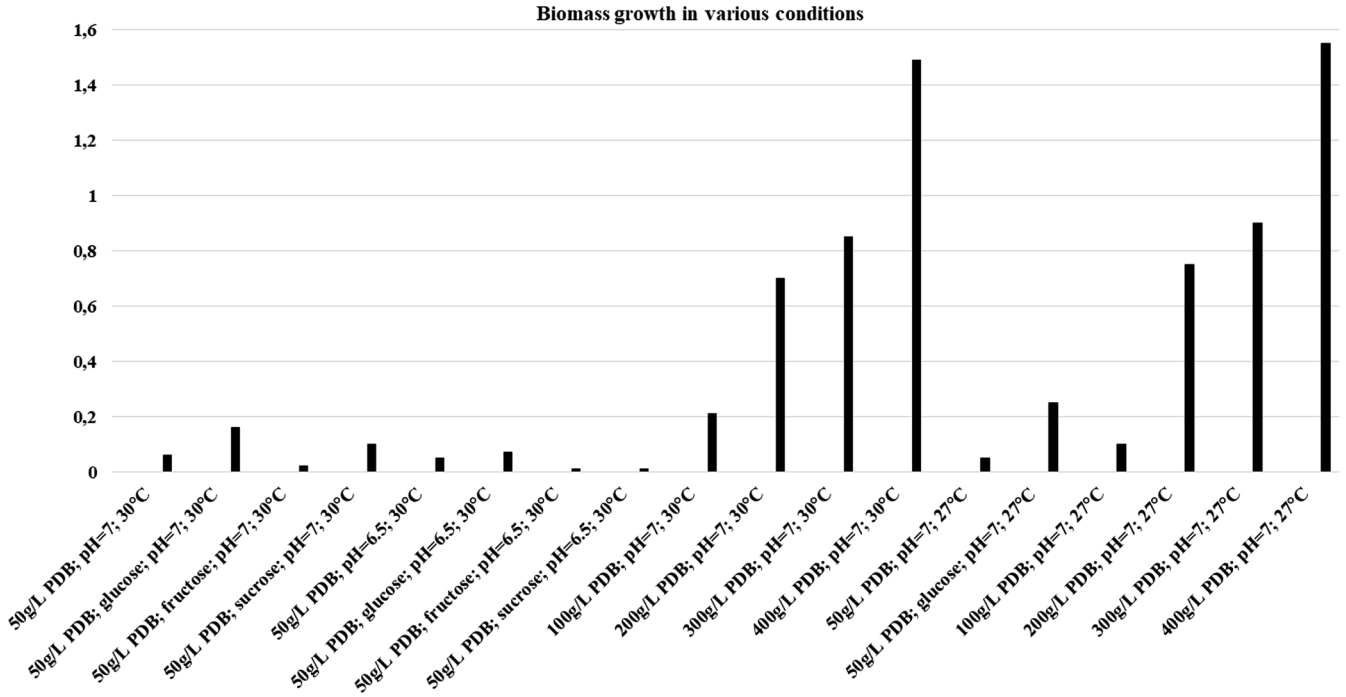


Fig. 1. Biomass growth in various conditions.

The highest increase in biomass was obtained using PDB at a concentration of 400 g/L at 27 °C. Dissolving the nutrient solution in the phosphate buffer solution at pH 7 was more beneficial for the growth of *P. oligandrum* cells. Moreover, it was noted that enriching the culture medium with glucose increased the biomass growth performance of the microorganism.

P. oligandrum cells multiplied in the fermentation method were used in the appropriate bio-reduction reaction. The biocatalyst was suspended in a phosphate buffer solution at pH 7 and pH 6.5. The whole mixture was incubated at 27 °C for 3 days. The obtained conversion degree of compound **1** was high—96.3% and 98.9% for the solutions at pH 7 and pH 6.5, respectively. However, compared with method I, an unexpected decrease in the enantiomeric purity of the product was observed. Compound **1′** was obtained with a 79-% and 93-% ee in the solutions at pH 7 and pH 6.5, respectively. It was decided to conduct the reduction of compound **1** catalyzed with the *P. oligandrum* cells multiplied in the fermentation method with an additional incubation step with the appropriate amount of fructose in the phosphate buffer solution at pH 6.5. After 3 days of biotransformation, an optically pure product was obtained with an over 90-% conversion. This observation clearly indicated that the presence of fructose is crucial for ensuring a high biotransformation selectivity. Interestingly, the optimal biotransformation conditions important for the catalytic activity of *P. oligandrum* cells are not similar to the optimal conditions for the growth of these cells.

Under optimized conditions, the reduction reaction was conducted for two derivatives of compound **1**: 1-(7-ethylbenzofuran-2-yl)ethanone (**2**) and 1-(3,7-dimethylbenzofuran-2-yl)ethanone (**3**). The results are presented in Table 4.

The enantioselectivity of *P. oligandrum* depends on the enzyme specificity and the substrate structure. In the case of compound **2**, the highest degree of conversion and the highest enantiomeric excess were obtained in the phosphate buffer solution at pH 6.5 with added glucose. The highest result was as follows: 94.4% of conversion degree and 94% of optical purity of (*S*)-1-(7-ethylbenzofuran-2-yl)ethanol (**2′**). However, compound **3** was reduced selectively in the system employing phosphate buffer at pH 6.5 with the addition of sucrose, and decomposed in the solution at pH 7. Finally, the biotransformation of the dimethyl derivative of compound **1** led to obtaining a secondary alcohol with the *S* configuration with a 99-% enantiomeric excess and a 72.6-% conversion. It can be concluded that the effectiveness of biotransformation of unsymmetrical ketones by the antifungal agent Polyversum® strongly depends on the structure of the substrates. The presence of substituents in the benzofuran ring has a significant influence on both the degree of conversion and the selectivity of the process. In the case of compound **3**, the degree of conversion was lower than that of the unsubstituted analog and amounted to approx. 70%. However, substitution of the aromatic ring at position 7 with an ethyl group results in a decrease in enantioselectivity, with a high enantiomeric excess of over 90% achieved in only one case. It can be suggested that Polyversum® presents an unusual catalytic ability to reduce benzofuranyl-methyl ketone derivatives. Under optimized conditions, an efficient and selective biotransformation of unsymmetrical aryl methyl ketones can be conducted.

Although the use of whole microbial cells in industrial processes is increasing, the development of an application design allowing a transfer of biotransformation reactions from the laboratory scale to the macro scale is limited due to the low efficiency of the reaction and difficulties in the isolation of the bioprocess products. The launch of production creates the necessity of constant precise control of the

Table 4
The efficiency and enantioselectivity of the bioconversion of compounds **2**, **3** by *P. oligandrum*.

Conditions	Degree of conversion [%][1]		ee [%][1]	
	2	**3**	**2′**	**3′**
Phosphate buffer; pH 7; glucose; 27 °C	86.3	dec.[2]	87	–
Phosphate buffer; pH 6.5; glucose; 27 °C	**94.4**	38.3	**94**	89
Phosphate buffer; pH 7; sucrose; 30 °C	71.4	dec.[2]	68	–
Phosphate buffer; pH 6.5; sucrose; 30 °C	93.6	**72.6**	79	**99**
Phosphate buffer; pH 7; fructose; 30 °C	59.3	dec.[2]	87	–
Phosphate buffer; pH 6.5; fructose; 30 °C	45.7	50.1	86	97

[1] The ee and degree of conversion were determined by HPLC.
[2] dec – decomposition.

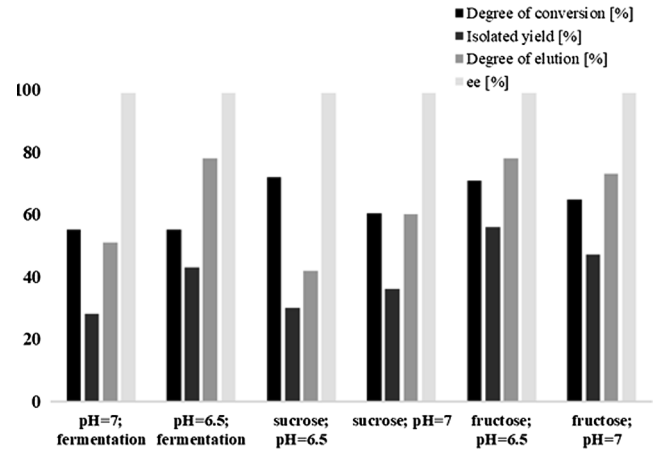


Fig. 2. The degree of conversion, the chemical yield, and the degree of isolation of compound **1′** from the post-reaction mixture.

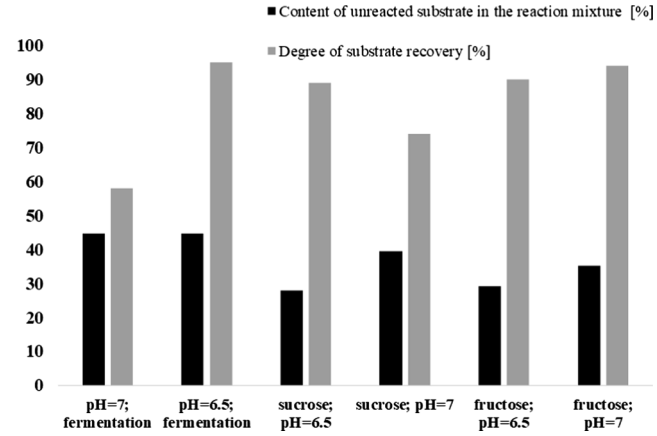


Fig. 3. The Content of unreacted substrate in the reaction mixture [%] and the degree of elution of the substrate **1** from the post-reaction mixture.

biotechnological process. Finally, products must be of a high chemical and biological purity and must not contain toxic substances or pathogenic microorganisms. The environmental aspect is also important, which limits the use of toxic reagents and forces methods that do not require drastic conditions. The microbiological method using *P. oligandrum* meets most of the requirements for the application project because it is easy to execute, ecologically friendly, and economical. In order to assess the feasibility of implementing this method in the biotechnological process, a bio-reduction reaction catalyzed with Polyversum® was conducted on a semi-preparative scale. It was checked whether the catalytic activity of the biocatalyst is maintained at higher concentrations of the reagent. The bio-reduction product was eluted from the aqueous phase and the solid phase (recovered biomass), and separated from the unreacted substrate on a preparative PLC plate. On this basis, the isolated product yield was calculated, and the degree of isolation of compound **1′** from the post-reaction mixture was assessed. The results are shown in Fig. 2.

In the semi-preparative scale in the more concentrated solution, formation of the substrate–enzyme complex was more difficult, which affected the conversion degree of the starting reagent. Consequently, the percentage of substrate conversion was lower than in the micro scale. The highest results were obtained in the system employing the phosphate buffer at pH 6.5 with the addition of fructose and sucrose (over 70%). The highest isolated yield as well as the highest degree of product recovery from the post-reaction mixture were obtained in the fructose solution. Generally, the amount of isolated product in most cases was over 50%, with a maximum value of approx. 78%. The optical purity of the enzyme transformation product in an enlarged scale was identical to that in the micro scale. In each case, compound **1′** was obtained with a 99% enantiomeric excess.

In the biotechnological process, the unreacted substrate should be recycled back to the transformation reaction. For this reason, the percentage of the original reagent that can be recovered after the reaction was also determined. Fig. 3 shows the content of unreacted substrate in the reaction mixture [%] and the degree of elution of the substrate from the post-reaction mixture.

Based on the obtained results, it can be concluded that biotransformation with the *P. oligandrum* microorganism may be of significance in the biotechnological process. This method is conducted under mild conditions, stereoselectively, with a high catalytic activity and a fairly good chemical yield of the reaction. This biocatalyst can be successfully used in biotechnology in the form of both free and immobilized cells in a technological process using a batch and/or flow reactor.

4. Conclusion

The study suggested that Polyversum® containing living cells of the *P. oligandrum* microorganism allows a stereoselective reduction reaction of benzofuranyl-methyl ketones. Microbiological biotransformation leads to the formation of chiral secondary alcohols with a high enantiomeric purity (94–99% ee). One of the prochiral hydride ions from the cofactor is selectively supplied by a bioreagent on the substrate molecule to one of the prochiral sides of the carbonyl moiety (*re* side), yielding an enantiomer with a predominantly *S* configuration.

The advantage of the whole-cell method is that there is no need to regenerate cofactors because they are contained in the cell. Fungal enzyme systems abound in various types of proteins, so they show a broad spectrum of activity even in relation to non-physiological substrates. The natural pathways of metabolizing microorganisms can be activated or inhibited by changing the reaction conditions. Moreover, biotransformation with the use of living organisms is non-toxic to the natural environment (environmentally friendly reagents, biodegradable biocatalyst) and economical. In the case of a reaction catalyzed by *P. oligandrum*, due to its good chemical yield, this method can be used in biotechnological processes.

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