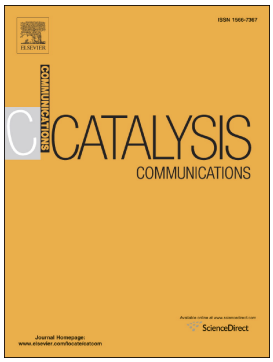

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Microbiological bio-reduction of prochiral carbonyl compounds by antimycotic agent Boni Protect

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ABSTRACT

The selective properties of the fungus *Aureobasidium pullulans*, in the antifungal agent Boni Protect, were investigated in the fermentative bioreduction of selected carbonyl compounds. Catalyzed by oxidoreductases contained in the microorganism *Aureobasidium pullulans* highly enantioselective biotransformation of prochiral ketones provides the secondary alcohols when the reaction is done in the presence of specific additives. *Aureobasidium pullulans* has also proved to be an effective bioreagent in the reduction of α- and β-keto esters. Optically pure hydroxy esters were obtained under fermentation conditions without the use of additives.

Keywords: biocatalysis; microbiological reduction; stereochemistry; antifungal agent; *Saccharomyces cerevisiae*; *Aureobasidium pullulans*

1. Introduction

Bio-reduction of the prochiral ketones is one of the methods for the synthesis of optically active secondary alcohols, versatile as building blocks in the synthesis of important biologically active compounds. Applying chemical methods for the asymmetric reduction requires the use of, e.g. chiral boron compounds [1, 2], borates with transition metal complexes containing the chiral P- or N-donor ligands [3-6], molecular hydrogen in the presence of Ru and Rh catalysts with chiral phosphine ligands [7, 8], and others hydrogen sources (e.g. HCOOH) in the presence of chiral complexes [9-12]. Often, the use of aforementioned methods does not lead to the satisfactory results (low enantioselectivity), is expensive, and contributes to environmental pollution. In this context, an interesting alternative is the application of bioreagents - living organisms in the form of tissue cultures, whole microbial cells, or isolated enzymes. The reduction methods utilizing the biocatalysts potential are cheap and simple means to obtain alcohols of high enantiomeric purity. The use of whole cell microorganisms suspended in the nutrient solutions is particularly economical because it does not require the regeneration of cofactors, necessary in the reduction reaction with oxidoreductases. Hence, a great interest in searching new, cheap, and easily accessible bioreagents, mainly among fungi that can be used in whole cell forms in the asymmetric bio-reduction of prochiral carbonyl compounds [13-16].

Aureobasidium pullulans, known as a black-yeast, is a commonly occurring oligotrophe, living in temperate as well as tropical climate, and also in saline water. It is used in biotechnology, especially for production of the polysaccharide called pullulan and the antimycotic aureobasidin A, and as a biocontrol agent in agriculture. Its antagonistic activity against a number of phytopathogenic fungi is used in fruit orchards to combat white mold [17-18].

In this work, the selective properties of the fungus *Aureobasidium pullulans* in the reduction of selected unsymmetrical ketones, and α- and β-keto esters are presented. For this purpose, a widely available antimycotic agent Boni Protect containing living cells of *Aureobasidium pullulans* was used.

2. Experimental Section

2.1. Asymmetric reduction by *Aureobasidium pullulans*

For a typical experiment, 0.14 g glucose ($7.6x10^{-4}$ mol) was added to a suspension of Boni Protect (2 g) in 30 mL of potassium phosphate buffer (pH 7.0) and the resulting suspension was stirred at 37 °C for 30 minutes. Then an additive compound ($1.25x10^{-5}$ mol; see Table 2) and substrate ($1.25x10^{-4}$ mol in 0.5 mL EtOH) were added and stirring was continued at the same temperature. The reaction progress was monitored by TLC (the solvent system used was hexane:ethyl acetate 3:1). After the reaction, hyflo-super celit and ethyl acetate were added and the mixture was filtered. The celit was washed with ethyl acetate and combined filtrates were extracted with ethyl acetate (5x20 mL). The organic portion was dried with $MgSO_4$. The solvent was evaporated under reduced pressure. The crude product was purified by PLC (Preparative Layer Plate) using hexane:ethyl acetate (3:1) as eluent. The enantiomeric ratios were determined on an HPLC system using a chiral column.

2.2. Asymmetric reduction by *Saccharomyces cerevisiae*

A suspension of baker's yeast (3.75 g) in 20 mL potassium phosphate buffer (pH 7.0) and 0.9 g ($2.6x10^{-3}$) sucrose was put into a 250 mL Erlenmeyer flask. The resulting mixture was stirred at 37 °C. After this stage of fermenting yeast (30 minutes), the solution of ketones/ketoesters ($1.25x10^{-4}$ mol in 0.5 mL EtOH) was added. The reaction progress was monitored by TLC. After 24 h the yeast cells were removed by filtration of celit filter. The yeast cells were washed with 2x25 mL water. The filtrate was saturated with NaCl and then extracted with 5x20 mL portions of ethyl acetate. The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to leave a residue of the crude product, which was purified by PLC using hexane:ethyl acetate (3:1) as eluent. The enantiomeric ratios were determined on an HPLC system using a chiral column.

3. Results and discussion

As we previously reported, the enantioselective microbiological reduction of prochiral ethyl (9-anthryl)glyoxylate in the presence of *Aureobasidium pullulans* (Boni Protect) afforded an appropriate

hydroxy ester with 99% ee [19]. The method applied to obtain the derivative which is used in the synthesis of chiral derivatizing agent, is simple, economical, and does not require an additional cultivation of the microbial cell.

Herein we report the microbiological reduction of selected ketones with the use of *A. pullulans* (Figure 1).

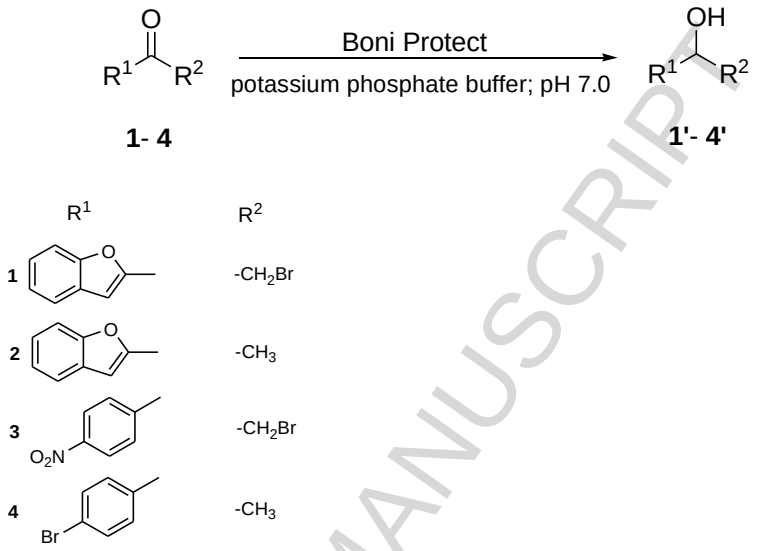

Figure 1. Bio-reduction of prochiral ketones **1**-**4** by *Aureobasidium pullulans*.

Fermentative bio-reduction allowed for obtaining the corresponding secondary alcohols in high yields. However, the reduction was either non-selective or with moderate selectivity. Oxidoreductases, contained in the microorganism, act competitively to each other by transferring the hydride (pro-*S* or pro-*R*) from the NAD(P)H cofactor to one of the sides of the prochiral carbonyl group (face *si* or *re*). The final enantiomeric excess of product results from the reactions occurring at different rates, carried out by a set of oxidoreductases. Furthermore, the selectivity of reduction depends on the substrate structure, particularly on the presence of additional functional groups or heteroatoms in it [20, 21]. Ketone **3** was reduced with the highest enantioselectivity 65%, while the reduction of **1** at the presence of *A. pullulans* occurred at a high rate but completely non-selectively (Table 1). The microbiological reduction of compounds **1**-**4**, catalyzed by *Saccharomyces cerevisiae* was also performed and compared with the fermentation method in the presence of *A. pullulans*. Alcohols **1'** and **4'** were obtained with higher enantiomeric excesses (45% and 72%, respectively) than compounds **2'** and **3'**

(36% and 62%, respectively). In each case, the product of the same configuration was preferentially produced, similarly to the reaction catalyzed by *A. pullulans*.

In order to improve the selectivity of the reaction, optimization of the process towards the reduction of enzyme incompatibility is necessary. The best known microbial reduction is the synthesis of alcohols from the corresponding ketones with the use of baker's yeast (*Saccharomyces cerevisiae*). The wide range of baker's yeast activity is conditioned by the presence of dehydrogenases of different stereopreferences and high substrate tolerance. Therefore, both in the fermentation method and in organic solvents, additional substances are used to improve the enantioselectivity, i.e. additives which inhibit oxidoreductases with a specific stereopreference [22, 23].

In the reduction with *A. pullulans*, ethyl chloroacetate, allyl bromide, and allyl alcohol were used as the additives which had already been applied earlier in the baker's yeast reduction [22, 23]) and also ionic liquid - [BMIM][PF_6], keto esters - ethyl 3-oxovalerate, ethyl (9-anthryl)glyoxylate (Table 2).

The use of the additives improved the microorganism selectivity in the reduction of tested ketones. Reduction of bromoketone **1** in the presence of ethyl (9-anthryl)glyoxylate gave the best results. The appropriate bromohydrin was obtained in quantitative yield and with 55% ee, whereas an optically pure **2'** was afforded in the presence of the following inhibitors: allyl alcohol, ethyl 3-oxovalerate, ethyl (9-anthryl)glyoxylate. The reactions were carried out within 72 h and alcohol **2'** was obtained in 39.5-46.9% yields. Ketone **3** was also selectively reduced (99% ee) and obtained in the highest yield (79.9%) when [BMIM][PF_6] was applied. As it can be seen from the Table 2, each of the additives allows for the preparation of **3'** with a high enantiomeric excess (74-99%). 4'-Bromoacetophenone (**4**) was reduced with very high enantioselectivity (99% ee) using [BMIM][PF_6], ethyl chloroacetate, and ethyl 3-oxovalerate, but in comparison to the reduction of **3**, these reactions occurred in yields less than 10%. The only reduction of **4** in the presence of ethyl (9-anthryl)glyoxylate provided alcohol **4'** with high enantioselectivity and in high yield (93% and 74%, respectively).

The absolute configuration of the obtained alcohols depends on the structure of starting prochiral ketones. In the case of ketones with a bulky substituent (benzofuran ring), the hydride attacks the carbonyl group preferentially from the *re* face. Hence, from ketones **1** and **2**, the alcohols of the

configuration *R* and *S*, respectively, were obtained. While the reduction of **3** and **4** with less sterical hindrance occurred from the *si* face and giving predominantly (*S*)-**3'** and (*R*)-**4'**.

In general, the use of the antifungal agent Boni Protect for the bio-reduction of ketones **2**-**4**, in the presence of inhibitors, allows receiving the appropriate alcohols with high enantiomeric excesses. The results are comparable to those obtained in chemical reduction methods. For example, alcohols **2'** and **4'** were afforded by the asymmetric transfer hydrogenation with 96.9% ee and 93% ee, respectively [3, 10, 24]. The compound **3'** was prepared with 73-92% ee in the borane-mediated asymmetric reduction [4, 5, 6]. However, the chemical reduction of ketone **1** gave a product with higher enantiomeric excess (91%) [12] than in bioreduction.

In the next step, the selected α- and β-keto esters were subjected to the reduction with Boni Protect fungicide (Figure 2).

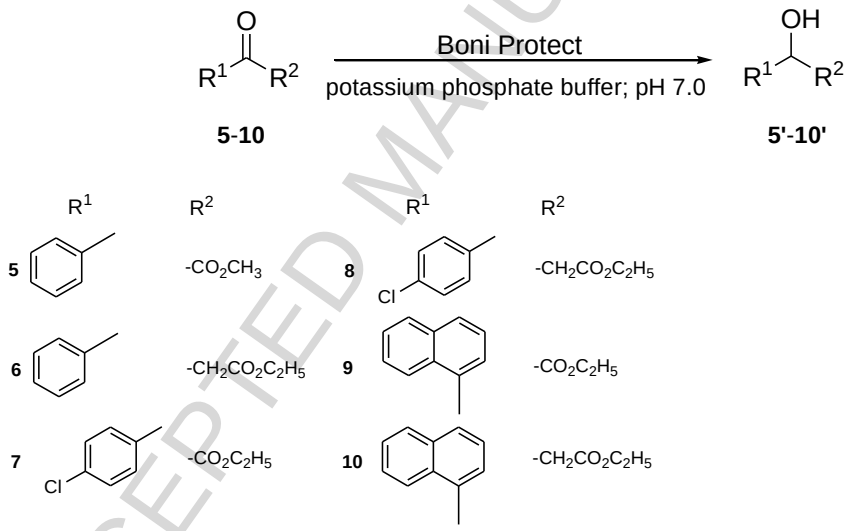

Figure 2. Bio-reduction of prochiral ketoesters **5**-**10** by *Aureobasidium pullulans*.

The results obtained from the reduction of keto esters by *A. pullulans* and from the fermentation method (reduction in the presence of *S. cerevisiae*) are summarized in Table 3.

The reduction of methyl 2-oxo-2-phenylacetate (**5**) in the presence of baker's yeast leads to the appropriate hydroxy ester of *R* configuration (according to the literature [25]). In the applied fermentative method, without the additive (methyl vinyl ketone), the product **5'** was obtained with 63% ee. While in the bio-reduction reaction with Boni Protect, the same compound was formed in the

opposite configuration with 99% ee. Similarly, ethyl 3-oxo-3-phenylpropanoate (**6**) was selectively reduced in the presence of *A. pullulans* to β-hydroxy ester **6'** of *S* configuration. In contrast, the bio-reduction by baker's yeast allowed for the formation of (*R*)-product with 84% ee. The presence of chlorine in the *para* position of phenyl ring did not affect the selectivity of the fungus *A. pullulans*. From both, α- and β-keto esters (**7** and **8**) the appropriate optically pure hydroxy esters of *R* and *S* configuration, respectively, were obtained. Using baker's yeast under fermentation conditions, without the use of dehydrogenase inhibitors of a specific stereopreference, the corresponding hydroxy esters were formed with lower enantioselectivity. However, for the reduction of compound **9**, baker's yeast occurred to be the best biocatalyst. The reaction in the presence of *S. cerevisiae* gave **9'** with 98% ee whereas in the presence of *A. pullulans* this product was afforded with lower enantiomeric excess (77%). The β-keto ester **10** was reduced in the reaction catalyzed by *A. pullulans*, alike the aforementioned α-keto ester **9**, to the product of *S* configuration. The hydroxy ester **10'** was formed in high chemical yield, as well as optical yield. In contrast, in the presence of baker's yeast, **10'** was produced in trace amounts with 69% ee.

Antimycotic agent Boni Protect shows high selectivity in the reduction of α- and β-keto esters. In the majority of tested compounds, the hydride ion selectively attacks the prochiral carbon atom on the *re* face giving predominantly the hydroxy esters of *S* configuration. Compared to the reduction by *S. cerevisiae*, the fermentative bio-reduction in the presence of *A. pullulans* leads to the optically pure hydroxy esters in high yields and does not require the use of additives to improve the reaction selectivity.

α-Keto esters **5**, **7**, and **9** were reduced by chemical methods with comparable high enatioselectivity, respectively compound **5** with 90.9-96.3% ee [8, 26, 27], compound **7** with 69.9-96.1% ee [8, 27], and compound **9** with 83.7-98.35% ee [27]. Similarly, β-keto esters **6** and **8** were reduced by the asymmetric hydrogenation to the optically pure hydroxy esters (compound **6** with 86-99% ee [7, 11, 28-30]; compound **8** with 95-99.9% ee [11, 28-30]). As it can be seen from the above comparison, the biochemical methods can compete with typical chemical methods. These reactions are highly selective and more ecological, inexpensive and simple.

4 Conclusion

Aureobasidium pullulans as an antifungal formulation Boni Protect was successfully used for the enantioselective bio-reduction of selected ketones, and α- and β-keto esters. Chiral alcohols and hydroxy esters were obtained in high optical purity. The reduction method is simple, economical, and does not require the cultivation of the bioreagent.

Acknowledgments

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Table 1 Reduction of **1**-**4** by *Aureobasidium pullulans* and *Saccharomyces cerevisiae*.

Microorganism	**1'** [%][a]	ee [%][a]	**2'** [%][a]	ee [%][a]	**3'** [%][a]	ee [%][a]	**4'** [%][a]	ee [%][a]
Aureobasidium pullulans	100[b]	*rac*	92.9[b]	61 (*S*)	75.5[b]	65 (*S*)	68.3[c]	51 (*R*)
Saccharomyces cerevisiae	96.5[c]	45 (*R*)	76.8[c]	36 (*S*)	65.0[b]	62 (*S*)	47.9[c]	72 (*R*)

[a] The ee and yield were determined by HPLC. [b] 24h. [c] 72h.

Table 2 Reduction of **1**-**4** by *Aureobasidium pullulans* with additives.

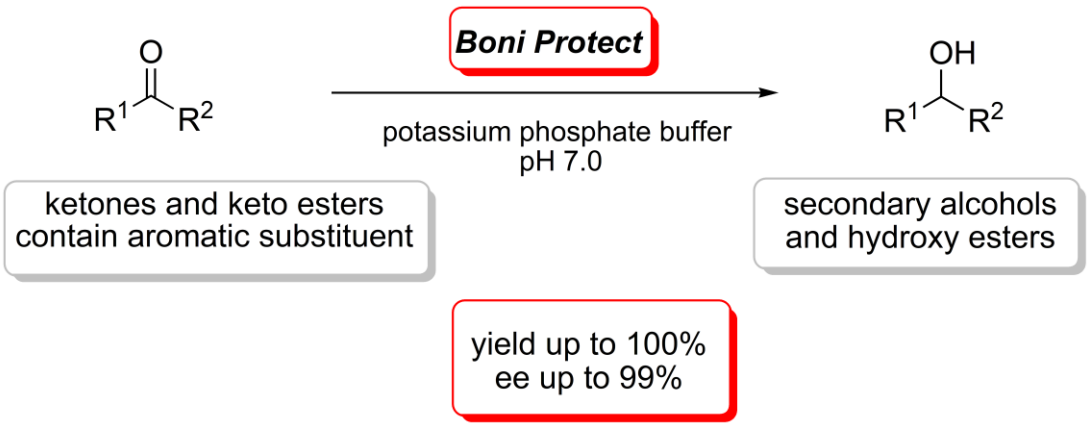
Additives	**1'** [%][a]	ee [%][a]	**2'** [%][a]	ee [%][a]	**3'** [%][a]	ee [%][a]	**4'** [%][a]	ee [%][a]
[BMIM][PF_6]	100[b]	*rac*	8.9[b]	77	79.9[b]	99	9.0[d]	99
Ethyl chloroacetate	98.6[b]	33	26.4[b]	69	65.3[b]	99	1.5[d]	99
Allyl bromide	78.7[c]	43	-	-	74.7[b]	99	55.8[d]	76
Allyl alcohol	99.6[c]	21	46.9[d]	99	84.0[b]	74	33.7[d]	52
Ethyl (9-anthryl)gloxylate	100[c]	55	40.8[d]	99	75.3[b]	96	74.0[d]	93
Ethyl 3-oxovalerate	100[c]	12	39.5[d]	99	66.8[b]	99	7.8[d]	99

[a] The ee and yield were determined by HPLC. [b] 24h. [c] 2h. [d] 72h.

Table 3 Reduction of **5**-**10** by *Aureobasidium pullulans* and *Saccharomyces cerevisiae*.

Microorganism		**5'**	**6'**	**7'**	**8'**	**9'**	**10'**
Saccharomyces cerevisiae	Yield [%][a]	100	100	20.0	25.0	100	5.0
	ee [%][a]	63 (*R*)	84 (*R*)	60 (*R*)	29 (*S*)	98 (*S*)	69 (*S*)
Aureobasidium pullulans	Yield [%][a]	100	100	100	80.0	100	100
	ee [%][a]	99 (*S*)	99 (*S*)	99 (*R*)	98 (*S*)	77 (*S*)	99 (*S*)

[a] The ee and yield were determined by HPLC.



Graphical abstract

Highlights

- Antimycotic agent Boni Protect was used for the enantioselective bio-reduction.
- The microbiological reduction of ketones, and keto esters were investigated.
- Chiral alcohols and hydroxy esters were obtained in high optical purity.