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Diastereoselective reduction of 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)-propyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one to Ezetimibe by the whole cell catalyst *Rhodococcus fascians* MO22

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ABSTRACT

The asymmetric microbial reduction of 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)-propyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one to 1-(4-fluorophenyl)-3(R)-[3(S)-hydroxy-3-(4-fluorophenyl)-propyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one (Ezetimibe) by *Rhodococcus fascians* MO22 is described. The catalytic capability of the microorganism for reduction has been examined also with protected ketone, an intermediate from chemical synthesis of Ezetimibe. Various parameters of the bioreduction have been optimized: the strain converted 94.8% of ketone and 63% of protected ketone into Ezetimibe with the same de of 99.9%. In the later case, two chemical steps are replaced with a single biotransformation.

1. Introduction

Ezetimibe **2** is 2-azetidione that selectively and potently inhibits the adsorption of biliary and dietary cholesterol and related plant sterols from the small intestine without affecting the adsorption of fat-soluble vitamins, triglycerides or bile acids. The chiral alcohol Ezetimibe **2** is an effective option for treating patients with primary hypercholesterolemia, reducing the risk of coronary heart disease [1,2]. A deprotection and reduction of the respective ketone to alcohol belong among key steps of synthesis of Ezetimibe **2**. The usual methods employed in the preparation of optically active alcohols are asymmetric reductions of ketones by chemical [3,4] or chemoenzymatic syntheses [5,6]. Several reviews deal with the use of enzymes in organic syntheses of the drugs [7–11] in which the chirality is a key factor in their efficiency. For pharmaceutical industry, the production of a single stereoisomer of the chiral molecule is of great importance.

Recently a number of microbial strains having ketone reductase (KR) activity have been reported and exploited for the stereoselective reduction of ketones. KRs show a broad substrate range and an excellent stereodifferentiation. However, specific catalyst must be developed for each substrate as these compounds are not natural substrates for the microbial enzymes [12]. The advantages of reactions catalyzed by enzymes over chemical syntheses are that enzymatic reactions are often highly stereoselective, regioselective and the biocatalysis is a process that is environment friendly [13].

In this paper, we describe the biotransformation of 1-(4-fluorophenyl)-3(R)-[3-oxo-3-(4-fluorophenyl)-propyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one **1** or its carboxybenzyl-protected form **3** to 1-(4-fluorophenyl)-3(R)-[3(S)-hydroxy-3-(4-fluorophenyl)-propyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one **2** by the nature isolate *Rhodococcus fascians* MO22 [14] with high Ezetimibe **2** yield and excellent diastereoselectivity (Scheme 1). The alternative enzyme-based method for the last step or two steps of synthesis of Ezetimibe **2** was described.

2. Experimental

2.1. Chemicals and general methods

A ketone **1**, Ezetimibe **2**, protected ketone **3**, and protected Ezetimibe **4** were synthesized in the Development Department, Zentiva Group, k.s., Czech Republic.

Media components were purchased from Hi-media and Difco and solvents were obtained from Merck and Chromasolv.

Thin layer chromatography (TLC) was made on plastic sheets Silica gel 60 F254 from Merck and the detection was done under UV (λ = 254 nm). Elution system, the mixture of ethylacetate:hexane = 1:1 was used; rf for ketone **1** = 0.5 and rf for alcohol **2** = 0.3.

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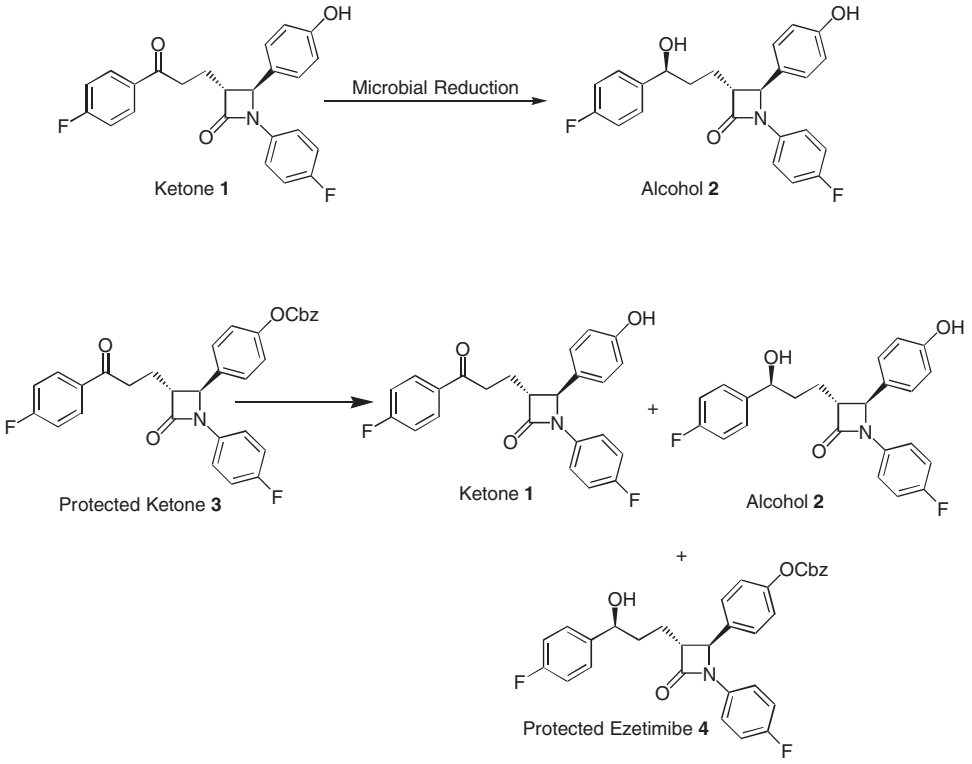

Scheme 1. Preparation of Ezetimibe **2**. Cbz is a carboxybenzyl protecting group.

HPLC analysis was made on Dionex Summit with PDA detector. Column Luna C18 5 μm was used for determination of the degree of conversion and column Chiralpak AD-H (Daicel) for de determination.

To determine quantitatively the percentage of conversion of the substrate **1** to the product **2**, the reaction mixtures containing the product **2** were also analyzed by HPLC. Mobile phase was 0.02 M KH_2PO_4 buffer (pH 3.0):acetonitrile = 3:7, isocratic elution with flow rate of 1.3 mL/min, column temperature of 20 °C, detection spectrophotometer λ = 220 nm, injection volume of 10 μL, and run time 6 min. The retention time of **2** and **1** was 3.13 and 4.07 min, respectively.

In the case of isolates capable of reduction and exhibiting more than 4% conversion of the substrate, the diastereomeric excess of Ezetimibe **2** was determined. Mobile phase used was hexane:ethanol = 1:1 + 0.5 mL/L of diethylamine. The isocratic elution was performed as follows: the flow rate of 1 mL/min, column temperature of 40 °C, injection volume of 10 μL, run time 12 min, and the reactants were detected at the wavelength λ = 275 nm. The retention time of **2** and R-alcohol was 4.7 and 6.6 min, respectively (Fig. 1).

For isolation of Ezetimibe **2** after biotransformation of ketone **1**, 30 mL of 20% cell suspension was saturated with NaCl and the reaction mixture was treated with ethylacetate (4 × 15 mL). Ethylacetate fractions were collected, dried out with Na_2SO_4 and evaporated on a rotavap. The Ezetimibe **2** and ketone **1** were purified by flash chromatography on Silica gel 60 from Fluka (elution system ethylacetate:hexane = 1:1).

2.2. Microorganisms

Microorganisms were obtained from the Culture Collection of Industrial Microorganisms, the Research Institute of Food Industry, Prague, and the Collection of Microorganisms of the Laboratory of Enzyme Technology, the Institute of Microbiology ASCR, v.v.i., Prague. Microorganisms were maintained as frozen glycerol stock cultures at −70 °C.

2.3. Culture conditions and screening procedures

Samples of the cell suspensions used in biotransformations were prepared by batch cultivations in 50-mL shake flasks containing

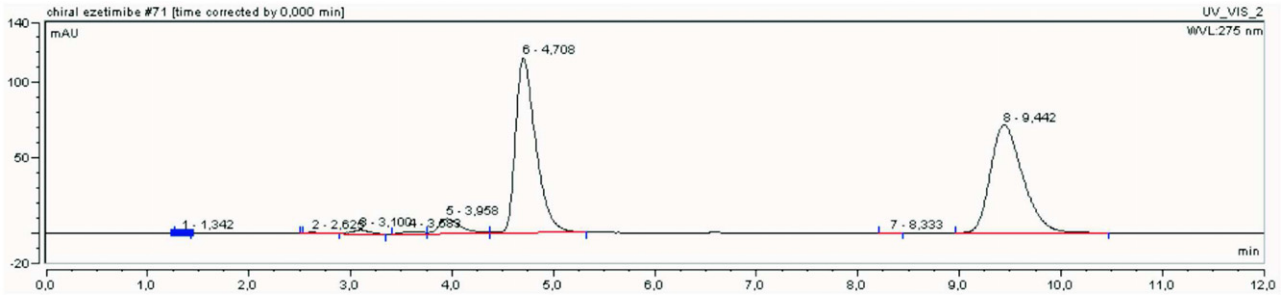

Fig. 1. Example of chromatogram from biotransformation. The retention time of Ezetimibe **2** was 4.7 min and 9.4 min for ketone **1**. A negligible peak around 6.6 min was R-alcohol.

15 mL of the culture medium. The cultures of bacteria and yeasts were grown in LB medium (1.0% tryptone, 0.5% yeast extract, 1.0% sodium chloride, pH 7.0) and YPD medium (1.0% yeast extract, 1.0% peptone, 1.0% glucose, pH 5.5), respectively. The value of pH in a medium was adjusted before sterilization. One vial of the frozen stock culture of each strain was used as inoculum and cultures were performed on an orbital shaker (200 rpm) for 48 h and at 28 °C. In the course of a cultivation or microbial reduction, pH was not controlled. To prepare the cell suspensions for biotransformations, a biomass was harvested from the cultures by centrifugation (10 000 × *g*, 15 min, 10 °C), washed with 50 mM potassium phosphate buffer (pH 7.0, buffer A), frozen overnight at −20 °C, and used for the reduction of **1** after resuspending in a required volume of the buffer A.

Screening for microorganisms capable of the diastereoselective reduction of the ketone **1** was performed as follows: the volume of 5 mL of 10% suspension of cells (cell wet weight/v) in buffer A was added into 25 mL Erlen-Mayer flasks. The reactions were performed at 200 rpm, 28 °C and initiated by the addition of 0.1 mL of solution of **1** in ethanol (25 mg/mL) so that the final concentration of **1** equaled 0.5 g/L. After 24 h, a sample of the reaction mixture (0.5 mL) was removed and extracted for 15 min with 0.5 mL of ethylacetate. The sample was centrifuged (10 000 × *g*, 5 min) and the organic layer was used for the detection of **1** and **2** by TLC.

*2.4. Optimization of conditions for biotransformation of **1** by cells of R. fascians MO22*

2.4.1. The influence of the growth phase of a culture

The microbial strain *R. fascians* MO22 was grown in a 10 L stirred bioreactor with a working volume of 8.0 L. The culture medium R contained 0.2% yeast extract, 0.3% beef extract, 0.5% bacteriological peptone, 0.7% tryptone, and 1.0% sodium chloride (pH 7.2).

Two-stage inoculum (F1 and F2) was prepared. Inoculum F1: a vial of the glycerol stock culture was inoculated into 100 mL of medium R in a 500 mL flask. The cultivation was carried out on the orbital shaker (200 rpm) for 24 h at 28 °C. 5 mL of the culture F1 was used to inoculate 100 mL of the medium R, and the inoculum F2 (2 flasks) was incubated for 34 h at 28 °C.

The bioreactor containing 8 L of the medium R was inoculated with 200 mL of the F2 inoculum and the batch culture was conducted under the following conditions: cultivation temperature of 28 °C, stirring frequency of 500 rpm, aeration rate of 10 L/min, and cultivation time 58 h. During fermentation, the cells were harvested periodically from 2 mL samples of the culture by centrifugation (10 000 rpm, 15 min, 10 °C), washed with buffer A, and kept at −20 °C. To evaluate the bioconversion ability of cells, the frozen biomass in a vial was resuspended in 1 mL of buffer A and the reaction was started by addition of the ethanol solution of ketone **1** (final concentration 0.5 g/L). The vials were shaken (200 rpm, 28 °C) for 4 h and the sample of the reaction mixture was removed and processed as described earlier. The organic layer was used for the determination of concentrations of **1** and **2** by HPLC.

2.4.2. The effect of reaction pH, temperature and cofactor regeneration

To find pH optimum for the bioreduction, the reaction was carried out at the pH ranging from 2 to 10, using 0.1 M buffers as follows: the citrate buffer for the pH range from 2 to 6, the phosphate buffer for the pH 7 and 8, and the Tris–HCl buffer for the pH 9 and 10. The temperature optimum was determined by incubation of the resting cell suspension in buffer A at temperature ranging from 25 °C to 40 °C. To improve the efficiency of the microbial reduction, the effect of routinely used substrates for cofactor regeneration was studied: the conversion of **1** was performed in the buffer A supplemented with glucose, glycerol, isopropanol, and formate at concentrations 10, 50 and 100 g/L. 10% (cell wet weight/v) suspension of cells was used in these experiments. The following parameters optimized in this work were used in the conversion experiments with cofactor regeneration: pH 7.0 and temperature of 30 °C.

Table 1
Asymmetric microbial reduction of ketone **1** to Ezetimibe **2**.

Microorganism	Yield (%)	de of **2** (%)
Saccharomyces cerevisiae No. 3	8.6	99.9
Saccharomyces cerevisiae No. 4	8.4	99.8
Saccharomyces cerevisiae No. 13	4.8	95.8
Torulopsis azyma CCY 26592	4.3	n.d.
Candida tropicalis RIFIS 216	4.7	n.d.
Rhodococcus sp. No.654	24.3	99.2
Rhodococcus fascians MO22	41.5	99.9

Yield is expressed as a percentage (w/w) of the ketone converted to Ezetimibe, n.d.: not determined.

3. Results and discussion

A number of yeasts and bacteria [15–18] were reported as ketoreductase positive microorganisms. To detect the useful biocatalyst for the production of Ezetimibe **2** with high conversion and de, a number of microorganisms have to be analyzed. As a total, we screened 230 microbial strains for the asymmetric reduction of the ketone **1** to alcohol **2**. Among them, eight yeast and nine bacterial strains were detected and isolated as KR positive microorganisms. The conversion and de of the product **2** were determined by HPLC with cell suspensions of the strains exhibiting at least a conversion of 4% of the ketone into the product. The results are shown in Table 1. The highest conversion of 41.5% and de of 99.9% were achieved with the strain *R. fascians* MO22 (collection of microorganisms of the Laboratory of Enzyme Technology) that was isolated from the population of microorganisms growing in a biofilter of the exhaust gas installed to remove pollutants the styrene and acetone.

The growth of *R. fascians* MO22 in a stirred bioreactor in the medium R and bioconversion of the ketone **1** by culture samples withdrawn at regular intervals from the bioreactor were conducted to study the effect of the culture growth phase on the bioconversion (Fig. 2). The culture growth was completed in 44 h when the culture reached the stationary phase of growth. The bioconversion increases in the course of the exponential phase of growth and reaches the maximum of 45% in the 22nd h of cultivation. The same conversion was maintained for the period of time of 22 h in the course of which the growth rate of the culture ceased. Later on, the fast decrease of the bioconversion was observed, but cells

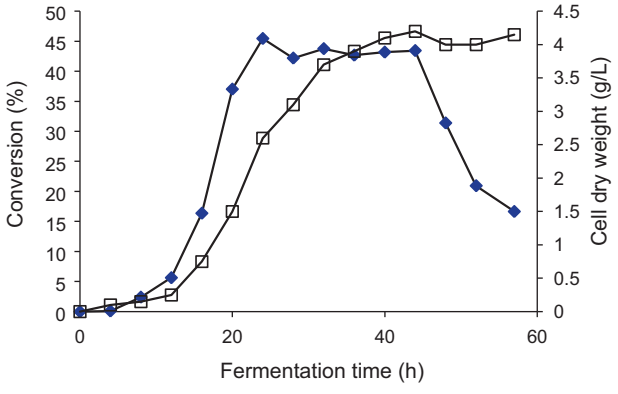

Fig. 2. The time-course of the biomass concentration (□: cell dry weight in g/L) in a batch culture of the strain *R. fascians* MO22 performed in a stirred bioreactor and the growth phase-dependent capability of the cells for diastereoselective reduction of ketone **1** to Ezetimibe **2** (♦: conversion in %).

Table 2
The effect of pH on the microbial reduction of ketone **1** to Ezetimibe **2** by cell suspension of *R. fascians* MO22.

pH	2	3	4	5	6	7	8	9	10
Conversion (%)	4.8	47.9	86.0	90.0	95.6	97.0	82.1	83.1	83.1

Conditions of biotransformation: $T = 30\,^{\circ}C$, $t = 4$ h, cells were resuspended in the buffer of a given pH value.

exhibited high diastereoselectivity: de of **2** was 99.9% in the course of the whole cultivation.

The effects of the temperature and pH on the asymmetric microbial reduction of the ketone **1** to Ezetimibe **2** were evaluated using 10% cell suspension of *R. fascians* MO22 (cell wet weight/v) and 0.1 g/L of **1** in a buffer in the absence of a substrate for cofactor regeneration. An effect of pH of the reaction mixture on the bioreduction was followed at pH value ranging from 2 to 10.

It is evident (Table 2) that the process of biotransformation reaches similar degree of conversion at the pH range from 4 to 10. The pH optimum for the conversion lies between the pH values 6 and 7. A distinct decrease of the conversion occurs on the acidic side of the range of pH values.

The effect of the reaction temperature on the conversion process was followed at temperatures ranging from 25 °C to 40 °C (Fig. 3). The optimum temperature for the bioreduction of the ketone **1** corresponded to 30 °C. The percentage of conversion at other temperatures was markedly reduced: the conversion was reduced by 50% and 70% at the temperature of 25 °C and 35 °C, respectively. In all cases, the maximum conversion was reached in 4 h. We presume that any internal source of energy has been depleted after 4 h of the reaction and no regeneration of cofactor occurred in a reaction buffer.

At temperatures up to 30 °C, de was 99.9% but the parameter decreased to 86.4% at temperature of 35 °C. Since biotransformations were performed with whole cells (not with the purified enzyme) we cannot exclude the presence of alternative KR with lower stereoselectivity which may be more stable or more active at higher temperatures.

Percentage of conversion can be affected by the depletion of the cofactor NADH or NADPH. The effect of addition of glucose, glycerol, isopropanol, and formate, substrates routinely used for the regeneration of cofactors of KRs, is shown in Fig. 4.

The glucose had a marked positive effect on the percentage of the conversion of **1** at the concentration of 50 g/L: in comparison to the control experiment, the percentage increased by 17% after 4 h. The effect of glycerol on the percentage of the conversion of **1** was positive in the whole concentration range: in comparison to the control experiment, the percentage increased by 22% after 4 h even at the concentration of 100 g/L.

A similar effect was observed if isopropanol (50 g/L) or formate (10 g/L) was added to the reaction buffer. While the formate seems to be a good candidate for regeneration of the cofactor, isopropanol

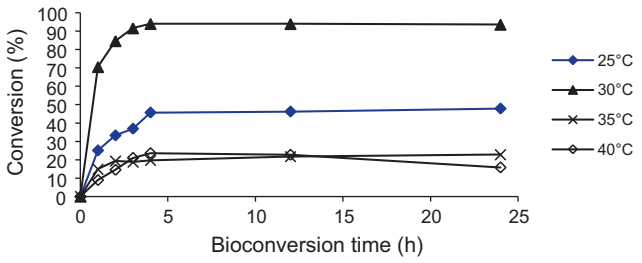

Fig. 3. The effect of temperature on the microbial reduction of ketone **1** (0.1 g/L) to Ezetimibe **2** by cell suspension of *R. fascians* MO22. The time course of the processes was studied in buffer A.

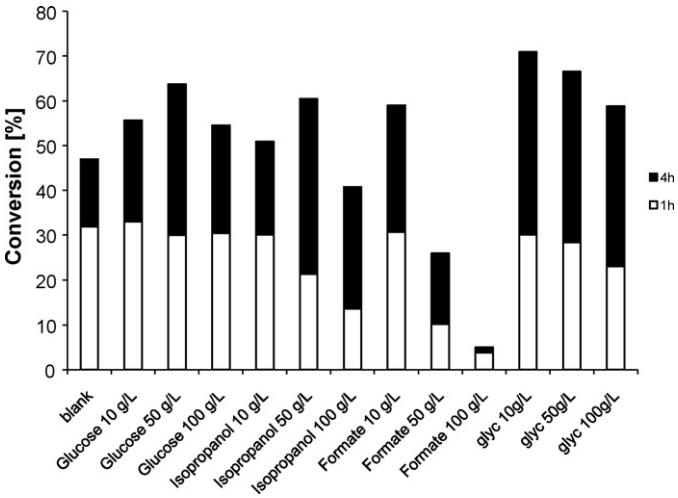

Fig. 4. Concentration effect of the glucose, isopropanol, formate and glycerol as substrates for the regeneration of the cofactor on the conversion of ketone **1** (0.1 g/L) to Ezetimibe **2** by cell suspension of *R. fascians* MO22 (pH 7.0, 25 °C). Conversion was determined after 1 and 4 h of biotransformation.

could reduce the rate of conversion at this concentration (a lower percentage reached after 1 h).

Deprotection of Cbz protecting group of **3** is a chemical reaction preceding microbial diastereoselective reduction of **1** to **2**. Bioreduction of **3** to **2** was determined with 10% or 20% (cell wet weight/v) suspension of the biomass withdrawn from the bioreactor culture of the strain *R. fascians* MO22 at the 22nd h of the cultivation. Optimal conditions for the biotransformation of **1** to **2** (buffer A supplemented with 50 g/L of glucose, 30 °C) were applied and the protected ketone **3** was used at concentrations ranging from 0.1 to 4.0 g/L. The suspensions were able to transform **3** directly into Ezetimibe **2** with conversion of 63%, with a few percents of **1** and protected Ezetimibe **4** (Table 3).

Biotransformation of **1** to **2** was evaluated under identical conditions as with protected ketone **3** (Table 4). The maximum conversion of 90 and 95% in 24 h was reached with 10% and 20% suspension of cells, respectively. As regards the productivity of the process, the highest value was obtained with 20% cell suspension at 0.5 g/L of ketone **1** within the 1st h of reaction: 175 mg/L h.

After 24 h, the reduction of **1** gives higher conversions at lower concentrations of the substrate (0.1–1.0 g/L) while the biotransformation of **3** gives higher conversions at concentration range from 1.0 to 4.0 g/L.

To isolate the product **2** and determine the yield of isolation, 30 mL of 20% suspension of cells was shaken for 48 h at optimal

Table 3
Biotransformation of protected ketone **3** used at different concentrations by 10% or 20% cell suspension of *R. fascians* MO22 at 30 °C.

Cell suspension	Protected ketone **3** (g/L)	Conversion after 24/48 h (%)		
		Ezetimibe **2**	Ketone **1**	Protected Ezetimibe **4**
10%	0.1	44/56	2/0	0/0
	0.5	42/49	4/3	4/0
	1	29/41	16/4	7/0
	2	27/39	19/6	8/3
	4	24/29	21/6	8/5
20%	0.1	53/63	1/5	0/0
	0.5	44/44	3/6	4/2
	1	39/43	5/9	9/3
	2	36/38	12/10	11/3
	4	28/37	19/11	10/4

Table 4
The time course of the biotransformation of ketone **1** used at different concentrations to Ezetimibe **2** by 10% or 20% cell suspension of *R. fascians* MO22 at 30 °C.

Cell suspension	Ketone **1** (g/L)	Conversion (%)			
		1 h	4 h	24 h	48 h
10%	0.1	62.0	88.5	89.8	86.5
	0.5	28.6	41.2	58.4	82.1
	1	2.5	7.3	24.4	41.1
	2	1.9	6.2	23.5	36.7
	4	1.1	3.9	17.8	23.5
20%	0.1	72.3	93.9	94.8	90.0
	0.5	35.0	62.6	84.4	92.1
	1	7.3	20.5	44.8	50.2
	2	3.6	11.4	37.3	47.2
	4	2.6	8.6	26.3	33.4

conditions for the biotransformation of **1** to **2** (buffer A supplemented with 1.5 g of glucose, 15 mg of ketone **1**, 30 °C). The reaction mixture was processed and the reactants were purified by flash chromatography from 330 mg of brown tar obtained after evaporation. The conversion reached 84%, yield of isolated Ezetimibe **2** was 68% (10.2 mg) and recovery of ketone **1** equaled 14% (2.1 mg).

4. Conclusion

Only 7% of the screened microbial strains reduced the ketone **1** to Ezetimibe **2**. Among them, the highest yield of the conversion was achieved with the strain *R. fascians* MO22.

The optimum conditions for the biotransformation of **1** to **2** are as follows: phosphate buffer (pH 7, 20 mM), temperature of 30 °C and the glucose (50 g/L) or glycerol (10 g/L) as substrates for cofactor regeneration. The formate is the alternative substrate for regeneration at concentration of 10 g/L.

The maximum conversion of 95% in 24 h was reached with 20% suspension of cells. The highest productivity of 175 mg/L h was obtained with 20% cell suspension at 0.5 g/L of ketone **1** after 60 min of the reaction (Table 4).

To our knowledge, this is the first report on achievement of excellent de and a high degree of conversion of **1** to Ezetimibe **2** using a whole cell catalyst.

The use of the extraction step and protocol for isolation of Ezetimibe **2** at industrial scale requires further optimization because the yield of **2** (68%) is low when compared to conversion of 84%.

Biotransformation of protected ketone **3** could replace one more synthetic step, usually a deprotection by hydrogenation on a paladium catalyst, and can avoid Ezetimibe **2** contamination with the heavy metal. Maximum conversion of 63% in 48 h was reached with 20% suspension of cells.

Acknowledgments

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