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Whole cell application of *Lactobacillus paracasei* BD101 to produce enantiomerically pure (*S*)-cyclohexyl(phenyl) methanol

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Abstract

In this study, a total of 10 bacterial strains were screened for their ability to reduce cyclohexyl(phenyl)methanone **1** to its corresponding alcohol. Among these strains, *Lactobacillus paracasei* BD101 was found to be the most successful biocatalyst to reduce the ketones to the corresponding alcohols. The reaction conditions were systematically optimized for the reducing agent *L paracasei* BD101, which showed high enantioselectivity and conversion for the bioreduction. The preparative scale asymmetric reduction of cyclohexyl(phenyl)methanone (**1**) by *L paracasei* BD101 gave (*S*)-cyclohexyl(phenyl)methanol (**2**) with 92% yield and >99% enantiomeric excess. The preparative scale study was carried out, and a total of 5.602 g of (*S*)-cyclohexyl(phenyl)methanol in high enantiomerically pure form (>99% enantiomeric excess) was produced. *L paracasei* BD101 has been shown to be an important biocatalyst in asymmetric reduction of bulky substrates. This study demonstrates the first example of the effective synthesis of (*S*)-cyclohexyl(phenyl)methanol by the *L paracasei* BD101 as a biocatalyst in preparative scale.

KEYWORDS

asymmetric reduction, biocatalyst, biotransformations, chiral aryl methanols, enantioselectivity

1 | INTRODUCTION

Chirality is a key factor in the safety of many drugs, and therefore, the synthesis of enantiopure drugs has become increasingly important issue in the pharmaceutical industry.[1] The interaction of chiral compounds with chiral environments may result in biologically different enantiomers that may have deteriorative effects. The modern pharmaceutical industry demands for the obtaining of biologically active chiral compounds in their enantiomerically pure forms for the production of drugs and other chemicals found in biological systems. So the enantiomeric purity of the synthesized product is of great importance.[2,3] The enantioselective formation of carbinols is one of the most essential strategies in modern organic chemistry as they are commonly used as intermediates for the synthesis of drugs and other industrially important compounds.[4,5] Heteroaryl methanols can be used for the production of pharmaceutically significant molecules that might have antihistaminic, anesthetic, diuretic, antidepressive, antiarrhythmic, and anticholinergic roles.[6] Thus, chiral carbinols can act as precursors of many biologically and pharmacologically relevant compounds,[7] and important examples of these compounds are (*R*)-orphenadrine,[8] (*S*)-phenyl(pyridin-2yl)methanol,[9] and carbinoxamine[10] (Figure 1). Generally, production of these compounds is

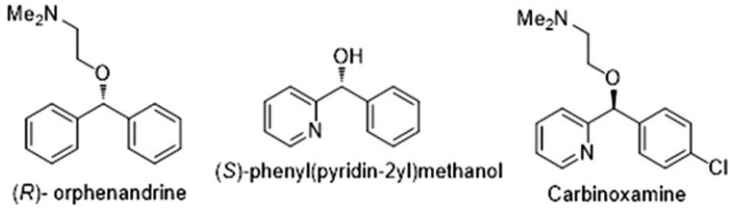


FIGURE 1 Chiral carbinol-based drugs

carried out by classic synthetic procedures that use catalysts such as metal hydrides, but the product obtained in these reactions ate in their non-enantiomerically form. Although classic chemical methods for the enantioselective synthesis of chiral carbinols have been comprehensively developed, the synthesis of some of these compounds is still extremely complicated, toxic, and expensive.[11] Biocatalytic synthesis is a very successful technique as a green chemistry application for preparing chiral compounds. However, enantioselective reduction of aryl heteroaryl ketones in the presence of biocatalyst is still challenging.[12,13] Diaryl ketones are usually known as "hard-to-reduce" substances for biocatalyst owing to their big steric obstacle and similar of two aromatic groups.[14,15] Biocatalysts have many superiorities compared with classic chemical catalysts. Chemical catalysts might produce toxic waste and a large range of by-products, whereas biocatalysts are biodegradable products and provide a clean and environmentally friendly process to carry out these reactions under mild reaction conditions with high selectivity for the substrate.[16] Therefore, highly selective and environmentally friendly biocatalytic process to synthesis optically active carbinols is the utilization of isolated enzymes and whole cell microorganisms.[17] Whole cell biocatalysts compared with the pure enzyme are advantageous as they are usually cheap and more stable. Whole cells contain various dehydrogenases that are able to accept a wide range of unnatural substrates. All enzymes and cofactors are well protected within their natural cellular environments. Moreover, the use of whole cell biocatalysts avoids enzyme purification and cofactor addition.[18-20] Enantiomerically pure carbinols are useful building blocks for the synthesis of complex molecules as the alcohol functional group can be easily converted, without racemization, into other functional groups.[21-24]

Sales of single enantiomeric drug products are expanding at an alarming rate every year in the world. Therefore, enormous efforts have been made in recent years to detect enantioselective routes to enantiomerically pure carbinols. Enantiopure diarylmethanols are important structural scaffolds in the synthesis of pharmaceuticals and bioactive compounds.[25-30] There are a number of studies in the literature that contain cyclohexyl(phenyl) methanone **1** in high enantiomeric selectivity using chemical catalysts[31-34]; however, there are very limited studies involving reduction of this compound using biocatalyst, and in these examples, the corresponding chiral secondary alcohols were obtained by low enantiomeric excess (ee) and conversion. Ema and coworkers obtained (*S*)-**2** with 59% ee by the lipase enzyme catalyst.[35] In the literature, it has been reported that cyclohexyl(phenyl)methanone is reduced to (*R*)-**2** by 75% ee and 55% conversion with yeast *Candida magnoliae* as biocatalyst.[36] The use of [α]-branched aromatic ketones as a biocatalyst for whole cell or pure enzymes as a biocatalyst has been reported in the literature in which the asymmetric reductions are generally of low selectivity or low conversion.[1] Recently, we performed asymmetric reduction of acetophenones and piperonyl methyl ketone by high enantioselectivity and conversion using *Lactobacillus paracasei* BD101 as biocatalyst.[37,38]

In this study, we report the asymmetric reduction of cyclohexyl(phenyl)methanone **1** to the (*S*)-cyclohexyl(phenyl)methanol **2** by *L paracasei* BD101 with >99% ee and 92% yields. Moreover, to the best of our knowledge, this is the first report on asymmetric reduction of cyclohexyl(phenyl)methanone **1** using biocatalyst in the enantiomerically pure form, high yield, and scale. Scale-up bioreduction of cyclohexyl(phenyl)methanone **1** was reduced to the (*S*)-cyclohexyl(phenyl)methanol **2** in excellent yield and enantiopure form using *L paracasei* BD101 as a biocatalyst. The effects of the reaction conditions in terms of pH, temperature, and agitation speed on the yield, conversion, and ee were also optimized for the bioreduction reaction.

2 | MATERIALS AND METHODS

2.1 | General

All chemicals and all solvents were purchased from Sigma-Aldrich (purity of >99%). The growth mediums for bacterial growth were purchased from Merck. Reactions were monitored by thin-layer chromatography (TLC) performed by using plates (aluminum, silica gel 60 F_{254} Merck, 0.25 mm) and hexane to ethyl acetate (4:1, v/v) as eluent. For analysis purpose, a small fraction of the product was prepared by preparative TLC. Purification of (*S*)-cyclohexyl(phenyl)methanol was performed by column chromatography filled with silica gel (0.063-0.2 mm), and the product was eluted with

a mixture of hexane to ethyl acetate (90:10, v/v). High-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1260 systems equipped with a UV and chiral detector. The racemic **2** was obtained by reducing the **1** with $NaBH_4$ in methanol at room temperature (RT). Optical rotation was measured with a Bellingham + Stanley, ADP220, 589 nm spectropolarimeter. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on Bruker 400 MHz spectrometer in $CDCl_3$. (*S*)-cyclohexyl(phenyl)methanol[39]: White solid, m.p.: 63-65°C, ^1H NMR (400 MHz, $CDCl_3$) δ = 7.36-7.28 (m, 5H), 4.39 (d, *J* = 7.2 Hz, 1H), 2.01-1.61 (m, 6H), 1.42-0.94 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 143.6, 128.2, 127.4, 126.6, 79.4, 45.0, 29.3, 28.8, 26.4, 26.1, 26.0; $[\alpha]_D^{25} = -39.5$ (c 0.4, $CHCl_3$) >99% ee; HPLC, Chiralcel OD column, hexane/*i*-PrOH, 95:5, flow rate of 0.5 mL/min, 254 nm, t_R (*S*) 21.3 minutes. HPLC condition of **1**: Chiralcel OD column, *n*-hexane/*i*-PrOH, 95:5, flow rate of 0.5 mL/min, 254 nm, 10.7 minutes (Supporting Information).

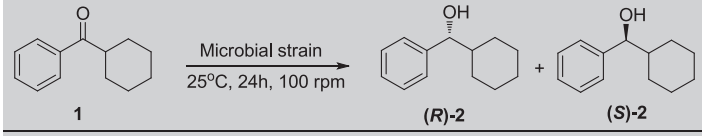
2.2 | Bacterial strains and culture conditions

The microorganism used in this study was isolated previously from boza, a cereal-based fermented beverage (Table 1, entries 1–7) and Turkish sourdough (Table 1, entries 8–10).[40] Bacterial strains were propagated from their glycerol stocks by inoculation to 10-mL MRS broth medium followed by overnight growth at 30°C. From these cultures, exponentially grown bacterial cells were inoculated to 1-L MRS broth medium at 10% concentration and incubated 2 days at 30°C under aerobic conditions. Following the growth of the bacteria in the flasks, bacterial cells were centrifuged, and following the wash process, whole cells were obtained freeze-drying (Labconco, TR) and stored at RT.

2.3 | General procedure for the asymmetric reduction of cyclohexyl(phenyl)methanone

Forty-milligram dry bacterial strain was added to 100-mL MRS broth and stirred on an orbital shaker at 150 rpm and 30°C. After 2 hours, pH was adjusted 6 and shaken 2 hours, followed by the addition of 1-mmol cyclohexyl(phenyl)methanone **1** directly to the medium, and incubated on a shaker (150 rpm) at 30°C for 48 hours. At the end of the incubation period, the cells were separated by centrifugation at 6000*g* for 5 minutes at 4°C and the supernatant was saturated with NaCl and then

TABLE 1 Screening of bacteria strains for the bioreduction of **1**



Entry	Microbial strain[a]	Conversion, %[b]	Yield, %[c]	ee, %[d,e] (*S*)
1	*Lactobacillus plantarum* BY14	61	54	52
2	*Lactobacillus fermentum* BY35	58	51	63
3	*Enterococcus faecium* BY48	54	47	48
4	*Lactobacillus paracasei* BD28	64	54	65
5	*Lactobacillus paracasei* BD101	74	70	81
6	*Lactobacillus paracasei* BD71	62	56	72
7	*Lactobacillus paracasei* BD87	54	48	15
8	*Leuconostoc mesenteroides* N6	70	64	51
9	*Weissella paramesenteroides* N7	21	15	62
10	*Weissella cibaria* N9	10	5	45

Abbreviations: ee, enantiomeric excess; HPLC, high-performance liquid chromatography. Reaction condition: substrate 1 mmol/100 mL; temperature, 25°C; time, 24 h; 100 rpm.

[a]Comparison of the best microbial strain.

[b]The conversions were determined by HPLC.

[c]Isolated yield.

[d]Determined by HPLC using Chiralcel OD column.

[e]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

extracted with dichloromethane. Dichloromethane extracts were combined and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was identified by NMR analysis followed by the purification of this product by column chromatography on silica gels using hexane to ethyl acetate as eluents (90:10). The absolute configuration was determined by sign of specific rotation. The conversion was determined by chromatography on a chiral OD column on HPLC (*n*-hexane/*i*-PrOH, 95:5, flow rate of 0.5 mL/min, 254 nm) after filtering the crude product with a column containing small silica gel and comparing the alcohol peaks with the ketone peaks. The ee of the products was determined by HPLC analysis using chiral OD column.

2.4 | Scale-up asymmetric reduction of cyclohexyl(phenyl)methanone

The 400-mg dry *L paracasei* BD101 was inoculated into 1-L sterilized fresh MRS broth as working volume in 5-L Erlenmeyer flask under sterile conditions and stirred on an orbital shaker at 30°C, 150 rpm for 2 hours. After 2 hours, pH was adjusted to 6 and the flask was shaken 2 hours followed by the addition of 32 mmol cyclohexyl(phenyl) methanone **1** to the medium and the reaction mixture was incubated at 30°C, for 56 hours under agitation at 150 rpm. At the end of the incubation period, the cells were separated by centrifugation at 6000*g* for 5 minutes at 4°C and the supernatant was saturated with NaCl and then extracted with dichloromethane. Dichloromethane extracts were combined and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified on a silica gel column and eluted using hexane to ethyl acetate (10:1, v/v) to obtain the product.

3 | RESULTS AND DISCUSSION

Ten different bacterial strains were evaluated for the reduction of **1** to **2**. The reactions for screening process were carried out in 200-mL Erlenmeyer flask containing 100 mL of fresh culture medium (MRS broth). The microbial reduction was performed by suspending the dry cells (40 mg) in a 100-mL fresh medium; then substrate **1** (1 mmol, 188.27 mg) was added directly, and the mixture was incubated on conditions of 100 rpm and 25°C for 24 hours. The bioreduction progress was monitored TLC, and a chiral HPLC column was used for the determination of the ee of product **2** and conversion of **1** to **2**. As can be seen in Table 1, the bacterial strains used in this study reduced **1** to **2** at levels of 5% to 70% ee. The best result for the asymmetric bioreduction was obtained when whole cell of *L paracasei* BD101 was used. The bioreduction was taken place with high conversion and produced **2** at 81% ee with this strain (Table 1, entry 5). One of the primary requirements of a microbial biotransformation is the optimization of the reaction conditions.[41] Therefore, we decided to determine the performance of the microorganism under optimized reaction conditions. To detect the optimal reaction conditions for the asymmetric reduction of **1** with *L paracasei* BD101, pH, temperature, incubation period, and agitation speed were examined. The first parameter that was investigated was the effect of pH on the production of **2** from **1**. Table 2 shows the results for the effect for the pH range from 4.0 to 7.0 on the product's ee and bioconversion of **1**. The reactions were carried out for 24 hours using 1 mmol of **1** in an orbital shaker at 25°C at 100 rpm. The pH performs a key role in the biocatalytic reactions when using whole cells due to the fact that it affects the activity and enantioselectivity of the enzymes involved in the reaction, alters the ionic state of substrates, products, and enzymes involved in the reaction, and affects the binding of enzyme's active site to substrates, especially for several isoenzymes with different enantioselectivities. As can be seen from Table 2, the bioreduction of **1** was importantly affected by effects of pH. The results indicate that ketone **1** could be reduced to alcohol **2** at pH 6.0 (Table 2, entry 4). Above or below these values, low ee and mild conversion were observed. Probably, the three-dimensional structure of the enzyme changes as the pH changes, and it can be said that conversion and ee have changed as a result of the interaction of the enzyme and, also, the solubility of the substrate may have affected the selectivity of the biocatalyst.[37]

TABLE 2 Effects of different pHs on the reduction of **1** by *Lactobacillus paracasei* BD101

Entry	pH	Conversions, %[a]	ee, %[b,c] (*S*)
1	4.5	67	51
2	5	76	59
3	5.5	71	65
4	6	78	84
5	6.5	70	79
6	7	64	10
7	7.5	20	5

Abbreviations: ee, enantiomeric excess; HPLC, high-performance liquid chromatography.

Substrate 1 mmol.

[a]The conversion was determined by HPLC.

[b]Determined by HPLC using Chiralcel OD column.

[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

The highest conversion (78%) with 84% ee was obtained when the pH was at 6.0. At this pH, the bioreduction ability of the *L paracasei* BD101 was the best for the reduction of ketone **1**. This pH value was further selected as the optimized pH value, and all further analysis was performed at pH 6.0. The second parameter that was investigated was the effect of reaction temperature on the reduction of **1** (Table 3) as temperature is a critical factor for chemical reactions.

Table 3 shows the effects of temperature on the bioreduction ability of *L paracasei* BD101 in which temperatures from 25°C to 34°C were tested. Our findings revealed that the maximum conversion (86%) and ee (99%) values were achieved at 30°C. Temperatures above 30°C resulted in a rapid decline in conversion and ee values (Table 3, entries 4 and 5). This can be originated from the fact that the reductive activity of the enzyme was inhibited partially at the elevated temperature.[42] The optimum temperature for the reduction of ketone **1** to alcohol **2** was set at 30°C, and hence, all the subsequent reduction experiments were carried out at 30°C (Table 3, entry 3). The effect of incubation period on the reaction rate was the third parameter investigated, and the results are given in Table 4. Different incubation times were studied to monitor the progress of the bioreduction reaction. The complete conversion of **1** was observed after 72 and 96 hours, but the ee of **2** decreased from 80% to 61%, respectively (Table 4, entries 3 and 4). Conversely, the conversion increased up to 94% with 99% ee when the incubation period was 48 hours (Table 4, entry 2). Probably, it is possible to say that selectivity decreases due to the degradation of the active area of the enzyme depending on the longer incubation.[40] Therefore, the optimized condition for the incubation period was determined to be 48 hours. The effect of agitation speed on the reaction rate was the last parameter investigated, and the results are given in Table 5. Agitation speeds from 100 to 250 rpm were chosen for the experiments. The results obtained from the experiments showed that the conversion of **1** was at a maximum of 150 and above revolutions per minute. However, the ee dropped when the agitation speed was increased. This decrease can be attributed to the effect of shear stress on the *L paracasei* BD101 cells at high agitation speeds, which can alter the cell internal structure and might lower their activity.[43] The best yield of alcohol **2** with high ee was obtained at an agitation rate of 150 rpm (Table 5, entry 2). In light of this, an agitation speed of 150 rpm was used for subsequent studies. The optimum condition for the asymmetric bioreduction of **1** to **2** was established at pH of 6.0, temperature of 30°C, incubation period of 48 hours, and agitation speed of 150 rpm. Under the optimum condition, the effects of dry biocatalyst amount on the conversion and ee of **1** reduction were investigated. The best ee (>99%) and conversion (>99%) were obtained when 40-mg dry biocatalyst was applied. When the amount of dry biocatalyst is 20 and 60 mg,

TABLE 3 Effects of temperature on the reduction of **1** by *Lactobacillus paracasei* BD101

Entry	Temperature, °C	Conversions, %[a]	ee, %[b,c] (*S*)
1	25	78	84
2	28	80	88
3	30	86	99
4	32	71	74
5	34	45	40

Abbreviations: ee, enantiomeric excess; HPLC, high-performance liquid chromatography.

Substrate 1 mmol.

[a]The conversion was determined by HPLC.

[b]Determined by HPLC using Chiralcel OD column.

[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

TABLE 4 Effects of incubation periods on the reduction of **1** by *Lactobacillus paracasei* BD101

Entry	h	Conversions, %[a]	ee, %[b,c] (*S*)
1	24	86	99
2	48	94	99
3	72	99	80
4	96	99	61

Abbreviations: ee, enantiomeric excess; HPLC, high-performance liquid chromatography.

Substrate 1 mmol.

[a]The conversion was determined by HPLC.

[b]Determined by HPLC using Chiralcel OD column.

[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

TABLE 5 Effects of agitation speeds on the reduction of **1** by *Lactobacillus paracasei* BD101

Entry	rpm	Conversions, %[a]	ee, %[b,c] (*S*)
1	100	94	99
2	150	99	>99
3	200	99	60
4	250	99	45

Abbreviations: ee, enantiomeric excess; HPLC, high-performance liquid chromatography.

Substrate 1 mmol.

[a]The conversion was determined by HPLC.

[b]Determined by HPLC using Chiralcel OD column.

[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

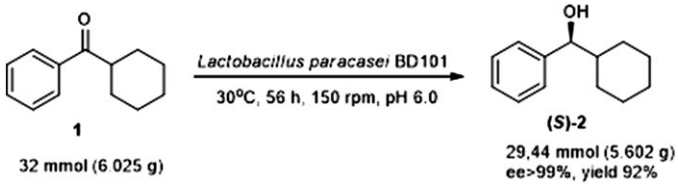


FIGURE 2 Laboratory scale-up synthesis of (*S*)-**2**

the enantioselectivity decreases to 75% and 70%, respectively. This can be attributed to the alteration of the amount of NADPH in the medium, which can affect the selectivity, and so activity of the enzyme may have changed.[42] With the optimum conditions in hand, we carried out the scale-up bioreduction of cyclohexyl(phenyl) methanone **1**. Preparative scale production of (*S*)-cyclohexyl(phenyl)methanol **2** was performed over a 1-L scale in a 5-L Erlenmeyer (Figure 2). Asymmetric reduction of **1** (45 mmol, 8.47 g) after 60 hours resulted in complete conversion, but the ee of the product was extremely low (65%). This could be due to the reduction in the enzyme activity, suggesting substrate toxicity and inhibition as reported previously.[44] Based on this knowledge, the substrate concentration was investigated with slight changes. The best result was obtained with a substrate concentration of 32 mmol (6.025 g). Complete conversion and ee of >99% were achieved after 56 hours of incubation period. Then the mixture was extracted with dichloromethane and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the product **2** was purified on silica gel column. As shown Figure 2, substrate **1** was easily reduced to the corresponding chiral carbinol in excellent yield and with its enantiopure form. This result show that *L paracasei* BD101 can be used on a large scale in the synthesis of important carbinols.

4 | CONCLUSION

In summary, screening of 10 bacterial strains has been carried out for the asymmetric reduction of cyclohexyl(phenyl)methanone **1**. Among the whole bacterial cells employed for the asymmetric reduction of **1**, *L paracasei* BD101 was found to be the best biocatalyst. Importantly, optimization parameters such as pH, temperature, incubation time, and agitation speed were systematically optimized for maximum product yield with perfect enantioselectivity of (*S*)-cyclohexyl(phenyl)methanol (**2**), and enantiomerically pure form was produced in gram scale with both excellent conversion (>99%) and yield (92%), through a cheap, environmentally friendly, and efficient process. Furthermore, this study demonstrates the first example of the effective synthesis of (*S*)-cyclohexyl(phenyl)methanol by the *L paracasei* BD101. *L paracasei* BD101 can be a good candidate for the whole cell biocatalytic preparation of other carbinols in organic chemistry. Also, *L paracasei* BD101 has been shown to be an important biocatalyst in asymmetric reduction of bulky substrates. Bulky groups might be better stabilized in the enzyme active site in such a way to increase the catalytic efficiency of the enzyme. Compared with the previous reports, cyclohexyl(phenyl)methanone **1** was reduced to **2** in a perfect conversion, yield, and ee. Therefore, this biocatalysis is apparently practical for preparation of highly enantiopure carbinols. The results confirmed that this effective and eco-friendly biocatalytic system exhibits excellent enantioselectivity and can be applied for the synthesis of important enantiopure secondary alcohols, which can be significant intermediate products for the synthesis of drugs and biologically active compounds.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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