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Discovery and Application of New Bacterial Strains for Asymmetric Synthesis of *L*-Tert-Butyl Leucine in High Enantioselectivity

Jian-Zhong Jin · Dong-Liang Chang · Jie Zhang

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Abstract Discovery of new bacterial strains with fast identification in a miniaturized system was performed for the synthesis of optically active *L*-tert-butyl leucine. With tert-butyl leucine amide as nitrogen source, one bacterial strain with high conversion and high enantioselectivity was discovered among 120 isolated microorganisms from local soils and identified as *Mycobacterium* sp. JX009. Glucose and ammonium chloride were examined as the good carbon source and nitrogen source for the cells' growth separately. The cells grew better at 30 °C and at pH 7.5 with higher activity of 2,650 U/l in comparison with other conditions. Cells' stability was improved by immobilization on synthetic resin 0730 without pretreatment. Tert-butyl leucine amide (30 mM) was successfully hydrolyzed by immobilized cells and examined as the highest chemical concentration that cells could endure. After six reaction cycles, the immobilized cells retained 90% activity with production of *L*-tert-butyl leucine in 98% ee. The results firstly reported the application of new bacterial strain in the hydrolysis of tert-butyl leucine amide to produce optically active *L*-tert-butyl leucine in an efficient way with investigation in detail.

Keywords *L*-tert-butyl leucine · Screening · *Mycobacterium* sp. JX009 · Hydrolysis · Immobilized on resins · Repeated usage

Introduction

The unnatural amino acids play an important role in the pharmaceutical, agrochemical, food additives and cosmetic industry for their special importance [1, 2]. Optically active *L*-tert-butyl leucine could be taken as an example, which is a nonproteinogenic chiral α-amino acid

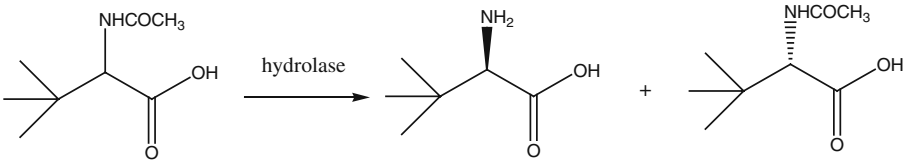
J.-Z. Jin (✉)
College of Biological and Environmental Engineering, Zhejiang Shuren University, Hangzhou 310015, People's Republic of China
e-mail: hzjjz@sohu.com

D.-L. Chang · J. Zhang
Jiaxing Research Center for Applied Chemistry, Chinese Academy of Sciences, Jiaxing 314006, People's Republic of China

widely used for the preparation of pharmaceutically active peptides, pesticides, and chiral ligands in organic synthesis [3, 4]. With its special importance, many methodologies, including chemical ways and enzymatic resolutions, were developed for its preparation in the past decades. In 1953, the optically active *L*-tert-butyl leucine was firstly prepared from purified hog kidney amidase with the complicated chemical workup in low yield and low enantioselectivity [5]. Resolution of racemic tert-butyl leucine with 10-camphorsulfonic acid was demonstrated as an alternative way instead of amidase hydrolysis afterwards [6]. In 1992, two tert-butyl leucine enantiomers were synthesized by Corey with expensive chiral ligands in a complicated procedure [7]. Meanwhile, enzymatic resolutions were furtherly developed for their high catalytic efficiency and high regio-stereoselectivity. Lipozyme from *Mucor miehei* was ever used in the resolution of (±)-2-phenyl-4-tert-butyloxazolin-5(4 H)-one for the production of *L*-tert-butyl leucine by two-step hydrolysis [8]. Afterwards, penicillin G acylase from *Escherichia coli* was immobilized to hydrolyze N-Phac-(±)-tert leucine, which was considered as a feasible process for industry [9, 10]. Among the reported methods, the Degussa company AG-mediated process was the most famous one for the production of *L*-tert-butyl leucine on industrial scale [11–13]. Leucine dehydrogenase was employed to catalyze reductive amination of trimethylpyruvate acid coupled with NADH regeneration by formate dehydrogenase.

Chemical resolution could be easily carried out on a large scale; however, the tedious process in low yield and the difficulties in the racemization of the opposite enantiomer were also observed. The asymmetric metal-catalyzed synthesis attracted much attention for its high efficiency; yet, the synthesis of complex chiral ligands in complicated procedures and the performance in the strict conditions made it unpractical. Although Degussa company AG-mediated reductive amination of trimethylpyruvate acid was successful, purified leucine dehydrogenase together with cofactor regeneration made the production costs higher. The reported enzymatic methods were not satisfying due to low activity or low enantioselectivity or complicated isolation procedure observed in the bioprocess. Thus far, no full paper about the enzymatic hydrolysis of *DL*-tert-butyl leucine amide to produce *L*-tert-butyl leucine in high enatioselectivity has been published. As an alternative, to discover the ideal biocatalysts from nature such as soil samples would be an effective choice. In the past, we developed successfully the high-throughput screening in a miniaturized system for the discovery of new alcohol dehydrogenases [14]. On the base, we have performed a new hydrolase screening by fast identification with tert-leucine amide as the nitrogen source for the production of *L*-tert-butyl leucine (Scheme 1).

Nature provides the huge amounts of microorganisms, such as bacteria, fungi, and yeasts, which contain many hydrolases that could be used as the ideal biocatalysts. In the screening, tert-butyl leucine amide was first synthesized and taken as the nitrogen source. With the efficient instruments, we performed the screening and successfully discovered the good strain, *Mycobacterium* sp. JX009, for the synthesis of optically active *L*-tert-butyl



tert-butyl leucine amide *L*-tert-butyl leucine

Scheme 1 Enzymatic synthesis of *L*-tert-butyl leucine by kinetic hydrolysis

leucine in high enantioselectivity. The carbon sources, nitrogen sources, the culture temperature, and medium pH were investigated to increase the hydrolase production. To improve hydrolase stability, cells were immobilized onto synthetic resin 0730 and used in the hydrolysis repeatedly. After six times, the residual hydrolase still kept initial 90% activity together with the *L*-tert-butyl leucine formation in 98% ee. Thus, we firstly investigated the discovery and application of a new bacterial strain in the hydrolysis of tert-butyl leucine amide to produce optically active *L*-tert-butyl leucine in high enantioselectivity in full paper.

Materials and Methods

Materials

DL-tert-butyl leucine was purchased from XinHua Pharmaceutical Company (Changzhou, China). *DL*-tert-butyl leucine amide was synthesized according to literature [15]. Acetonitrile and isopropanol used for high performance liquid chromatography (HPLC) were purchased from Merck Company (Shanghai, China). Synthetic resin 0730 was brought from Huazhen Technical Company (Shanghai, China). Chiral thin-layer chromatography was from Germany (CHIRAL-PLATE silica gel RP modification coated with Cu^{2+} and chiral reagent; Nacherey and Nagel, Duren, Germany). All other chemicals, unless noted otherwise, were commercial products of the highest purity available. Soil samples were taken at random from Jiaxing city, Zhejiang Province.

Growth Media

Growth media were autoclaved for 20 min at 121 °C. Glucose, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, 2 mM tert-butyl leucine amide, vitamin solution, and trace element solution were added separately after cooling of the medium by sterile filtration. For solid media, 1.5% agar (*w*/*v*) was added. The medium comprised of 1.95 g KH_2PO_4, 2.5 g K_2HPO_4, 1.0 g NaCl, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot H_2O$, 2.0 g glucose, 2.0 g tert-butyl leucine amide, 0.8 ml trace element solution, and 2.5 ml vitamin solution in 1,000 ml with adjustment of pH 7.0. The trace element solution was prepared as described in literature [16]. The composition of the vitamin solution was taken from Schlegel [17].

Screening Procedure

A 25-ml culture medium containing 2 mM tert-butyl leucine amide as nitrogen source was inoculated with a sample from natural water sources or a suspension of soil in Jiaxing city. The flasks were shaken at 25 °C with 200 rpm for 4 days. Depending on the turbidity of solution which indicated microbial growth, an aliquot was taken and transferred into a 30-ml fresh medium. After five transfers, the cultures were diluted by sterilized water and inoculated on agar plates with the same medium at 25 °C. The bacterial strain colonies were observed after several days. The single colonies were picked and purified by plating until pure bacterial cultures were obtained. Pure cultures were maintained on agar plates at 25 °C and grown in 30-ml liquid media in shaking flasks for the culture of certain amount of cells. The growth medium was mixed with 50% sterilized glycerol in 1:1 and put into the 96 deep-well plates and stored at −80 °C. The isolated strains in the 96 deep-well plates were transferred to the growth medium by the replicator according to the literature [14] with 2 mM tert-butyl leucine

amide as the substrate. After the workup, the samples were analyzed by chiral thin-layer chromatography (TLC) for the discovery of new bacterial strains.

Chiral Thin-Layer Chromatography

Plates were heated for 15 min at 100 °C for the detection of new bacterial strains. The samples were taken from the screening, analyzed by chiral TLC with mixture of solvents (methanol/water/acetonitrile=1:1:4) as the eluent, and visualized by 0.3% ninhydrin (ninhydrin in 2-methylpropanol).

Analysis of Tert-Butyl Leucine Enantiomers by HPLC

The enantiomers of tert-butyl leucine were measured by HPLC (Kromasil 100 C18: 250× 4 mm; 5 μm; buffer A, 23 mM sodium acetate, pH 6.0; buffer B, acetonitrile/H_2O 10/1.5) after derivatization with o-phthalaldehyde and N-isobutyryl-L-cysteine (0.1 M sodium borate buffer, pH 10.4) as described by Krieg [12]. *L*-tert-butyl leucine was eluted at 17.3 min and *D*-tert-butyl leucine, at 18.7 min.

Growth of Promising Bacterial Strains

The cell growth cultures were prepared by inoculation of 30 ml above medium without agar in a 250-ml flask with cells grown on an agar slant, incubated at 30 °C, and shaken at 200 rpm. The cells were harvested at the late exponential phase stages. Cell concentration was determined by measuring the optical density at 600 nm and calibrated to dry cell weight. The composition of other media was described in the corresponding figure or table legends.

Bacterial Strains' Activity Test

Bacterial activity test was performed in 10 ml Tris buffer (pH 7.0) with 2 mM tert-butyl leucine amide as substrate. It was shaken at 200 rpm and 25 °C for 0.5 h. The cells were removed by centrifugation and the supernatant was detected with HPLC for the conversion and enantioselectivity test.

Preparation of Immobilized Cells

Cells' immobilization was accomplished by adding 2 g of synthetic resin 0730 to 10-ml culture supernatant. The mixture was shaken for 2 h at 30 °C and 200 rpm. The whole cell-loaded resin was then filtered, washed thoroughly with 200 mM Tris–HCl buffer, and stored at 4 °C prior to use.

The activity of the immobilized cells was measured in a Tris–HCl buffer system with tert-butyl leucine amide as substrate. To 20 ml, 0.2 M Tris–HCl buffer containing the immobilized cells was added to 5 mM tert-butyl leucine amide. The reaction was carried out at 30 °C and 200 rpm in a 100-ml flask. The residual substrate concentration was calculated from the areas of the two enantiomer peaks with a calibration curve.

Stability and Temperature Experiments

The pH stability was examined by adjusting the culture to the specified pH (5.0, 6.0, 7.0, and 8.0), incubating at 30 °C for 5 h. The residual enzyme activity was measured

spectrophotometrically with the same method above. The initial activity measured immediately before incubation was defined as 100%.

To examine the effect of temperature on stability of the hydrolase, culture supernatant was performed for 24 h at 25 °C, 30 °C, and 35 °C before measuring residual activity.

Results and Discussion

Discovery of New Bacterial Strains by Screening

Sixty samples were collected from soils at random in Jiaxing City, Zhejiang Province. One hundred fifty-one single colonies with hydrolase activity were isolated and purified with addition of 2 mM tert-butyl leucine aminde on agar. Among those, 31 strains could not grow in liquid media. The other 120 strains were further tested for the hydrolysis of tert-butyl leucine amide. With the enantioselectity determination by a chiral thin-layer chromatography, positive results were observed in the 23 strains catalysis in high conversion. Among them, 20 strains produced the tert-butyl leucine in *L* structure while the other three strains produced *D* structure. With HPLC analysis, more than 95% ee in *L* form was observed in three strains catalysis. However, no strain produced *D*-tert-butyl leucine above 95% ee. The screening result showed that the majority of the isolated strains had a *L*-amidase since *L*-amino acids commonly exist in nature.

Three isolated *L*-amidase producers with high ee were identified by 16S RNA. The results as well as activity data were shown in Table 1. A comparison with other two strains, D003 and G011, showed that the isolated A009 had a higher specific activity and higher ee (98%). It was identified as *Mycobacterium* sp. JX009 and considered as the good biocatalyst for further research.

Effect of Carbon Source on Hydrolase Activity and Cell Growth

A range of different carbon sources such as olive oil, dextrin, glycerol, Tween-80, and glucose were tested for their capacity to benefit cell growth and hydrolase production of *Mycobacterium* sp. JX009. Ammonium sulfate was used as nitrogen source to avoid interference in organic nitrogen sources (Table 2).

From the table, we know that cells had better growth and higher activity when glucose was supplemented as carbon source. Supplementation with olive oil, dextrin, glycerol, or Tween-80 yielded hydrolase activities of 250 U/l, 280 U/l, 650 U/l, and 730 U/l, respectively. The cells grew slowly with lower activity with olive oil as carbon source. A possible reason is that high oleic oil could inhibit cell metabolism and hydrolase production. Glucose is usually used as carbon source for cell growth, which also happened

Table 1 Identification of three isolated *L*-amidase producers

Strain	Identification	Activity (U/l)	Conversion (%) (5 h)	ee (%)
A009	*Mycobacterium* sp. JX009	1,100	34	98
D003	*Variovorax* sp. JX003	510	16	95
G011	*Klebsiella* sp. JX011	860	27	96

The reaction was performed in 10 ml Tris–HCl buffer with 2 mM tert-butyl leucine amide as the substrate, shaken at 200 rpm and 30 °C for 5 h. Activity was determined in the first 30 min

Table 2 Effect of carbon source on hydrolase activity and cell growth

Carbon source (20 g/l)	Dry cell weight (g/l)	Hydrolase activity (U/l)
Olive oil	2.5	250
Dextrin	3.0	280
Glycerol	3.2	650
Tween-80	3.8	730
Glucose	4.8	1,220

The medium was comprised (g/l) of 5 g $(NH_4)_2SO_4$, 1.95 g KH_2PO_4, 2.5 g K_2HPO_4, 1.0 g NaCl, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot H_2O$, 0.8 ml trace element solution, 2.5 ml vitamin solution, and 2.0 g of each carbon source with adjustment to pH 7.0. The cultures were carried out in 250-ml flasks containing 30-ml medium, shaken for 24 h at 30 °C and 200 rpm

to the cultivation of *Mycobacterium* sp. JX009. The cells grew well, with the highest hydrolase activity of 1,220 U/l with the addition of 2% glucose. Thus, glucose was chosen and used as the sole carbon source in subsequent cultures.

Effect of Nitrogen Source on Lipase Production and Cell Growth

Several nitrogen sources such as $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, and peptone to yeast extract (YE) were tested for the cell growth with 2% glucose as carbon source. Both the cell growth and hydrolase activity were greatly affected (Table 3). With the addition of NH_4Cl as the nitrogen source, the hydrolase activity could be achieved, highest of 1,620 U/l with less biomass. Among other nitrogen sources, the hydrolase activity varied from 720 U/l on peptone to 1,220 U/l on $(NH_4)_2SO_4$. There is slight difference between the cell growth and activity with peptone and YE. In the addition of $(NH_4)_2SO_4$, a possible reason may be that more nitrogen source was provided by $(NH_4)_2SO_4$. Peptone and yeast extract are complex nitrogen sources and the cells could utilize it easily and totally. Thus, higher cell densities were observed during the cells' growth in those two cases. However, the hydrolase activities were the lowest (720 U/l and 780 U/l separately); probably, the other enzymes inside the cells could also make use of complex medium. In comparison, NH_4Cl gave the highest activity of 1,620 U/l together with less cell density. Based on that, it was chosen as the optimal nitrogen source due to its better capacity of hydrolase production. Then, we

Table 3 Effect of nitrogen source on hydrolase production and cell growth

Nitrogen source (5 g/l)	Dry cell weight (g/l)	Hydrolase activity (U/l)
$(NH_4)_2SO_4$	4.8	1,220
NH_4Cl	4.1	1,620
NH_4NO_3	4.3	930
Peptone	5.1	720
Yeast extract (YE)	5.2	780

The culture medium was comprised (g/l) of 1.95 g KH_2PO_4, 2.5 g K_2HPO_4, 1.0 g NaCl, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot H_2O$, 0.8 ml trace element solution, 2.5 ml vitamin solution 2.0 g glucose, 5.0 g of each nitrogen source with adjustment to pH 7.0. The cultures were carried out in 250-ml flasks containing 30-ml medium, shaken for 24 h at 30 °C and 200 rpm

could make a conclusion that the *Mycobacterium* sp. JX009 would produce more hydrolase, with high activity during the cultivation with specific nutrients.

Effect of pH

The stability of the hydrolase inside the cells was shown to decrease with either increasing or decreasing pH values (Fig. 1). It was relatively stable at pH 6.0–7.0 after cultivation for 5 h at 30 °C. The activity decreased more sharply at high pH than at low pH, the residual activity being 41% at pH 5.0, but only 20% at pH 9.0. The data in the figure showed that hydrolase had stability in the narrow pH range. Cells' immobilization would be thought to increase the hydrolase stability in the hydrolysis reaction. The medium at a pH of 6.0–7.0 was considered as suitable for hydrolase production. The cultivation pH between 5.0 and 9.0 caused much larger differences in hydrolase activity (350–1,780 U/l) than in biomass (3.1–5.2 g/l). At pH 7.0, the maximum biomass and hydrolase activity obtained was 5.2 g/l and 1,780 U/l, respectively. It decreased to 650 U/l at pH 5.0 or 350 U/l at pH 9.0, indicating that cells preferred a neutral pH for hydrolase production. Thus, pH 7.0 was considered as the optimal pH for the cultivation of *Mycobacterium* sp. JX009.

Effect of Carbon/Nitrogen Ratio on Hydrolase Production and Cell Growth

Based on the above results, glucose and ammonium chloride were chosen as carbon and nitrogen sources, respectively, and the cells were cultivated at pH of 7.0. To optimize the ratio of glucose to ammonium chloride, different concentrations of ammonium chloride were tested at a given concentration of glucose (2.0 g/l; Fig. 2).

From the figure, it was concluded that the biomass and hydrolase activity increased when ammonium chloride concentration was increased. With a constant glucose concentration (2.0 g/l), an increase in ammonium chloride concentration resulted in significant growth of cells, which means that ammonium chloride was a limiting growth factor. The hydrolase activity changed from 450 to 2,140 U/l when ammonium chloride concentration was increased from 0.5 to 6.0 g/l. There was no significant change when the ammonium chloride concentration increased furtherly. On the contrary, the hydrolase activity decreased when the nitrogen source was above 8.0 g/l. It was assumed that some inhibition

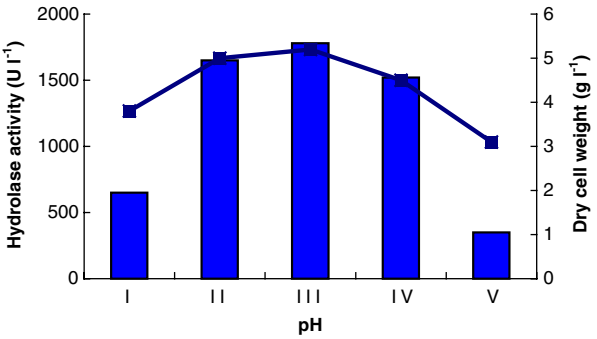


Fig. 1 Effect of medium pH on hydrolase production and cell growth. The growth medium was adjusted to different pH values (5.0, 6.0, 7.0, 8.0, and 9.0) with 1 mol/l HCl or 1 mol/l NaOH. The cultures were carried out in 250-ml flasks containing 30-ml medium and shaken for 24 h at 30 °C and 200 rpm. *Square* hydrolase activity (U/l), *Line* dry cell weight (g/l). (*I* pH=5.0, *II* pH=6.0, *III* pH=7.0, *IV* pH=8.0, *V* pH=9.0)

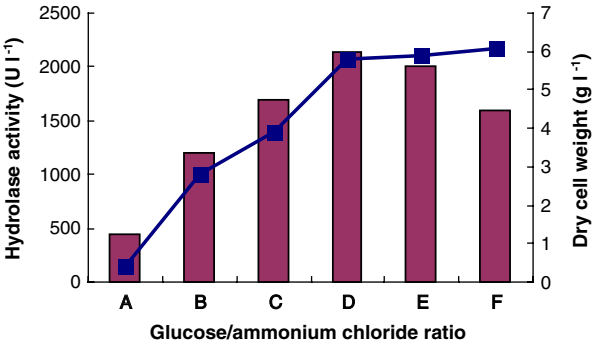


Fig. 2 Effect of carbon/nitrogen ratio on hydrolase production and cell growth. Cultures were carried out in 250-ml flasks containing 30-ml medium and shaken for 24 h at 30 °C and 200 rpm. *Filled squares* hydrolase activity, *filled lines* dry cell weight (*A*=4:1, *B*=1:1, *C*=1:2, *D*=1:3, *E*=1:4, *F*=1:5)

happened during cell growth. Finally, the ratio of 2.0 g/l glucose and 6.0 g/l ammonium chloride was considered as the best choice for the cell growth with highest cell density and highest hydrolase activity.

Effect of Temperature

With the glucose and ammonium chloride as the carbon source and nitrogen source, respectively, the cells were cultivated at 35 °C, 30 °C, 25 °C, and 20 °C parallelly for the test of temperature influence. From Fig. 3, we know that hydrolase activity increased when the cells were cultivated at lower temperature. After the incubation for 24 h, hydrolase activity increased from 1,900 U/l to 2,650 U/l from 35 °C to 30 °C, which implied that a lower cultivation temperature might favor hydrolase accumulation. Furtherly, when the temperature decreased from 30 °C to 20 °C, the hydrolase activity increased slightly from 2,650 U/l to 2,900 U/l. It showed that temperature influences hydrolase production during the cell growth to a certain extent.

The cell growth was also influenced greatly by temperature. Higher cell density of 8.6 g/l could be obtained when the cells grew at high temperature of 35 °C. In a lower temperature from 30 °C to 20 °C, the dry cell weight changed from 7.6 g/l to 3.7 g/l. Figure 3 showed that cells grew slowly in lower temperature with higher hydrolase activity. The reason for this effect of cultivation temperature on the hydrolase activity may be ascribed to the poor stability of the hydrolase in the higher temperature and

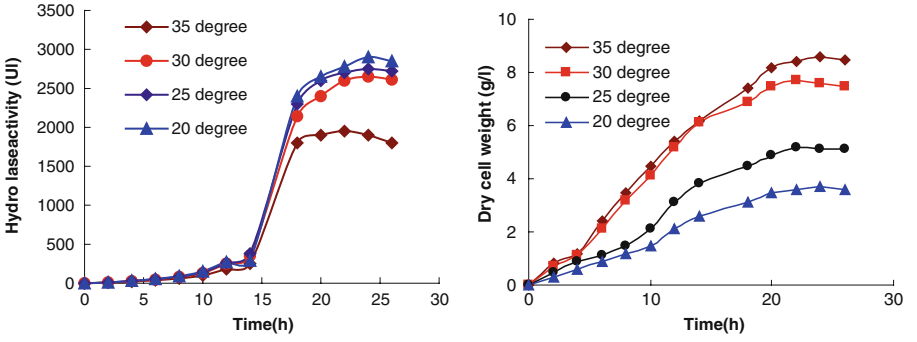


Fig. 3 Time courses of hydrolase production and cell growth at different temperatures. Cultures were carried out in 250-ml flasks containing 30-ml medium with glucose and ammonium chloride as carbon source and nitrogen source, respectively, shaken for 24 h at 200 rpm

Table 4 Optimization of maximal substrate concentration catalyzed by immobilized cells

Ten-milliliter culture supernatant immobilized onto 2.0 g resin. The reaction was carried out in 250-ml flasks containing 20 ml Tris–HCl buffer, shaken for 12 h at 30 °C and 200 rpm

Substrate concentration (mM)	Conversion (%)	ee (%)
5	50	99
10	50	99
20	49	99
30	49	98
50	40	90
60	35	82

the release of more protease. Thus, considering the enzyme activity and dry cell weight, we chose 30 °C to be the optimal temperature for cell growth and hydrolase production.

Maximal Chemical Concentrations that Immobilized Cells Could Catalyze and Their Repeated Usage

The results above show that the pH stability and thermal stability of the hydrolase in culture were not good enough. For this reason, the cells were immobilized by adsorption of cultivation supernatant, without any pretreatment, onto a synthetic resin 0730. The immobilized cells were used to catalyze tert-butyl leucine amide from 5 mM to 60 mM on 20-ml scale (Table 4). The table showed that the optically active *L*-tert-butyl leucine could be produced in 99% ee in the catalysis of 5-mM and 20-mM substrates. Ninety-eight percent ee with 49% conversion could also be obtained in the catalysis of 30 mM tert-butyl leucine amide. However, when the substrate concentration increased to 50 mM, the product ee decreased to 90% together with lower conversion of 40%. The worst result was observed in the catalysis of 60 mM tert-butyl leucine amide, which means that the substrate inhibition happened. The hydrolase inside the cells was damaged with lower activity in the high concentration of chemicals. Thus, 30-mM substrate was considered the highest concentration of tert-butyl leucine amide that immobilized cells could catalyze.

To test its stability, the immobilized cells were repeatedly used in the hydrolysis of 30 mM tert-butyl leucine amide in Tris–HCl buffer (pH 7.0). After six reaction cycles (Fig. 4), the residual hydrolase still retained about 90% original activity with the *L*-tert-butyl leucine production in 98% ee, which indicates the high enantioselectivity of hydrolase inside the cells. Thus, the immobilized *Mycobacterium* sp. JX009 would be thought as a potential new biocatalyst for industrial application.

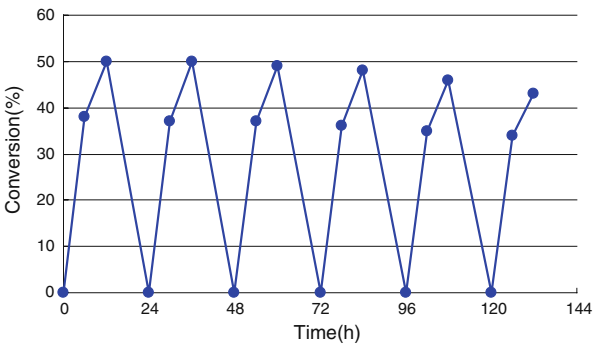


Fig. 4 Reuse of immobilized cells in the hydrolysis of 30 mM tert-butyl leucine amide. The reaction was carried out in 250-ml flasks containing 20 ml Tris–HCl buffer, shaken for 12 h at 30 °C and 200 rpm

Conclusions

In this study, we did the screening to discover new bacterial strains for the synthesis of the optically active *L*-tert-butyl leucine by the hydrolysis of tert-butyl leucine amide. A promising strain with high efficiency was isolated and studied furtherly, which was identified as *Mycobacterium* sp. JX009. Production of the hydrolase inside the cells was influenced by several factors, including the carbon source, nitrogen source, medium pH, and cultivation temperature. In this work, hydrolase production was markedly enhanced by optimization of medium composition, culture pH, and temperature. The immobilized cells were shown to be efficient biocatalysts for the synthesis of *L*-tert-butyl leucine and could be reused at least six times with 90% activity retained. As we know, for the first time, the newly discovered strains by screening from nature were immobilized onto synthetic resins and applied in the synthesis of *L*-tert-butyl leucine in high efficiency with investigation in detail.

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References

1. Menzel, A., Werner, H., Altenbuchner, J., & Groeger, H. (2004). *Engineering in Life Sciences, 4*, 573–576.
2. Bruggink, A., Roos, E. C., & Vroom, E. (1998). *Organic Process Research & Development, 2*, 128–133.
3. Helimchen, G., & Pfaltz, A. (2000). *Accounts of Chemical Research, 33*, 336–345.
4. Jarvo, E. R., & Miller, S. J. (2002). *Tetrahedron, 58*, 2481–2495.
5. Izumiya, N., Fu, S. C. J., Birnbaum, S. M., & Greenstein, J. P. (1953). *The Journal of Biological Chemistry, 205*, 221–230.
6. Viret, J., Patzelt, H., & Collet, A. (1986). *Tetrahedron Letters, 27*, 5865–5868.
7. Corey, E. J., & Link, J. O. (1992). *Journal of the American Chemical Society, 114*, 1906–1908.
8. Tuner, N. J., Winterman, J. R., McCague, R., Parratt, J. S., & Taylor, S. J. C. (1995). *Tetrahedron Letters, 36*, 1113–1116.
9. Liu, S. L., Song, Q. X., Wei, D. Z., Zhang, Y. W., & Wang, X. D. (2006). *Preparative Biochemistry & Biotechnology, 36*, 235–241.
10. Liu, S. L., Wei, D. Z., Song, Q. X., Zhang, Y. W., & Wang, X. D. (2006). *Bioprocess and Biosystems Engineering, 28*, 285–289.
11. Grabley, S., Keller, R., & Schlingmann (1987). EP 0 141 223.
12. Kragl, U., Vasic-Racki, D., & Wandrey, C. (1996). *Bioprocess Engineering, 14*, 291–297.
13. Krieg, L., Ansorge-Schumacher, M. B., & Kula, M. R. (2002). *Advanced Synthesis & Catalysis, 344*, 965–973.
14. Zhang, J., Duetz, W. A., Witholt, B., & Li, Z. (2004). *Chemical Communications, 18*, 2120–2121.
15. Laumen, K., Ghisalba, O., & Auer, K. (2001). *Bioscience, Biotechnology, and Biochemistry, 65*, 1977–1980.
16. Joeres, U., & Kula, M. R. (1994). *Applied Microbiology and Biotechnology, 40*, 599–605.
17. Schlege, H. G. (1985). *Allgemeine Mikrobiologie* (6th ed.). Stuttgart: Thieme Verlag. 174.