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Whole-cell biocatalytic of *Bacillus cereus* WZZ006 strain to synthesis of indoxacarb intermediate: (*S*)-5-Chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester

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Abstract

In this study, a newly isolated strain screened from the indoxacarb-rich agricultural soils, *Bacillus cereus* WZZ006, has a high stereoselectivity to racemic substrate 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester. (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester was obtained by bio-enzymatic resolution. After the 36-hour hydrolysis in 50-mM racemic substrate under the optimized reaction conditions, the $e.e._s$ was up to 93.0%
and the conversion was nearly 53.0% with the *E* being 35.0. Therefore, *B cereus* WZZ006 performed high-level ability to produce (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester. This study demonstrates a new biocatalytic process route for preparing the indoxacarb chiral intermediates and provides a theoretical basis for the application of new insecticides in agricultural production.

KEYWORDS

chiral intermediate, enantioselectivity, enzymatic resolution, indoxacarb, whole cell biocatalyst

1 | INTRODUCTION

Chiral pesticides with specificity and environmental safety have captured continuously with wide attention in agriculture,[1,2] because of the emphasis on environmental pollution and the advocacy of green chemistry. Indoxacarb (see Figure 1 for structure), a new chiral oxadiazine pro-insecticide,[3] being converted into the active compound by removal of a CO_2Me in the insect gut (discovered and developed by the E.I. DuPont Company), has shown to possess high activity, commercial availability, and environmental compatibility to administered to lepidopteran and hemipteran pests.[4,5] An essential characteristic of indoxacarb is its novel bioactivation mechanism by blocking insect voltage-dependent sodium channels, which is associated with high insecticidal activity.[6-8] The 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester is a crucial chiral intermediate for the preparation of indoxacarb. Studies have shown that the *S*-enantiomer of indoxacarb is active as the racemate in insects, while the *R*-enantiomer is inactive.[3] Therefore, the synthesis of (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester has sparked heated interest in the pesticide industry.

Currently, the standard procedure for synthesizing (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester is limited to organic synthetic methods. Although these chemical methods are popularly used due to mature technology and can achieve a rapid

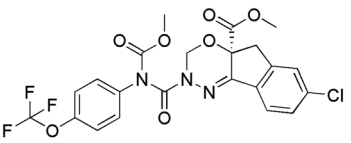


FIGURE 1 The structure of indoxacarb

and efficient synthesis, the reactions are still complex, toxic, and not conducive to large-scale industrial production.[9] The purity of the obtained (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester is only about 90%. McCann et al[10] found that the (*S*)-isomer of 54% enantiomeric excess (*e.e.*) was obtained with the Sharpless asymmetric hydroxylation reagent AD-mix-β. With the chiral N-camphorsulfonyl oxaziridine reagents developed by Davis et al,[11] the yield could be increased to 85%, but the *e.e.* value was only 35%. Under the condition of using the chiral alkaloid cinchonine as a catalyst and *tert*-butyl hydroperoxide (TBHP) as an oxidant,[12] 85% yield and 50% *e.e.* were obtained. The level of *e.e.* directly affects the quality of the final product and the activity of pesticides,[13-15] so exploring its new synthetic method has a long-term application significance.

Nowadays, the biocatalytic approach using microorganisms or isolated enzymes, from the perspective of environmental and industrial application, has increasingly to be a recent focus on chemical, agricultural, and pharmaceutical industries. Because of the potential for the preparation of chiral compounds with high optical purity, the biocatalytic approach has gradually become a research hotspot for the synthesis of chiral compounds. In comparison with chemical synthesis, the biocatalytic approach has many superiorities because of high selectivity, broad substrate range, environment-friendly process, and mild reaction conditions. In particular, dynamic kinetic resolution (DKR) that couples a racemization reaction with a conversion of the unwanted enantiomer into the product, can increase the yield up to 100%.[16-19] Also, whole-cell biocatalysts compared with isolated enzyme shows the advantages of cheap, stable, less laborious, and accessible. The whole microbial cells as excellent catalysts,[20] avoiding cofactor addition and enzyme purification, have the advantages of high substrate specificity, good stereoselectivity, mild reaction conditions, and low pollution. The whole cell biocatalytic synthesis has continually emerged as an outstanding method to replace traditional chemical methods in some fields.[21-25] Few bacterial strains like, *Lactobacillus paracasei* BD101 were used as whole cell biocatalyst for pure (*S*)-cyclohexyl (phenyl)methanol.[26] Also, Vitale et al[27] used *Lactobacillus reuteri* DSM 20016 whole cells for the production of (*S*)-rivastigmine. Samuel et al[28] used *Bacillus subtilis* 168 whole cells for efficient production of 2,3-butanediol. As far as we know, only a few studies have reported the whole-cell biocatalytic process for producing (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester. Therefore, it would be valuable to develop its bioprocess.

Herein, we aimed to explore a convenient and efficient bioprocess to obtain (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester by a newly isolated strain *Bacillus cereus* WZZ006.

2 | MATERIALS AND METHODS

2.1 | General

Indoxacarb-rich agricultural soils samples used to screen bacteria with hydrolytic activity were collected from various places of Zhejiang, Anhui, and Henan Provinces, China. (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester (98%) was prepared from 5-chloro-1-indanone in our laboratory, according to reported procedures.[29-31] All chemicals used were analytical reagents.

2.2 | Analytical methods

Nuclear magnetic resonance (NMR) was recorded on Bruker Ultrashield spectrometer (Bruker Ltd.,Swissland) operating at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in $CDCl_3$. Gas chromatography mass spectrometry (GC-MS) analysis was performed on an Agilent 7980A GC system equipped with an HP-5MS Agilent 19091S-433 column.

The progress of the hydrolysis reaction was monitored by high-performance liquid chromatography (HPLC, Waters, 1525), and the biotransformation yield and enantioselectivity were quantitatively determined. The determination was carried out by using an HPLC method on an Ultimate Cellud-Y (5 μm, 4.6 × 250 mm, Yuexu, China) column with a mobile phase of chromatographic grade n-hexane and isopropanol (80:20, v/v) at 0.5 mL/min flow rate, UV detection wavelength was set to 254 nm, column temperature was 25°C and injection volume was 10 μL. The retention time of 5-chloro-1-indanone, (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester, and (*R*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester was 17.49, 18.86, and 23.50 minutes, respectively (Figure 2).

One unit of esterase activity was defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol of substrate per minute at 30°C, pH 7.0. The peak

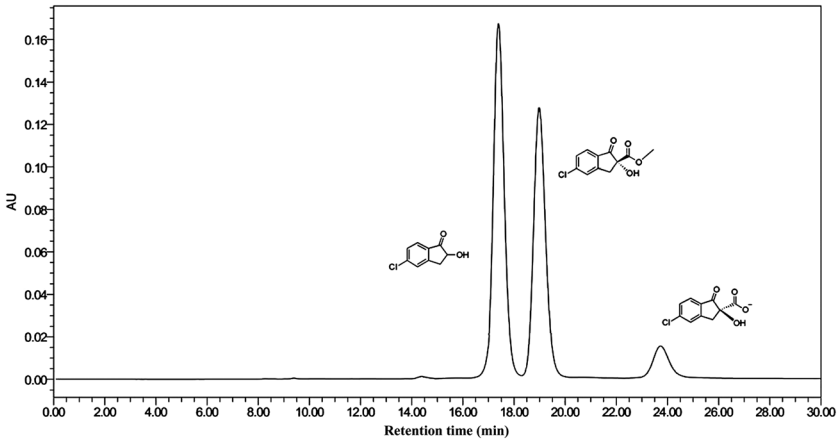


FIGURE 2 High-performance liquid chromatography (HPLC) of 5-chloro-1-indanone and (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester

areas of the chromatograms based on the (*S*)- and (*R*)-configuration substrates and reaction products were used to calculate the substrate optical purity and enantioselectivity. The percentage enantiomeric excesses of the substrate ($e.e._s$), conversion (C), and enantioselectivity (E) were calculated according to equations as below[32]:

$$e.e._s = \frac{[S]-[R]}{[S]+[R]} \times 100\% \quad , \tag{1}$$

$$C = \frac{C0 - Ct}{C0} \times 100\% \quad , \tag{2}$$

$$E = \frac{\ln[(1-C)(1-e.e._s)]}{\ln[(1-C)(1+e.e._s)]} \tag{3}$$

In the formula, [*S*] and [*R*] represent the peak areas of the (*S*)- and (*R*)-configuration substrates, respectively; C_0 represents the initial concentration of the substrate (mM), and C_t represents the substrate concentration remaining after the reaction (mM).

2.3 | Screening of strains for enantioselective hydrolysis of 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester

Bacteria with enzymatic resolution activity of 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester were isolated from indoxacarb-rich agricultural soils through two rounds of screening. In the first round, strains with ester hydrolysis activity were screened, and then strains were further screened for high enantioselectivity. An enrichment culture procedure was carried out previous to screening as follows: a little fresh soil sample was suspended in 50-mL enrichment medium with a composition (per liter) of 0.5-g (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester, 3.0-g $NaNO_3$, 1.0-g KH_2PO_4, 0.5-g KCl, 0.5-g $MgSO_4{\cdot}7H_2O$, and 0.01-g $FeSO_4{\cdot}7H_2O$ (pH 7.0). The reaction mixtures were incubated at 30°C for 5 days on a rotary shaker at 200 rpm. Then, when the optical density at 600 nm (OD_{600}) reached 4 to 5, 1-mL cultures were transferred to fresh medium with the same ingredients after the evidence of microbial growth. The procedure above had three replications. Then, 100-μL enrichment cultures were diluted and spread onto agar medium plates with the same composition except the agar to isolate pure colonies. The single colonies were grown aerobically at 30°C for 24 hours at 200 rpm in the medium that composed (per liter) 10.0-g glucose, 10.0-g peptone, 6.0-g $KH_2PO_4{\cdot}3\ H_2O$, 3.0-g beef extract, 3.0-g KH_2PO_4, 0.5-g $MgSO_4{\cdot}7H_2O$, and 0.5-g NaCl (pH 7.0). Following cultivation, wet cells were centrifuged at 10,000 rpm for 10 minutes and resuspended in 950-μL phosphate buffer (200 mM, pH 7.0). 20-mM (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester was added to the reaction and stirred at 200 rpm and 30°C for 24 hours. Samples were acidified with 4-M hydrochloric acid (HCl), extracted with ethyl acetate, dried by a vacuum rotary evaporator, and combined with HPLC analys is to measure the substrate conversion and enantiomeric excess ($e.e._s$).

2.4 | Identification of the isolated strain

The isolated WZZ006 strain, was grown on beef extract peptone agar medium at 30°C for 24 hours, was identified according to its cell morphology and genus information. Then, the single colony was picked for observing the morphological characterization via an optical microscope (Nikon Eclipse E100). For genotypic identification, the chromosomal DNA of strain WZZ006 was extracted according to the procedure of Wilson[33] with the SK8255 Column Bacterial Genomic DNA Extraction Kit (Sangon

Biotech [Shanghai] Co., Ltd.). The 16S ribosomal DNA (rRNA) gene was polymerase chain reaction (PCR) amplified using the 27F (5′-AGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers.[34,35] The reaction was cycled with an initial denaturation for 94°C for 4 minutes and followed by 30 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s), elongation (72°C for 90 s), and then a final elongation step at 72°C for 10 minutes. The PCR products were purified with SanPrep Column DNA Gel Extraction Kit (Sangon Biotech [Shanghai] Co., Ltd.). The sequencing was carried out by Sangon Biotech (Shanghai) Co., Ltd. The final gene sequence data were analyzed with the BioEdit program and compared with the BLAST network services provided by the National Center for Biotechnology Information (NCBI). The Biolog OmniLog Identification System (Biolog GENIII)[36] was assessed for its ability to identify genospecies of strain WZZ006.

2.5 | Production of an esterase from *B cereus* WZZ006

The *B cereus* WZZ006 was cultivated in a 250-mL shake flask to produce an esterase. The medium included the following constituents (per liter): 10-g glucose, 10-g peptone, 3-g beef extract, 6-g $K_2HPO_4{\cdot}3H_2O$, 3-g KH_2PO_4, 0.5-g $MgSO_4{\cdot}7H_2O$, and 0.5-g NaCl, pH 7.The reaction mixture was incubated at 180 rpm, 30°C for 24 hours. The cultures were withdrawn periodically to evaluate the biomass (OD_{600}), enzyme activity and enantioselectivity of *B cereus* WZZ006. The whole cells were harvested after fermentation by centrifugation under 4°C at 12,000 rpm for 10 minutes and were lyophilized for further use.

2.6 | General procedure for chemical synthesis of racemic 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester[29-31]

2.6.1 | Synthesis of 5-chloro-2,3-dihydro-1oxan-1-indanone-carboxylic acid methyl ester (compound II)

In a 250-mL four-necked flask, 6.12-g sodium methoxide, and 60-mL dimethyl carbonate (DMC) were added in sequence. The reaction was stirred at 90°C under nitrogen. When the fraction (DMC) was eluted, 60-mL 5-chloro-1-indanone (9.17 g, 0.0552 mol) of DMC solution was slowly added dropwise. After the titration was completed, the reaction was carried out for 1 hour. The reaction was monitored by HPLC, and cooled to room temperature after the reaction was completed. 2-M HCl solution was added to adjust the pH to 7. The organic phase was separated and collected, then dried with anhydrous sodium sulfate, and filtered and evaporated to give a brown solid. The creamy-white solid was obtained by recrystallization with isopropyl alcohol.

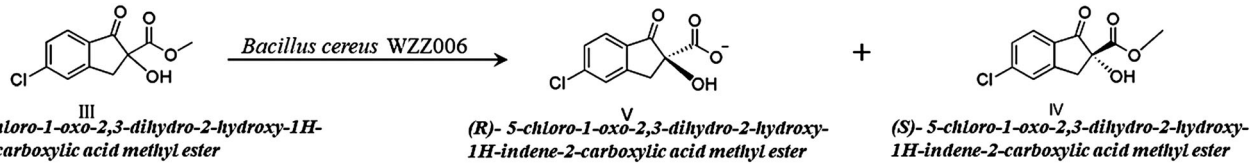
2.6.2 | Synthesis of methyl 5-chloro-2-hydroxy-1-oxo-2,3-dihydro-1*H*-indene-2-carboxylate (compound III)

Compound **II** (5.0 g, 22 mmol) was dissolved in 50 mL of 1,4-dioxane, and 150 mL of water and potassium hydrogen persulfate complex salt (Oxone) (15 g, 24.5 mmol) was added at 60°C. The solution slowly became clear and monitored by HPLC.

After the reaction was completed, it was cooled to room temperature and extracted with 200 mL of dichloromethane. The organic phase was washed once with saturated sodium thiosulfate and brine (200 mL), and the organic phase was collected, dried with anhydrous sodium sulfate, and filtered and evaporated at reduced pressure (0.1 MPa). The crude product was recrystallized from cyclohexane to give a brown solid. The structure was determined by NMR data interpretation.

2.7 | Enzymatic hydrolysis of (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester

The enzymatic resolution of (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester was carried out in a 250-mL three-necked flask, which contained 2 g of wet cells of *B cereus* WZZ006, 200 mg of the racemic substrate, and 40 mL of phosphate buffer (200 mM, pH 7.0). The reaction was stirred at 30°C, 180 rpm for 48 hours. The hydrolysis reaction scheme is shown in Scheme 1. Aliquots of the reaction sample were withdrawn at a fixed time interval, acidified with 4-M HCl, extracted with ethyl acetate, dried by a vacuum rotary evaporator, and further determined by HPLC. At the end of the reaction period, the aqueous phase was acidified with 4-M HCl then extracted with ethyl acetate. Ethyl acetate was removed at reduced pressure. A preparative thin-layer chromatographic (TLC) method on silica gel was applied to separate and purify the crude product with petroleum ether and ethyl acetate (3:2, v/v) as solvent.



SCHEME 1 Route of resolution of (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester by *Bacillus cereus* WZZ006

3 | RESULTS AND DISCUSSION

3.1 | Identification of stereoselective ester hydrolase producing bacteria

In this experiment, seven strains with superior stereoselective hydrolysis activity were screened from the freeze-dried bacteria powder, which were isolated and purified from more than 500 indoxacarb-rich agricultural soil samples collected from different places of Zhejiang, Anhui, and Henan Provinces, China. Among them, strains WZZ006, C26, Pt9, X225, S14, and X075 selected to hydrolyze the R-isomer of the substrate, and CM008 hydrolyzed S-isomer. As showed in Table 1, strain WZZ006 exhibited the highest hydrolysis activity and enantiomeric activity to the racemic substrate, so strain WZZ006 was selected for activation and preservation as a subsequent experimental strain.

The selected strains were inoculated into a beef paste peptone solid medium and cultured at 30°C for 24 hours to observe the colony morphology. As shown in Figure S1, a single cell is rod shaped, with a square end, and multiple cells can be connected to a growing chain. The colonies were grayish white, large, rough, flat, irregular, and with spores. After Gram staining, the cells were purple, indicating that the strain was a gram-positive bacterium. After full-length, sequencing, and analysis by Shanghai Shenggong Bioengineering Co., Ltd., the 16S rDNA sequence of this strain was 1,455 bp in length. The specific sequence is as Figure S2.

TABLE 1 The screening of microorganism for enantioselective hydrolysis of the substrate

Number	Strain Code	*e.e.*$_s$, %	Conversion, %	*E*
1	WZZ006	80.1	55.5	11.0
2	C26	52.2	58.9	3.5
3	Pt9	41.6	53.1	6.8
4	X225	38.7	46.2	3.8
5	S14	21.6	40.5	2.4
6	X075	18.5	37.9	2.2
7	CM008	73.3	60.7	5.9

Abbreviations: *E*, enantioselectivity; *e.e.*$_s$, enantiomeric excesses of the substrate.

The comprehensive analysis and identification of strain 1 by 16S rDNA and BIOLOG (Table S1) showed that the strain was in line with the characteristics of *B cereus*. Therefore, it was named *B cereus* WZZ006 and deposited in the China Center for Type Culture Collection under the accession number of CCTCC M 2017621.

3.2 | Effects of pH, temperature, rotating speed, cosolvent, and substrate concentration on enzymatic resolution of racemic indoxacarb intermediates

A change in the surface charge density and molecular structure of the cell or enzyme molecule is accomplished by changes in pH. This has led to the changes in the rate of entry and exit of the substance followed by activity and selectivity of the enzyme change.[37,38] Therefore, it is essential to regulate the pH of the reaction medium. As shown in Figure 3, the enzyme activity of the cells increased within the pH range of 5.0 to 7.0; the conversion rate increased from 0.4 ± 0.4% to 44.6 ± 0.7%, and *e.e.*$_s$ also increased sharply. When the pH at 7.0, the optical purity of the enzyme activity and the substrate

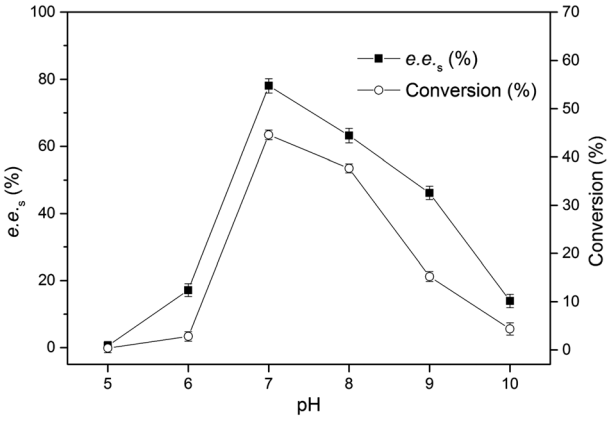


FIGURE 3 Effects of pH on the enzymatic resolution of racemic indoxacarb intermediate. Reaction conditions: 0.05-g *Bacillus cereus* WZZ006 freeze-dried powder, 50-μL 20-mM racemic substrate, 180 rpm, 30°C, 24 hours; 50-mM citrate-sodium citrate buffer, pH 5.0; 50-mM phosphate buffer, pH 6.0 to 8.0; 50-mM tris-HCl buffer, pH 9.0; 50-mM Na_2CO_3-$NaHCO_3$ buffer, pH 10.0.The hydrolysis of the substrate was determined by high-performance liquid chromatography (HPLC)

reached the maximum; the conversion rate was 44.6 ± 0.7%, and the $e.e._s$ was 78.1 ± 1.9%. When pH > 8.0, $e.e._s$ gradually decreased with the increase of pH, and the enantioselectivity of the enzyme was minimized. Therefore, pH 7.0 is the optimum pH for the reaction.

Temperature is a principle element affecting the activity of biocatalysts and the thermodynamic equilibrium of the reaction.[39] The enzymatic resolution of the racemic substrate at different temperatures is shown in Figure 4. As the temperature increases, the enzyme activity first rises and then decreases. Under low-temperature conditions (20°C-25°C), the enzyme activity is weak, in an inactive catalytic state. When the temperature is 25°C to 30°C, the activity of the enzyme increases significantly. At 30°C temperature, the enzyme activity was maximum; the conversion rate is 52.7 ± 1.2%, and the $e.e._s$ is 88.7 ± 1.9%. As the temperature is further increased, the activity of the enzyme is rapidly reduced, especially when the temperature is higher than 40°C, the activity of the enzyme is only 10%, which may be due to the high-temperature affects the molecular structure of the enzyme, resulting in a substantial reduction in enzyme

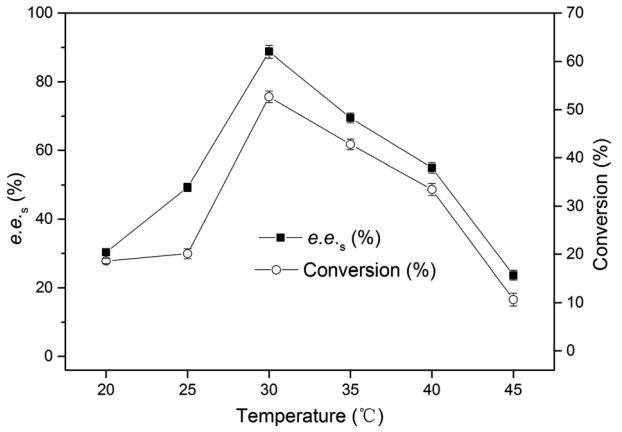


FIGURE 4 Effects of temperature on the enzymatic resolution of racemic indoxacarb intermediate. Reaction conditions: 0.05-g *Bacillus cereus* WZZ006 freeze-dried powder, 20-mM substrate and pH 7.0 PB buffer, under different temperature gradients (20°C, 25°C, 30°C, 35°C, 40°C, 45°C), 180 rpm, 24 hours. The hydrolysis of the substrate was determined by high-performance liquid chromatography (HPLC)

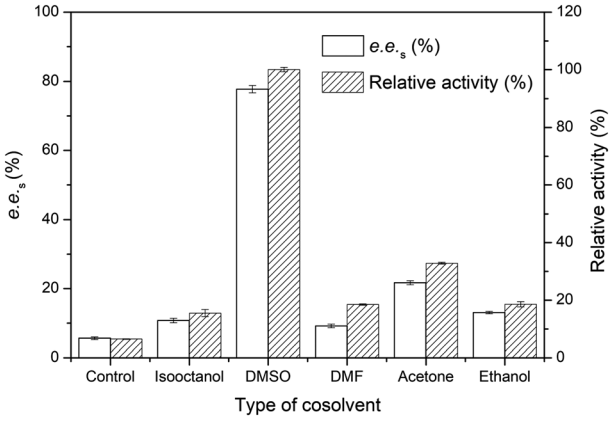


FIGURE 6 Effects of cosolvent on the enzymatic resolution of racemic indoxacarb intermediate. Reaction conditions: 0.05-g *Bacillus cereus* WZZ006 freeze-dried powder, 0.05 g of the substrate and pH 7.0 PB buffer; 5% different cosolvents (isooctanol, DMSO, DMF, acetone, ethanol) were added to 30°C, 180 rpm, 24 hours. Take no cosolvent as a control. Determine the hydrolysis of the reaction by high-performance liquid chromatography (HPLC), and define the highest enzyme activity under different cosolvent conditions to be 100%. Calculate relative vitality

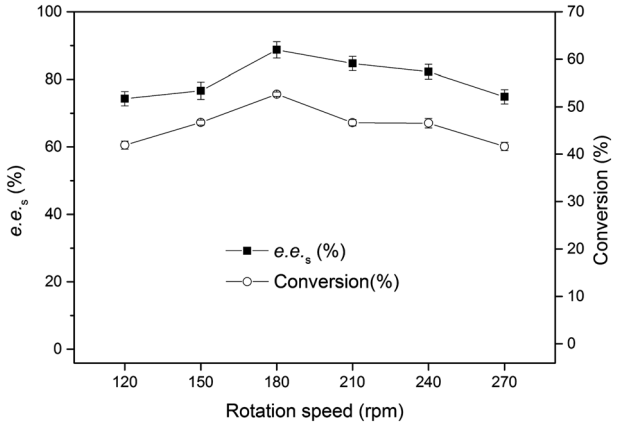


FIGURE 5 Effects of rotation speed on the enzymatic resolution of racemic indoxacarb intermediate. Reaction conditions: 0.05-g *Bacillus cereus* WZZ006 freeze-dried powder, 20-mM substrate and pH 7.0 PB buffer, 30°C, different speed (120, 150, 180, 210, 240, 270 rpm), 24 hours. High-performance liquid chromatography (HPLC) determined the hydrolysis of the substrate

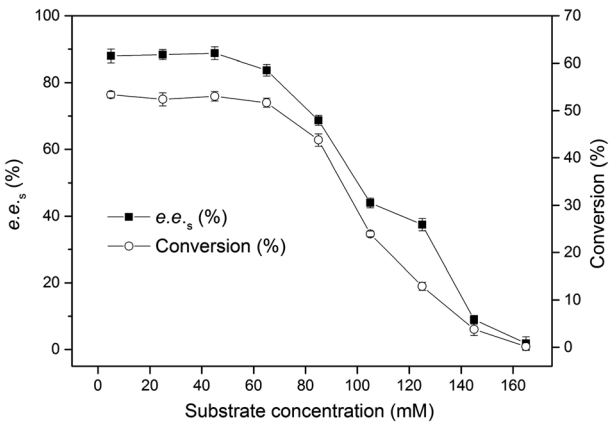


FIGURE 7 Effects of substrate concentrations on the enzymatic resolution of racemic indoxacarb intermediate. Reaction conditions: 0.05-g *Bacillus cereus* WZZ006 freeze-dried powder, different concentrations of substrate (5-165 mM) and pH 7.0 PB buffer, 30°C, 180 rpm, 24 hours. The hydrolysis of the substrate was determined by high-performance liquid chromatography (HPLC)

activity. In light of this, the temperature of the reaction should be controlled at 30°C.

The rotational speed has a tendency to affect the diffusion of the compound in the reaction system and the contact time of the cells or enzymes with the substrate. The experiment explored the effect of different rotation speed on the enzyme-catalyzed enzymatic resolution reaction. The results are presented in Figure 5. The conversion rate and *e.e.*$_s$ were significantly improved at speeds ranging from 120 to 210 rpm, while the *e.e.*$_s$ dropped when the speed increased. When the speed is 180 rpm, the conversion rate and *e.e.*$_s$ are the highest. Probably, it can show that high rotational speed can alter the internal structure of cells by shear stress.[40] Therefore, 180 rpm was used for subsequent researches, and the conversion rate and *e.e.*$_s$ at 52.7 ± 0.5% and 88.7 ± 1.4%, respectively.

For the bioconversion of ester compounds, the addition of a cosolvent can accelerate the reaction rate, and also some enzymes can exhibit better enantioselectivity in water-organic systems. The results in Figure 6 show that the addition of a cosolvent facilitates the reaction and accelerates the reaction rate. The effect of adding DMSO is the best, while isooctanol, DMF, and ethanol have little effect on the catalytic reaction. Therefore, DMSO was chosen as a cosolvent for subsequent experiments.

When the amount of biocatalyst added is constant, it is necessary to investigate the maximum saturated substrate concentration catalyzed by the enzyme. The effect of the substrate with a concentration range of 5 to 165 mM on the catalytic reaction was investigated. The results are shown in Figure 7. When the biocatalyst amount is 0.05 g and the concentration is 5 to 45 mM, the conversion rate remains unchanged, substantially higher than 50.0 ± 0.8%, and *e.e.*$_s$ reaches 88.0 ± 1.8%. When the substrate concentration is greater than 45 mM, the *e.e.*$_s$ and conversion rates drop sharply. This indicates that the high concentration may cause substrate inhibition, the catalysis of enzyme reached a saturation point, the reaction speed became slow, and the high concentration may destroy the molecular structure of the enzyme to affect enzyme activity. In summary, under certain conditions of enzymes, the appropriate substrate concentration should be selected; the concentration of 45 mM was used for subsequent experiments.

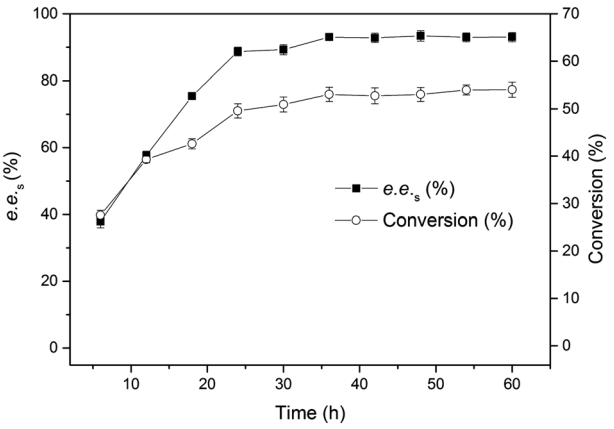


FIGURE 8 Time course of enzymatic resolution of racemic indoxacarb intermediate under optimized conditions

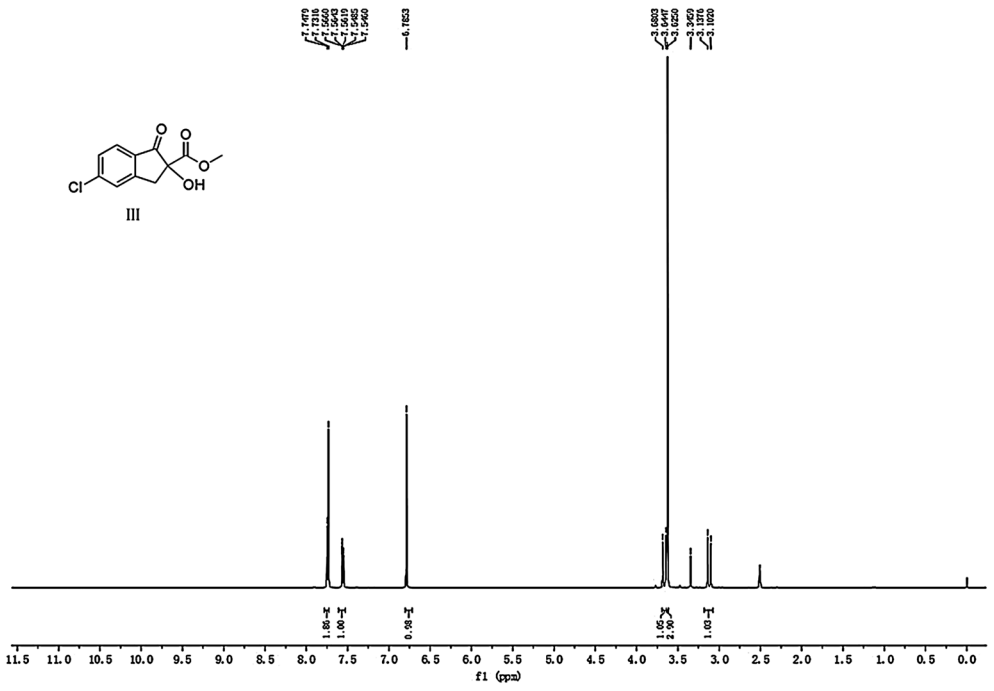


FIGURE 9 ^{1}H NMR of 5-chloro-2,3-dihydro-2-hydroxy-1-oxo-1*H*-indene-carboxylic acid methyl ester (**III**)

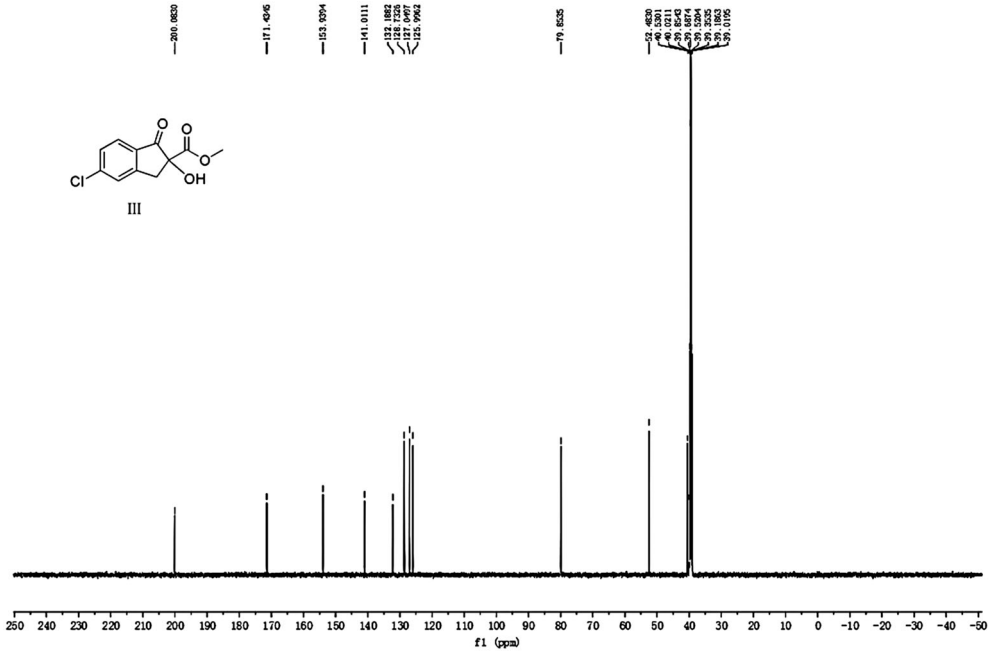


FIGURE 10 ^{13}C NMR of 5-chloro-2,3-dihydro-2-hydroxy-1-oxo-1*H*-indene-carboxylic acid methyl ester (**III**)

3.3 | Time course of whole-cell biocatalytic reaction

The time course of enantioselective hydrolysis of 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester using *B cereus* WZZ006 is shown in Figure 8. The reaction was carried out under the following optimized conditions: 0.05-g *B cereus* WZZ006 freeze-dried powder, 45-mM substrate, solution pH 7.0, reaction temperature 30°C, rotation speed of 180 rpm, cosolvent DMSO, respectively. After 36 hours, the hydrolysis was basically completed, and the $e.e._s$ and conversion rates reached the highest, respectively 93.0 ± 1.4% and 53.0 ± 1.6%.

3.4 | Analysis of the hydrolysis product

In this study, a simple chemical reaction was made to prepare a racemic substrate 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester (**III**, yield: 75%) with a purity of 98% using 5-chloro-1-indanone as a raw material. The ^{1}H and ^{13}C NMR spectrum were recorded in Figures 9 and 10.

Then, the enzymatic resolution of the racemic substrate was carried out and analyzed by HPLC. The three peaks in Figure 2 represented the product (**VI**), the *S*-isomer (**IV**, unreacted), and the *R*-isomer (**V**, transformed) of the substrate, respectively. The results showed that there was only a single reaction in the hydrolysate and no other side effects. Besides, it was found that the product was not acid, and its polarity was greater than that of the substrate ester (**III**) by observed the retention time. Based on the substrate structure, it was speculated that the product might be decarboxylated and converted into an alcohol compound: 5-chloro-2-hydroxy-1-indolone (**VI**). Also, the resulting product was separated by TLC and analyzed with AD-H chiral column (Figure S3). There were two close and equal peaks with similar retention times, which inferred that the product was a racemic compound, and it also indicated that the substrate was not ring opened or formed into other structures after being hydrolyzed, just decarboxylated. Furthermore, the product structure was then characterized by GC-MS

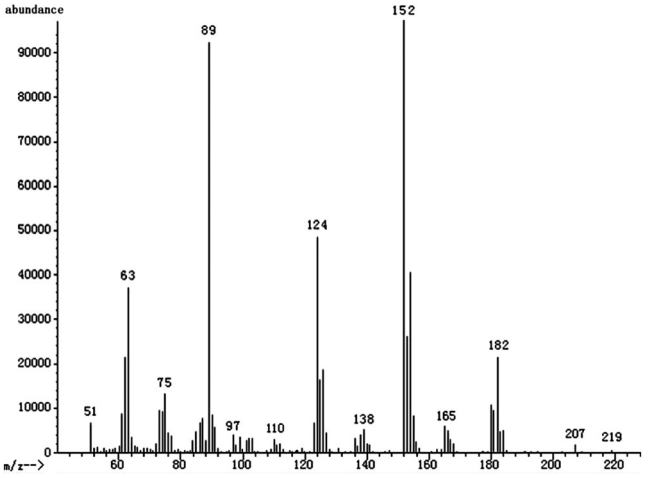
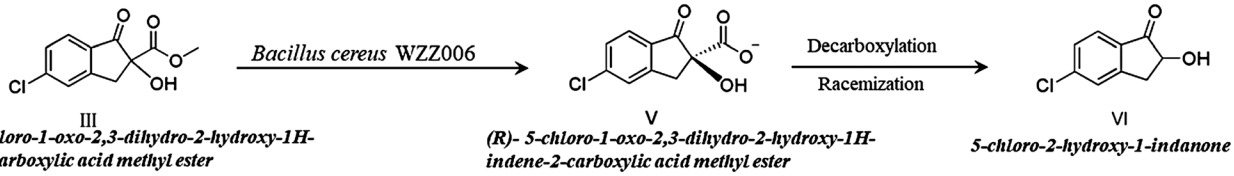


FIGURE 11 Analysis of the product by gas chromatography mass spectrometry



SCHEME 2 The production of the hydrolysis product 5-chloro-2-hydroxy-1-indanone

(Figure 11) and NMR (Figures S4 and S5). MS (ESI) *m/z*: 182 $[M + H]^+$, ^1H NMR (500 MHz, DMSO-d_6): $\delta = 7.65$ (t, $J = 7.65$, 1*H*), 7.49 (d, $J = 7.49$,1*H*), 5.89 (m, $J = 5.89$, 1*H*), 4.42 (m, $J = 4.42$, 1*H*), 3.49 (m, $J = 3.49$, 1*H*), 2.82 (m, $J = 2.82$, 2*H*); 13C NMR (125 MHz, DMSO-d_6): $\delta = 205.0$ (C), 152.9 (C), 140.2 (C), 133.2 (C), 128.2 (CH), 127.0 (CH), 125.1 (CH), 72.9 (CH), 35.4 (CH2). Finally, the GC-MS and NMR results also illustrated the product had a further decarboxylation after hydrolysis (Scheme 2) and was identified as 5-chloro-2-hydroxy-1-indanone (**VI**).

4 | CONCLUSION

In summary, we have demonstrated a whole-cell biocatalytic synthesis method of (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester. After screening various strains, a newly isolated strain of *B cereus* WZZ006 is the best biocatalyst. Furthermore, optimization reaction parameters such as pH, temperature, and substrate concentration were systematically optimized for perfect conversion with high optical purity of the racemic substrates. A conversion of 53.0% with 93.0% $e.e._s$ and 35.0 *E* for (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester was obtained at 30°C, 180 rpm for 36 hours, using *B cereus* WZZ006 as the catalyst.

Therefore, this biocatalytic process route has been proven to be efficient for the whole bacterial cell biocatalytic preparation of enantiopure (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester, which has laid an excellent theoretical foundation for the industrial production and application of indoxacarb. This approach offers a good starting point for discussion and further industrial production research.

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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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