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RESEARCH ARTICLE

Characterization of one novel microbial esterase WDEst9 and its use to make L-methyl lactate

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ABSTRACT

Chiral lactic acids and their ester derivatives are crucial building blocks and intermediates for the synthesis of a great variety of valuable functional materials and pharmaceuticals. Before our study, the reports about the enantioselective preparation of pure L-lactic acid and its ester derivatives through direct hydrolysis of racemic substrate were quite rare. Herein, we heterologously expressed and functionally characterized one novel microbial esterase WDEst9 from *Dactylosporangium aurantiacum*, which exhibited high resistance to diverse metal ions, organic solvents, surfactants, NaCl and KCl. We further utilized WDEst9 as a green biocatalyst in the kinetic resolution of (±)-methyl lactate through direct hydrolysis and generated L-methyl lactate with high enantiomeric excess (*e.e.* >99%) and high yield (>86%) after process optimization. Notably, the enantioselectivity of WDEst9 was opposite than that of two previously reported esterases PHE14 and BSE01701 that can generate D-methyl lactate though kinetic resolution of (±)-methyl lactate. Microbial esterase WDEst9 is a promising green biocatalyst in the preparation of valuable chiral chemicals and opens the door for the identification of useful industrial enzymes and biocatalysts from the genus *Dactylosporangium*.

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Biocatalysis; novel esterase; kinetic resolution; L-methyl lactate; opposite enantioselectivity

Introduction

Chiral α-hydroxy acids are one crucial class of chiral chemicals and chiral drug intermediates (Datta and Henry 2006; Gao et al. 2009). Due to the existence of one α-hydroxy functional group and one acid functional group, chiral α-hydroxy acids can be further converted to many valuable chiral chemicals, drugs and functional materials (Gao et al. 2009). So the preparation of a series of chiral α-hydroxy acids has been the hot spot of asymmetric synthesis.

Chiral lactic acids are very good representatives of chiral α-hydroxy acids. Both L-lactic acid and D-lactic acid can be utilized as the starting material for the synthesis of a great variety of functional materials through polymerization of the hydroxyl functional group and the acid functional group (Garlotta 2001). Using L-lactic acid or D-lactic acid, which harbours different chirality in the molecular, as the starting material can bring different chirality into final functional materials and further dramatically affect the physical properties of desired functional materials (Yáñez et al. 2003; Auras et al. 2010; Ma et al. 2014). Additionally, both L-lactic acid and D-lactic acid have been utilized as key intermediates for the synthesis of valuable chiral drugs and pesticides (Kricheldorf 2001; Gao et al. 2006; Tashiro et al. 2011). So the asymmetric synthesis of both L-lactic acid and D-lactic acid is very important for both material industry and pharmaceutical industry.

Chiral lactic acids can be synthesized through traditional organic synthetic method. However, traditional organic synthesis requires harsh working conditions and brings huge pollutions to our environments (Su et al. 2004; Hasegawa et al. 2008). Although L-lactic acid has been reported to be produced by microorganisms, those microorganisms can still produce undesired optical impurity D-lactic acid. Thus, tedious crystallization and chromatography are necessary to get rid of those unwanted impurities and obtain L-lactic acid with relative high optical purity (Ohara et al. 2001; Sakai et al. 2004). So researchers are

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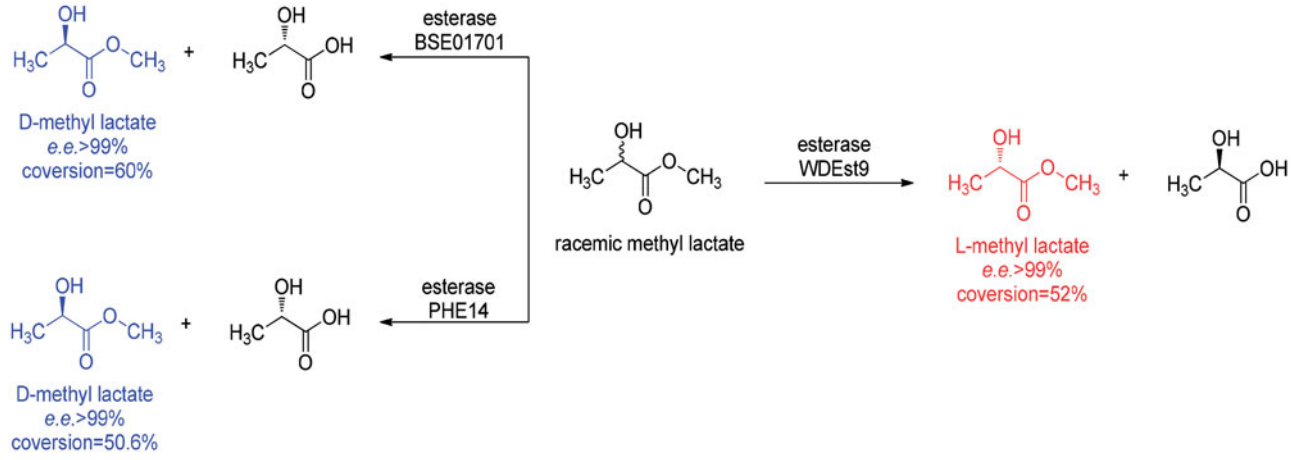

Scheme 1. Comparison of esterase WDEst9 with BSE01701 and PHE14 in the kinetic resolution of racemic methyl lactate through direct hydrolysis reactions.

investigating the synthesis of chiral lactic acids through green biocatalytic methods using enzymes. Due to the existence of one hydroxyl group in the molecular, chiral lactic acids should be able to be produced through bio-reductions of keto precursors using dehydrogenases. But the utilization of dehydrogenases in asymmetric synthesis requires expensive co-factors such as NADH or NADPH and the expensive co-factors need to be recycled in order to reduce the production costs (Liang et al. 2016).

Another biocatalytic method for the generation of chiral lactic acids is through kinetic resolutions using esterases/lipases. Ohara et al. (2011) reported that butyl L-lactate with an optical purity of 98.6% was obtained through asymmetric hydrolysis reaction using one commercially available immobilized lipase. Richard et al. (2013) also demonstrated the possibility of obtaining chiral L-lactic acid ester through trans-esterification at the of the free hydroxyl function of the lactate esters. Esterases (EC 3.1.1.1) are another important class of industrial enzymes which have been successfully used in the synthesis of chiral chemicals through trans-esterifications or direct hydrolysis reactions. Previously, we functionally characterized the functionalities of two novel deep-sea microbial esterases, BSE01701 from the Indian Ocean and PHE14 from the Western Pacific Ocean, in the asymmetric synthesis of chiral lactic acid. Both BSE01701 and PHE14 exhibited the same enantioselectivity and generated enantiomerically pure D-methyl lactate through direct hydrolysis of inexpensive racemic methyl lactate (Huang et al. 2016; Wang et al. 2016). Herein, we identified and functionally characterized another novel microbial esterase WDEst9 and utilized WDEst9 as a biocatalyst in the asymmetric synthesis of enantiomerically pure L-methyl lactate through direct hydrolysis of racemic methyl lactate. Interestingly, the enantioselectivity of WDEst9 was opposite to that of both BSE01701 and PHE14 during kinetic resolutions (Scheme 1).

Materials and methods

Microorganisms and plasmids

The strain *Dactylosporangium aurantiacum* subsp. *Hamdenensis* NRRL 18085 was obtained from Agricultural Research Service Culture Collection (NRRL). The plasmid pET-28a (+) (Novagen, GEOrge Town, KY) was used as the expression vector of WDEst9. *E. coli* DH5α and *E. coli* BL21(DE3) were used for plasmid construction and protein expression, respectively.

Microorganisms and plasmids

The deduced protein sequence of WDEst9 was analyzed using the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments of protein sequences were performed using the DNAMAN 7.0 program. Secretion signal was estimated by the SignalP 4.1 prediction tool from http://www.cbs.dtu.dk/services/SignalP/. The theoretical value of protein molecular and *pI* were predicted by expasy from http://web.expasy.org/compute_pi/.

Cloning, expression and purification of WDEst9

The coding DNA sequence of an esterase (named WDEst9) was amplified from the genomic DNA of *D. aurantiacum* subsp. *Hamdenensis* NRRL 18085. The primers for the cloning of *WDEst9* gene were 5′-CATGAATTCATGCCACTCGACCCGCAG-3′ and 5′-CAC AAGCTTTTAGGATCCGAACCACGC-3′ (*EcoR* I and *Hind* III restriction sites are given in italics). The PCR products were digested with *EcoR* I and *Hind* III, and then cloned

into the expression vector pET-28a (+). The recombinant plasmid was confirmed by DNA sequencing and then transformed into *E. coli* BL21(DE3) competent cells according to standard protocols. When OD_{600} of cells reached approximately 0.6–0.8, isopropyl-beta-D-thiogalactopyranoside was added at a final concentration of 0.3 mmol/L. After 20-h induction at 22 °C, the cells were harvested by centrifugation at 4000 r/min for 10 min, 4 °C, washed with phosphate buffer (50 mmol/L, pH 7.5) for two times and then resuspended in the same buffer. The cells were disrupted by sonication and the supernatants containing recombinant WDEst9 were collected by centrifugation at 10,000 r/min for 15 min, 4 °C. The recombinant protein was purified and desalted using nickel–nitrilotriacetic acid agarose resin (GE Healthcare Life Science, Shanghai, China) and PD-10 desalting columns (GE Healthcare Life Sciences) following manufacturers' protocols. Purified WDEst9 was tested by SDS-PAGE, using 12% polyacrylamide gels. Protein concentration was determined using the method of Bradford method (Bradford 1976), with bovine serum albumin as standard.

Microorganisms and plasmids

The standard reaction mixture containing 10 mmol/L substrate (*p*-nitrophenyl esters, *p*-NP) dissolved in 10 μL of acetonitrile, 275.7 U/L enzyme, 940-μL phosphate buffer (50 mmol/L, pH 7.5), and 40-μL ethanol was incubated at 35 °C for 5 min. The activity of the esterase was determined by detecting the absorbance (release) of *p*-NP at 405 nm. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-NP per minute from the *p*-NP esters.

Biochemical characterization of WDEst9

The substrate specificity of WDEst9 was determined by using various substrates (*p*-NP C2–C8) under standard reaction conditions. Kinetic parameters were measured using a Lineweaver–Burk plot under the optimal reaction conditions. The optimum pH for WDEst9 was determined by using *p*-PN butyrate (C4) as the substrate over a pH range 6.0–10.0. The buffers used were 50 mmol/L NaAc/HAc (pH 6.0), 50 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 6.5–7.5), 50 mmol/L Tris/HCl (pH 8.0–9.0), and 50 mmol/L Glycine/NaOH (pH 9.5–10.0). The pH stability was measured by incubating purified WDEst9 in the buffers with different pH values at 4 °C for 6 h and 12 h, respectively. The effect of temperature on the activity of WDEst9 was examined from 20 to 50 °C under the optimal pH. The thermostability was measured by incubating WDEst9 at a temperature range of 20–60 °C for a time range of 0–60 min by using *p*-PN acetate (C4) as the substrate under optimal pH value. The effect of salinity (NaCl and KCl) on WDEst9 activity was investigated by measuring the residual activity after incubation in solutions containing 0–4 mol/L NaCl or KCl for 12 h at 4 °C. The effect of metal ions (Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}) was determined by incubating WDEst9 in the presence of different metal ions (1 mmol/L) for 12 h at 4 °C. The effect of various organic solvents and surfactants were investigated by incubating WDEst9 in the presence of nine different organic solvents (10%, *v*/*v*) and four different surfactants (0.1%) for 12 h at 4 °C. The activity of the enzyme without any additive in the reaction mixtures was defined as 100%.

Optimization of the kinetic resolution of (±)-methyl lactate by WDEst9

The effect of pH on the resolution of (±)-methyl lactate was examined under a pH range of 6.0–9.0. The optimum temperature on the resolution of (±)-methyl lactate was measured at different temperatures ranging from 25 °C to 45 °C. Under the optimum pH value and temperature, the effect of organic solvents and surfactants on enzymatic reactions were carried out by adding nine kinds of organic solvents (10%, *v*/*v*) and four different surfactants (0.01%) as the reaction co-solvents. Reactions without the addition of organic solvents and surfactants were marked as 100%. Various concentrations (from 0.01 to 0.03 mol/L) of (±)-methyl lactate was carried out to determine the optimum substrate concentration on enzymatic reactions. The optimum enzyme concentration on the resolution of (±)-methyl lactate was measured by adding WDEst9 into the reactions at final concentrations of ranging from 0.3 to 3.5 g/L. Under optimum reaction conditions, the effect of reaction time on the resolution of (±)-methyl lactate was examined at different times ranging from 1 to 4 h. After the completion of the enzymatic reactions, samples were extracted with an equal volume of ethyl acetate and the organic phase was further analyzed to evaluate the enzymatic resolutions of (±)-methyl lactate by chiral GC. The enantiomeric excess (*e.e.*), conversion rate (*C*) and yield (*Y*) of hydrolytic reactions were calculated by using the equation of Chen et al. (1982).

Analytical methods of chiral GC

The *e.e.* of the reaction products was determined by using a Gas Chromatograph (FULI GC-9700II) equipped with H_2 flame ionization detector and 112-6632

CYCLOSIL-B chiral capillary column (30 m × 0.25 mm ID, 0.25 μm df). The retention times of L-methyl lactate and D-methyl lactate were 4.72 and 4.51 min, respectively.

Results and discussion

Sequence analysis of WDEst9

An open reading frame (ORF) of 936 bp, encoding a protein of 311 amino acids (WDEst9) without a signal peptide, was identified from the genome sequence of *D. aurantiacum* subsp. *Hamdenensis* NRRL 18085. Blast of the protein sequence of WDEst9 showed 88% similarity with one putative alpha/beta hydrolase from *Dactylosporangium aurantiacum* (WP_033361445), 63% similarity to one putative protein from *Longispora albida* (WP_018350597) and 63% similarity to one putative acetyl esterase from *Micromonospora narathiwatensis* (SBT42274). So, WDEst9 is a novel microbial esterase which exhibits low sequence identities with some putative esterases. The protein sequence analysis showed that WDEst9 belonged to the typical α/β hydrolase family with a typical catalytic center (Ser^{157}, Asp^{252} and His^{282}) (De Simone et al. 2000) (Figure S1). Interestingly, the enantio-selectivity of esterase WDEst9 was opposite than that of esterase BSE01701 and PHE14 during the kinetic resolution of (±)-methyl lactate from our previous report (Huang et al. 2016; Wang et al. 2016). The protein sequence analysis showed that both BSE01701 and PHE14 also belong to the α/β hydrolase family with the conserved signature (Gly-X-Ser-X-Gly) in the open reading frames. The three-dimensional structure of WDEst9 (consists of seven β strands and eight α helices) is different from those of BSE01701 (consists of eight β strands and eight α helices) and PHE14 (consists of five β strands and five α helices). Furthermore, the catalytic triads of BSE01701 and PHE14 are formed by Ser^{110}–Asp^{162}–His^{193} and Ser^{116}–Asp^{180}–His^{219}, respectively, which are different from WDEst9. The differences in protein sequences and structures of the three enzymes should be the main reasons leading to the different stereo-preference. However, we cannot predict the detailed biochemical characteristics, stereo-preference and stereo-selectivity of one enzyme just based upon protein sequence and structure analysis at the current stage. Thus, further investigation of the crystals of WDEst9, BSE01701 and PHE14 and the interaction of the two esterases with the two enantiomers of (±)-methyl lactate may provide more information for the explanation of opposite stereo-preference.

Expression and purification of WDEst9

The theoretical molecular weight and the *pI* of WDEst9 were calculated to be 36.5 kDa and 4.65 by http://web.expasy.org/compute_pi/. After the purification with Ni-NTA affinity chromatography column, the expected protein band was observed by SDS-PAGE (Figure S2).

Biochemical characterization of WDEst9

Substrate specificity of WDEst9

The preferred substrate for hydrolysis was *p*-PN butyrate (C4), and marked as 100% relative activity. The relative hydrolytic activities of WDEst9 toward *p*-PN (C2), *p*-PN (C6) and *p*-PN (C8) were below 30% (Figure S3). The results indicated that the preferred substrates of WDEst9 were short-chain *p*-PN esters rather than long-chain *p*-PN esters. The kinetic analysis of WDEst9 was performed using *p*-NP esters (C2-C6) as the substrates. The highest hydrolytic activity of WDEst9 toward *p*-PN (C4) was 96.4 ± 2.65 U/mg, with the lowest K_m value being 0.99 ± 0.05 mmol and the highest V_{max} value being 192 ± 3 μM mg^{-1} min^{-1}, respectively. Before our work, there were some similar reports about the substrate specificity of esterases, such as Est6 (104.41 U/mg) (Jiang et al. 2012), EstB (981 ± 25.4 U/mg) (Chu et al. 2008) and Est9X (61 ± 3.6 U/mg) (Fang et al. 2014). Above results further suggested that WDEst9 was characterized to be an esterase instead of a lipase (Arpigny and Jaeger 1999).

Effect of pH on the activity and stability of WDEst9

P-nitrophenyl butyrate (C4) was used as the substrate for further functional characterization of WDEst9. The optimal pH for the hydrolytic activity of esterase WDEst9 was 8.0 (Figure S4). The esterase WDEst9 exhibited very good stability in buffer of different pH values (pH 6.0–10.0) for 0–12 h, and kept highest activity and stability in Tris/HCl buffer of pH 8.0 with residual activity of 86.9 ± 3.7% (Figure S5).

Effect of temperature on the activity and stability of WDEst9

WDEst9 exhibited its highest hydrolytic activity at 30 °C (Figure S6). The thermostability analysis of WDEst9 showed it was stable and kept 76% (20 °C) and 80% (30 °C) of its highest activity after incubation for 60 min (Figure S7). However, the stability of WDEst9 decreased rapidly when the temperature was over 40 °C and remained 19% (50 °C) of its highest

activity after 60 min of incubation. One esterase EstIM1 from a metagenomic library of mountain soil could remain 80% of its original activity after being heated 30 °C for 1 h, but was inactivated when being heated 40 °C for 40 min (Ko et al. 2012). The esterase Est55 from *G. stearothermophilus* retained more than 90% of its original activity after 3 h treatment at 60 °C (Ewis et al. 2004) and esterase Est-XG2 from a metagenomic library retained more than 80% of its original activity after 2 h treatment at 80 °C (Shao et al. 2013).

Effect of salinity (NaCl and KCl) on the activity of WDEst9

The hydrolytic activity of WDEst9 was increased in the presence of 0.2–2 mol/L NaCl or KCl, and WDEst9 showed its maximum activity at 1 mol/L NaCl (159.9 ± 2.2%) or KCl (156.2 ± 3.5%) (Figure S8). Esterase WDEst9 remained more than 30% of its hydrolytic activity in the presence of 4 mol/L NaCl or KCl. Similar results were also observed in three other salt-tolerant esterases (EstKT4, EstKT7 and EstKT9) from a metagenomics library with more than 50% of their initial activities in the presence of two salts of 3 mol/L (Jeon et al. 2012). These results indicated that WDEst9 was an esterase with good tolerance to high salinity.

Effect of metal ions on the activity of WDEst9

The activity of esterase WDEst9 was increased by 1 mmol/L Mg^{2+} (121.4 ± 6.2%), Li^{+} (116.5 ± 0.7%) and Ca^{2+} (105.8 ± 0.9%), and it maintained more than 50% of activity under most of tested metal ions expect for 1 mmol/L Fe^{2+}, Cu^{2+} and Zn^{2+} (Table S1). Similar results were also observed in the case of one esterase H9Est from a metagenomics library which was inhibited by Cu^{2+} (67.4 ± 6.6%) and Zn^{2+} (32.3 ± 4%) (De Santi et al. 2015).

Effect of organic solvents and surfactants on the activity of WDEst9

Organic solvents and surfactants can greatly affect the activity of esterases, especially the activity and enantio-selectivity in biocatalysis (Choi et al. 2004). Activities of enzymes in organic solvents are important for biocatalysts used in organic synthesis (Shao et al. 2013). The results showed that different organic solvents and detergents had different impacts on the hydrolysis activity of WDEst9. The presence of DMSO, cyclohexane (10%, *v/v*) and sodium tripolyphosphate (0.01%) greatly increased the hydrolytic activity of WDEst9 with the residual activities being 145.3 ± 8.3%, 125.1 ± 0.2% and 116.7 ± 5.3%, respectively (Table S2). With the increasing demand of esterases under extreme conditions, esterase WDEst9 with high resistance to organic solvents and surfactants are highly desirable in industry.

Kinetic resolution of (±)-methyl lactate by WDEst9

Effect of pH and temperature on the kinetic resolution of (±)-methyl lactate

During enzymatic kinetic resolutions, both pH values and temperature could modify the state of the active center of enzymes, thus affecting both the enantioselectivity and the enzymatic activity. As shown in Figure 1, the optimal reaction pH on the kinetic resolution of (±)-methyl lactate was pH 8.5 with the

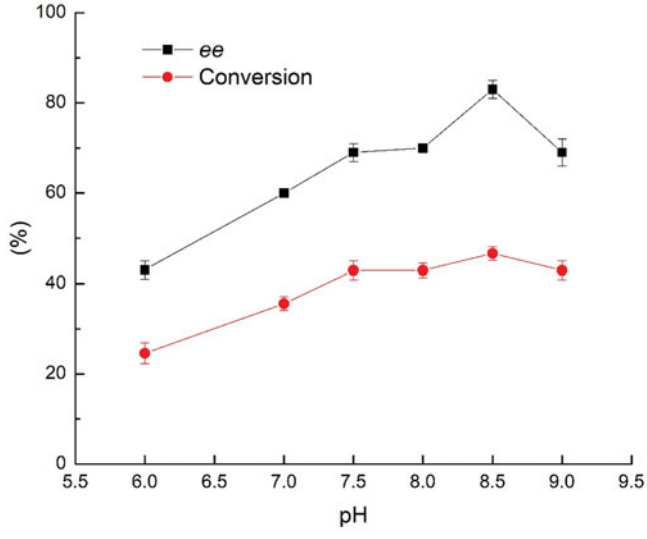

Figure 1. Effect of pH on the kinetic resolution of (±)-methyl lactate by WDEst9.

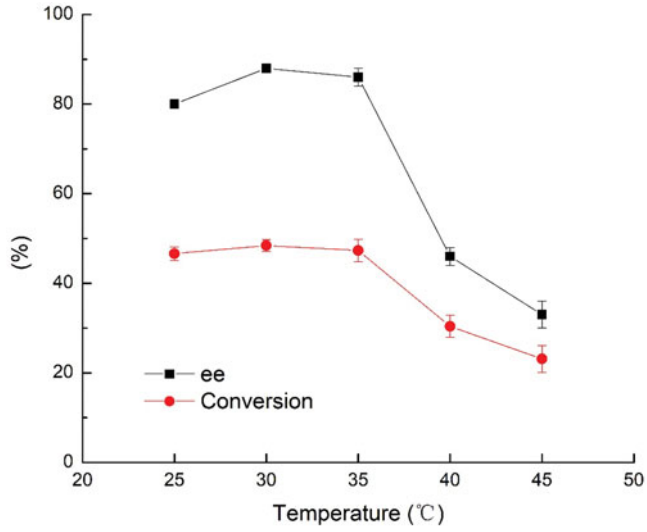

Figure 2. Effect of temperature on the kinetic resolution of (±)-methyl lactate by WDEst9.

highest *e.e.* being 83%. The best *e.e.* and conversion could reach 88% and 48.4%, respectively, at 30 °C, indicating that the optimal reaction temperature for the kinetic resolution of (±)-methyl lactate was 30 °C (Figure 2). However, the *e.e.* and the conversion decreased dramatically when the temperature was over 30 °C, indicating that the high temperature may greatly affect the kinetic resolutions of (±)-methyl lactate by WDEst9.

Effect of organic solvents and surfactants on the kinetic resolution of (±)-methyl lactate

The effect of organic solvents and surfactants on the kinetic resolution of (±)-methyl lactate were carried out by adding nine kinds of organic solvents (10%, *v/v*) and four different surfactants (0.01%) as the reaction co-solvents. The results showed that the addition of organic solvents and surfactants did not have beneficial effect on the increase of either the *e.e.ee* or the conversion in the kinetic resolution of (±)-methyl lactate by WDEst9 (Table 1), while there were some reports in which the addition of organic solvents increased both the *e.e.* and the conversion during enzymatic kinetic resolutions. Thus, further optimization of the kinetic resolution of (±)-methyl lactate by WDEst9 was studied without the addition of any organic solvent or surfactant.

Effect of substrate concentration and enzyme concentration on the kinetic resolution of (±)-methyl lactate

As shown in Figures 3 and 4, 0.015 mol/L substrate and 1.9×10^4 U/L enzyme were the optimum substrate concentration and enzyme concentration for the kinetic resolution of (±)-methyl lactate by WDEst9, with the *e.e.* being over 94% and the conversion being 51.3%. The results indicate that both the substrate concentration and the enzyme concentration played important roles in the kinetic resolutions of (±)-methyl lactate catalyzed by WDEst9.

Table 1. Effect of organic solvents and surfactants on the kinetic resolution of (±)-methyl lactate by WDEst9.

Organic solvents and surfactants	*e.e.* (%)	Conversion (%)
Control	88 ± 1	48.4 ± 1.5
Methanol	69 ± 2	33.7 ± 1.9
Ethanol	60 ± 2	35.2 ± 1.4
n-Decyl alcohol	53 ± 1	31.5 ± 1.4
Cyclohexane	38 ± 1	25.7 ± 1.5
n-Octane	60 ± 1	29.3 ± 1.4
n-Decane	65 ± 2	30.4 ± 2.3
DMSO	80 ± 2	40.0 ± 1.4
DMF	53 ± 1	28.6 ± 1.5
Methylbenzene	30 ± 1	20.9 ± 1.5
Triton X-100	80 ± 1	45.5 ± 1.9
Tween-20	79 ± 2	45.5 ± 2.3
Tween-80	72 ± 1	42.5 ± 1.5
Sodium Tripolyphosphate	85 ± 2	49.5 ± 2.2

Effect of reaction time on the kinetic resolution of (±)-methyl lactate

The effect of reaction time on the kinetic resolution of (±)-methyl lactate was investigated by carrying out enzymatic resolutions for different times (from 1 h to 4 h). The results showed that the optimal reaction time for the kinetic resolution of (±)-methyl lactate by WDEst9 was found to be 3 h, with the highest *e.e.* being over 99%, and the conversion and the yield being 52.1% and 86.7%, respectively (Figures 5 and 6).

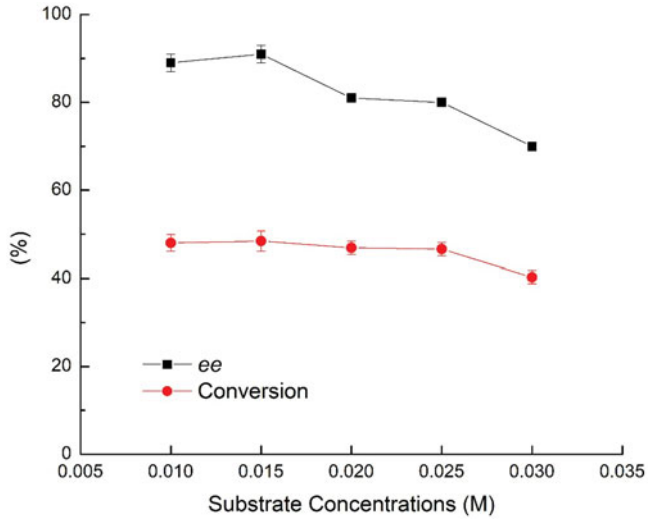

Figure 3. Effect of substrate concentration on the kinetic resolution of (±)-methyl lactate by WDEst9.

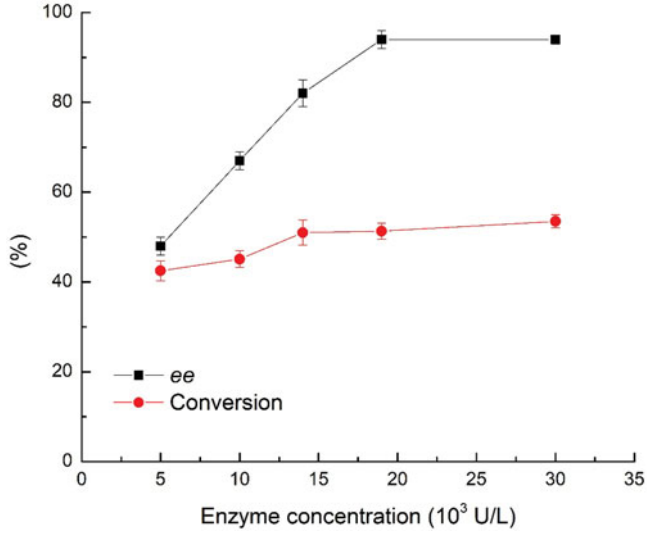

Figure 4. Effect of enzyme concentration on the kinetic resolution of (±)-methyl lactate by WDEst9.

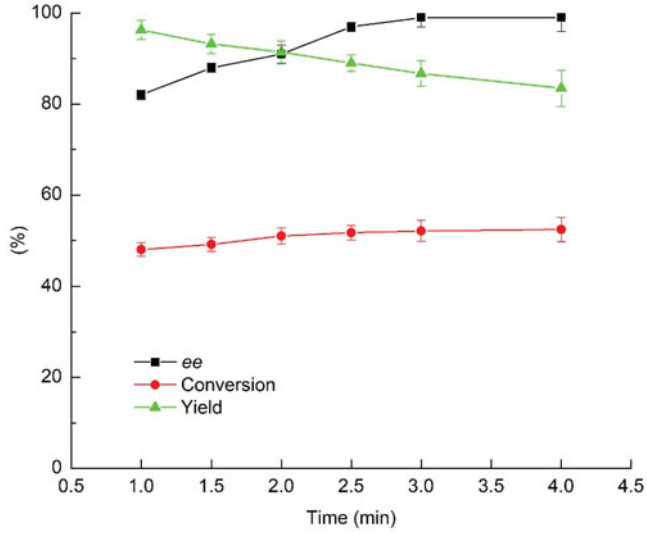

Figure 5. Effect of reaction time on the kinetic resolution of (±)-methyl lactate by WDEst9.

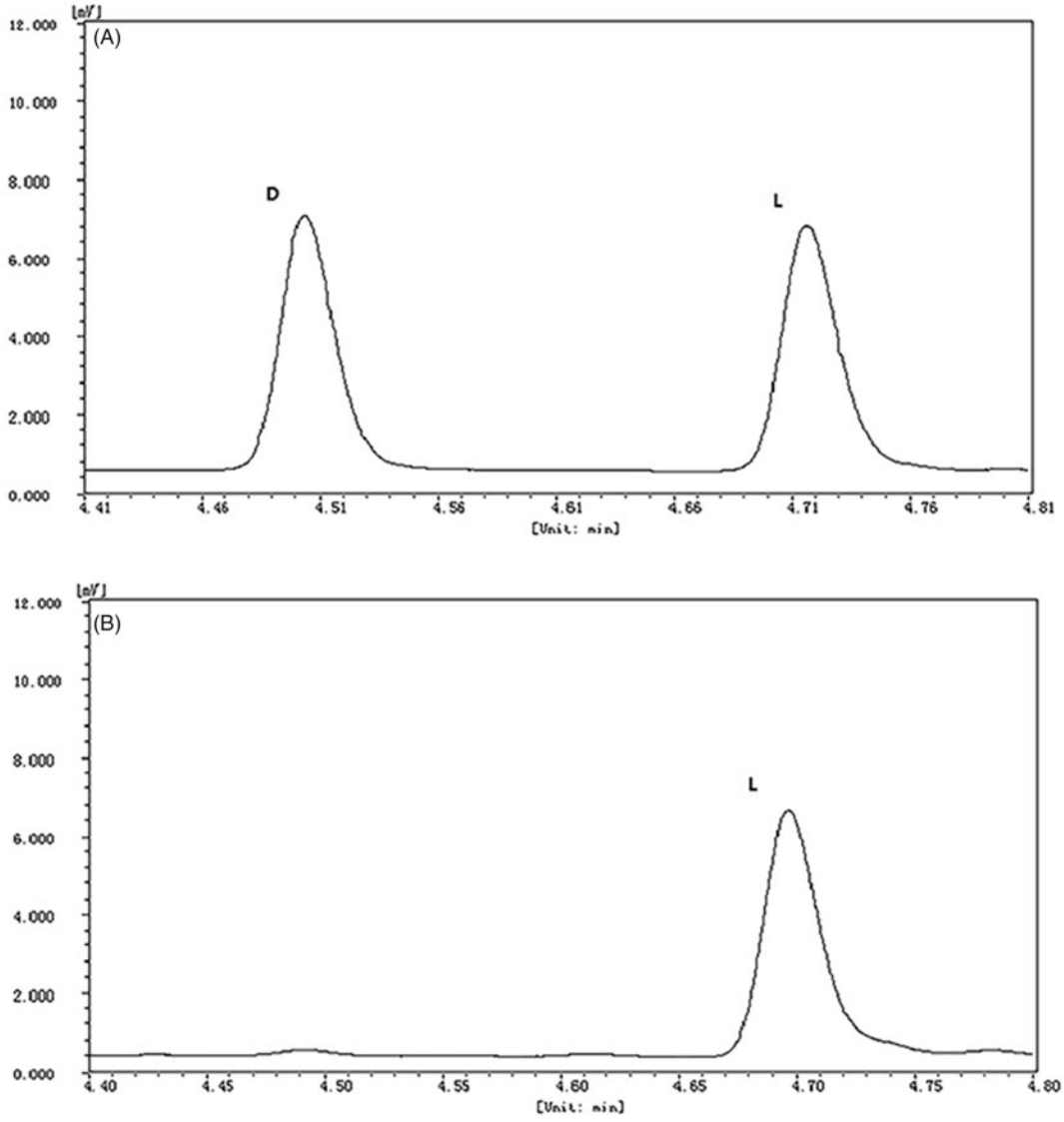

Figure 6. GC chromatogram of the kinetic resolution of (±)-methyl lactate by WDEst9. (A: GC chromatogram of (±)-methyl lactate; B: GC chromatogram of the generated L-methyl lactate by WEst9; L indicates L-methyl lactate and D indicates D-methyl lactate).

Comparation of WDEst9 and other esterases in the kinetic resolutions of (±)-methyl lactate

In our study, we identified and functionally characterized a novel microbial esterase WDEst9 and further utilized WDEst9 as a biocatalyst in the preparation of L-methyl lactate through kinetic resolution by direct hydrolysis, with the *e.e.* being over 99% and the yield being over 86% (Table S3). Notably, the enantioselectivity of WDEst9 was opposite to that of two other esterases, PHE14 and BSE01701, we had previously utilized for the kinetic resolution of (±)-methyl lactate (Huang et al. 2016; Wang et al. 2016). The optimal pH during kinetic resolution by WDEst9 (pH 8.5) was close to that of PHE14 and BSE01701 (pH 9.0) (Table 2). The optimal temperature during kinetic resolution by WDEst9 (30 °C) was close to that of PHE14 and BSE01701 (30 and 35 °C, respectively). Neither organic solvents nor surfactants had beneficial effect for the kinetic resolution of (±)-methyl lactate by WDEst9 and PHE14. However, both *n*-heptane and Triton X-100 stimulated the *e.e.* of chiral product in the kinetic resolution of (±)-methyl lactate by BSE01701. The optimal substrate concentration in the kinetic resolution catalyzed by WDEst9 was relatively lower than that of BS01701 and PHE14.

Conclusions

Dactylosporangium is a genus belonging to the phylum Actinobacteria. Previous studies related to *Dactylosporangium* mainly focus on the isolation of strains and diversity analysis. Some scientists also

Table 2. Comparation of WDEst9 and other esterases in the kinetic resolutions of (±)-methyl lactate.

Esterase	WDEst9	PHE14	BS01701
Configuration of product	L	D	D
Optimal pH	8.5	9.0	9.0
Optimal temperature (°C)	30	30	35
Effect of organic solvents	Inhibition	Inhibition	*e.e.* increased by *n*-heptane
Effect of surfactants	Inhibition	Inhibition	*e.e.* increased by Triton X-100
Optimum substrate concentration (mol/L)	0.015	0.06	0.1
e.e. (%)	>99	>99	>99
Conversion (%)	52.1	50.6	60
Yield (%)	86.7	88.7	–
References	This work	Wang et al. (2016)	Huang et al. (2016)

"–" denotes no calculation.

worked on the identification of active antibiotics from *Dactylosporangium* and elucidation of corresponding biosynthetic pathways. However, there were basically no reports about either the identification of industrial enzymes from *Dactylosporangium* or utilizaiton of those enzymes in biocatalysis. Industrial lipases/esterases are generally obtained from yeast or *Pseudomonas* and industrial proteases are generally obtained from *Bacillus*. We previously cloned and characterized two novel esterases (DAEst6 and WDEst17) from *Dactylosporangium aurantiacum* subsp. *Hamdenensis* NRRL 18085 and further developed those two esterases into biocatalysts for the generation of optically pure (*R*)-methyl mandelate and ethyl (*R*)-3-hydroxybutyrate with high *e.e.* and conversion, indicating the great potential of identification of useful industrial enzymes and biocatalysts from *Dactylosporangium* (Deng et al. 2016; Wang et al. 2018).

Herein, we identified and functionally characterized another novel esterase WDEst9 from the genome of *D. aurantiacum* subsp. *Hamdenensis* NRRL 18085. WDEst9 was characterized to be an esterase which exhibited high resistance to NaCl and KCl. WDEst9 was also further utilized as a green biocatalyst in the kinetic resolution of (±)-methyl lactate through kinetic resolution. Parameters such as pH, temperature, organic co-solvents, surfactants, substrate concentration, enzyme concentration and reaction time were further investigated for the kinetic resolution of (±)-methyl lactate by direct enzymatic hydrolysis. Notably, the enantioselectivity of the novel biocatalyst WDEst9 used in the kinetic resolution of (±)-methyl lactate was interestingly opposite than that of two other microbial esterases and could enzymatically prepared L-methyl lactate, rather than D-methyl lactate, with high enantiomeric excess (*e.e.* > 99%) and high yield (>86%). Before our studies of the three microbial esterases (WDEst9, BS01701 and PHE14), there were basically no report about enzymatic kinetic resolution of racemic methyl lactate through direct hydrolysis reaction, possibly because the two enantiomers of methyl lactate were very hard to be discriminated by esterases or lipases. Among the racemic ester chemicals we resolved through enzymatic kinetic resolutions, those chemicals with dramatic structural differences on either side of the ester bonds, such as 1-phenylethyl acetate and methyl mandelate, were relatively easier to be resolved using esterases/lipases. However, those chemicals with very little structural differences on either side of the ester bonds, such as methyl lactate and methyl 2-chloropropionate, were extremely hard to be resolved using esterases/lipases. We generally could only obtain one or two esterases/lipases, which could resolve those small racemic ester chemicals, after screening from an enzyme library of thousands of esterases/lipases. Thus, WDEst9 is a novel esterase which can recognize the small structural differences of racemic ester chemicals, and further investigation of the three-dimensional structures of WDEst9 as well as BS01701 and PHE14 are necessary for the elucidation of their abilities to discriminate small ester chemicals and also importantly, reverse stereoselectivities.

In conclusion, microbial esterase WDEst9 is a very promising green biocatalyst in the asymmetric synthesis of a great variety of chiral chemicals represented by L-methyl lactate for the preparation of functional materials and pharmaceuticals. Additionally, our work related to the enzymatic kinetic resolutions using esterases WDEst9, DAEst6 and WDEst17 from *Dactylosporangium* opens the door for the development of both industrial enzymes and biocatalysts from the genus *Dactylosporangium*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Arpigny JL, Jaeger KE. 1999. Bacterial lipolytic enzymes: classification and properties. Biochem J. 343:177–183.

Auras R, Lim LT, Selke SEM, Tsuji H. 2010. Mechanical properties. In: Grossman RF, Nwabunma D, editors. Poly(lactic acid): synthesis, structures, properties, processing, and application. Hoboken (NJ): John Wiley & Sons, Inc.

Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem. 72:248–254.

Chen CS, Fujimoto Y, Girdaukas G, Sih CJ. 1982. Quantitative-analyses of biochemical kinetic resolution of enantiomers. J Am Chem Soc. 104:7294–7299.

Choi YJ, Miguez CB, Lee BH. 2004. Characterization and heterologous gene expression of a novel esterase from Lactobacillus casei CL96. Appl Environ Microbiol. 70: 3213–3221.

Chu XM, He HZ, Guo CQ, Sun BL. 2008. Identification of two novel esterases from a marine metagenomic library derived from South China Sea. Appl Microbiol Biotechnol. 80:615–625.

Datta R, Henry M. 2006. Lactic acid: recent advances in products, processes and technologies – a review. J Chem Technol Biotechnol. 81:1119–1129.

De Santi C, Ambrosino L, Tedesco P, Zhai L, Zhou C, Xue Y. 2015. Identification and characterization of a novel salt-tolerant esterase from a Tibetan glacier metagenomic library. Biotechnol Prog. 31:1442–1442.

De Simone G, Galdiero S, Manco G, Lang D, Rossi M, Pedone C. 2000. A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase. J Mol Biol. 31: 1442–1442.

Deng D, Zhang Y, Sun AJ, Hu YF. 2016. Functional characterization of a novel *Dactylosporangium* esterase and its utilization in the asymmetric synthesis of (*R*)-methyl mandelate. Appl Biochem Biotechnol. 180:228–247.

Ewis HE, Abdelal AT, Lu CD. 2004. Molecular cloning and characterization of two thermostable carboxyl esterases from Geobacillus stearothermophilus. Gene. 329:187–195.

Fang Z, Li J, Wang Q, Fang W, Peng H, Zhang X, Xiao Y. 2014. A novel esterase from a marine metagenomic library exhibiting salt tolerance ability. J Microbiol Biot. 24: 771–780.

Gao C, Qiu JH, Li JC, Ma CQ, Tang HZ, Xu P. 2009. Enantioselective oxidation of racemic lactic acid to D-lactic acid and pyruvic acid by Pseudomonas stutzeri SDM. Bioresource Technol. 100:1878–1880.

Gao KL, Song QX, Wei DZ. 2006. Coupling of enantioselective biooxidation of DL-1,2-propanediol and bioreduction of pinacolone via regeneration cycle of coenzyme. Appl Microbiol Biotechnol. 71:819–823.

Garlotta D. 2001. A literature review of poly(lactic acid). J Polym Environ. 9:63–84.

Hasegawa S, Azuma M, Takahashi K. 2008. Stabilization of enzyme activity during the esterification of lactic acid in hydrophobic ethers and ketones as reaction media that are miscible with lactic acid despite their high hydrophobicity. Enzyme Microb Tech. 43:309–316.

Huang JL, Zhang Y, Hu YF. 2016. Functional characterization of a marine Bacillus esterase and its utilization in the stereo-selective production of D-methyl lactate. Appl Biochem Biotechnol. 180:1467–1481.

Jeon JH, Lee HS, Kim JT, Kim S-J, Choi SH, Kang SG, Lee J-H. 2012. Identification of a new subfamily of salt-tolerant esterases from a metagenomic library of tidal flat sediment. Appl Microbiol Biotechnol. 93:623–631.

Jiang X, Xu X, Huo Y, Wu Y, Zhu X, Zhang X, Wu M. 2012. Identification and characterization of novel esterases from a deep-sea sediment metagenome. Arch Microbiol. 194: 207–214.

Ko K-C, Rim S-O, Han Y, Shin BS, Kim G-J, Choi JH, Song JJ. 2012. Identification and characterization of a novel cold-adapted esterase from a metagenomic library of mountain soil. J Ind Microbiol Biotechnol. 39:681–689.

Kricheldorf HR. 2001. Syntheses and application of polylactides. Chemosphere. 43:49–54.

Liang JY, Zhang Y, Sun AJ, Deng D, Hu YF. 2016. Enantioselective resolution of (±)-1-phenylethanol and (±)-1-phenylethyl acetate by a novel esterase from Bacillus sp. SCSIO 15121. Appl Biochem Biotechnol. 178: 558–575.

Ma K, Maeda T, You H, Shirai Y. 2014. Open fermentative production of L-lactic acid with high optical purity by thermophilic Bacillus coagulans using excess sludge as nutrient. Bioresource Technol. 151:28–35.

Ohara H, Yamamoto M, Onogi A, Hirao K, Kobayashi S. 2011. Optical resolution of *n*-butyl D-and L-lactates using immobilized lipase catalyst. J Biosci Bioeng. 111:19–21.

Ohara H, Doi U, Otsuka M, Okuyama H, Okada S. 2001. Development of industrial production of poly-L-lactate from glucose. Seibutsu-kogaku kais. 79:142–148.

Richard G, Nott K, Nicks F, Paquot M, Blecker C, Fauconnier ML. 2013. Use of lipases for the kinetic resolution of lactic acid esters in heptane or in a solvent free system. J Mol Catal B-Enzym. 97:289–296.

Sakai K, Taniguchi M, Miura S, Ohara H, Matsumoto T, Shirai Y. 2004. Making plastics from garbage: a novel process for poly-L-lactate production from municipal food waste. J Ind Ecology. 7:63–74.

Shao H, Xu L, Yan YJ. 2013. Isolation and characterization of a thermostable esterase from a metagenomic library. J Ind Microbiol Biotechnol. 40:1211–1222.

Su W, Chang ZY, Gao KL, Wei DZ. 2004. Enantioselective oxidation of racemic 1,2-propanediol to D-(−)-lactic acid by *Gluconobacter oxydans*. Tetrahedron: Asymmetr. 15: 1275–1277.

Tashiro Y, Kaneko W, Sun Y, Shibata K, Inokuma K, Zendo T, Sonomoto K. 2011. Continuous D-lactic acid production by a novelthermotolerant Lactobacillus delbrueckii subsp. lactis qu 41. Appl Microbiol Biotechnol. 89:1741–1750.

Wang YL, Xu YK, Zhang Y, Sun AJ, Hu YF. 2018. Functional chacterization of salt-tolerant microbial esterase WDEst17

and its use in the generation of optically pure ethyl (*R*)-3-hydroxybutyrate. Chirality. 30:769–776.

Wang YL, Zhang Y, Sun AJ, Hu YF. 2016. Characterization of a novel marine microbial esterase and its use to make D-methyl lactate. Chinese J Catal. 37:1396–1402.

Yáñez R, Belén Moldes A, Alonso JL, Parajó JC. 2003. Production of D(−)-lactic acid from cellulose by simultaneous saccharification and fermentation using Lactobacillus coryniformis subsp. torquens. Biotechnol Lett. 25: 1161–1164.