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Research Article
Cloning, Expression and Characterization of a Mesophilic Catechol 1,2-dioxygenase from *Rhodococcus ruber* OA1

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Abstract

Background and Objective: Salicylic Acid (SA) is widely used in medicine and food. *Rhodococcus ruber* OA1 utilizes salicylate as the sole carbon and energy source for growth and catechol 1,2-dioxygenase (C12O) is detected in *R. ruber* OA1 grown on salicylate. However, C12O has not previously been physiologically or biochemically characterized in *R. ruber*. The aim of this study was to characterize C12O from *R. ruber* OA1 (OA1-C12O). **Methodology:** The catechol 1,2-dioxygenase gene (*cat*A) from *R. ruber* OA1 (OA1-*catA*) was cloned into the *pEASY*-E1 vector to obtain the recombinant plasmid *pEASY*-E1-*catA* and C12O was expressed in *Escherichia coli* BL21 (DE3). The heterologously expressed OA1-C12O was purified and its physiological and biochemical characteristics were further studied. **Results:** Based on the phylogenetic analysis of *catA* gene, it was found that OA1-*catA* clustered with other *catA* genes from Gram-positive bacteria including *Nocardia* sp. C-14-1, *Streptomyces ghanaensis* ATCC 14672 and *Amycolatopsis orientalis* HCCB10007. After the expression and enzymatic characterization of C12O, it was revealed that the expressed OA1-C12O had the ability to degrade catechol to cis,cis-muconic acid with a specific enzyme of 231.4 U mg^{-1} protein. The optimal reaction conditions of OA1-C12O were 25°C and pH 7.0. Besides, Mn^{2+} could increase the activity of OA1-C12O, while Mg^{2+} and NH_4^+ inhibited its activity. **Conclusion:** In this study the C12O from *R. ruber* OA1 was successfully expressed in *E. coli* BL21 (DE3) for the first time and its catalytic characteristics were explored in detail.

Key words: *Rhodococcus*, catechol 1,2-dioxygenase, heterologous expression, enzyme activity, physiological-biochemical characteristics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salicylic Acid (SA) is an important pharmaceutical compound used in the production of analgesics such as aspirin, anti-tuberculosis drugs such as sodium aminosalicylate and as an additive in some foods and cosmetics[1,2]. Due to the extensive use of SA, it has polluted the soil and water in the environment[3,4]. The SA is also an intermediate metabolite in the degradation of polycyclic aromatic hydrocarbons (PAHs)[5]. The SA degradation pathway has been studied in many different organisms. In the classical pathway, SA is converted by salicylate 1-hydroxylase (S1H) to form catechol (1,2-dihydroxybenzene)[6].

Catechol is not only an intermediate metabolite in SA biodegradation but is also an important industrial raw material in chemical and pharmaceutical production[7]. Catechol is toxic to some fungi[8], animals[9] and humans[10,11]. Catechol degradation is catalyzed by catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) to form cis cis-muconic acid and hydroxymuconic-semialdehyde, respectively[5]. C12O is a Fe(III) containing enzyme[12], which catalyzes cleavage of the catechol ring at the 1,2-(*ortho*) position to form cis, cis-muconic acid[13]. So far, *catA* from many different organisms, including *Pseudomonas putida* strain N6[13], *Rhodococcus opacus* 1CP[12], *Geobacillus* sp. G27[14], *Sphingomonas xenophaga* QYY[15] and *Acinetobacter* sp. DS002[16] have been cloned and expressed in *Escherichia coli*. However, the physiological and biochemical characteristics of C12O proteins have not been well studied.

Rhodococcus ruber OA1 is an aromatic compound degrading strain that was isolated from a pharmaceutical wastewater treatment plant in Zibo, China and has been deposited in China Center for Type Culture Collection (CCTCC) under the accession number CCTCC AB2015073. *Rhodococcus ruber* OA1 could utilize salicylate, benzoate, naphthalene, phenol and catechol as sole carbon and energy sources for growth. When grown on salicylate as the sole carbon source, OA1 exhibited C12O enzymatic activity (with a specific activity of 5.32 U mg^{-1}) but C23O activity was not detected. It implied that opening of the catechol ring in the process of salicylate degradation might happen at 1,2-(*ortho*) position in strain OA1. In order to study and exploit the enzyme that catalyzes opening of the catechol ring at the 1,2-(*ortho*) position in *R. ruber* OA1, a gene encoding C12O (*catA*) was cloned from strain OA1 and expressed in *E. coli* BL21 (DE3) cells according to referrence[17]. Then, the enzyme catalytic properties were further investigated. The study will contribute to the understanding of C12O in *Rhodococcus* and its use in the decontamination of environmental pollutants.

MATERIALS AND METHODS

Bacterial strains and plasmid: *Rhodococcus ruber* strain OA1 (CCTCC AB2015073) was used in this study as a source of the catechol 1,2-dioxygenase gene (*catA*). *Escherichia coli* Trans1-T1 (TransGen biotech, China) and *E. coli* BL21 (DE3) (TransGen biotech, China) were used to propagate plasmids and express target genes, respectively. The expression vector pEASY-E1 (TransGen biotech, China) carrying a C-terminal His6-tag was used to clone and express *catA*.

Cloning and sequencing of *cat*A from *R. ruber* OA1: *Rhodococcus ruber* strain OA1 was cultured in LB broth at 37°C for 2 days and genomic DNA was extracted using the Bacterial gDNA Kit (BIOMIGA, China) for use as a PCR template. The primers used in this study were designed with Primer Premier 5 based on the genome sequence of strain OA1 (GenBank accession number JXXO01000000). The *cat*A primer sequences were as follows: Forward primer 5′-ATGACCACCACCGAAAACCCC-3′ and reverse primer 5′-TCAGGCCTCGGGGTCGAGC-3′. The *cat*A gene was amplified by PCR using TransStart FastPfu DNA polymerase (TransGen biotech, China). The PCR products were separated by electrophoresis and finally purified by Gel/PCR Extraction Kit (BIOMIGA, China). The purified products were sequenced by Biosune Biotechnology Co., Ltd, Shanghai.

Sequence analysis of *catA*: Clustal 1.83[18] was used to perform a multiple alignment from which a phylogenetic tree of *catA* sequences was constructed using the Maximum Likelihood method embedded in MEGA 6[19]. The molecular weight and isoelectric point (pI) of the deduced OA1-C12O protein were predicted with ExPASy Compute pI/Mw server (http://web.expasy.org/compute_pi/). The nucleotide sequences of *catA* were aligned using DNAMAN software.

Construction of recombinant plasmid: The purified PCR products were ligated into the pEASY-E1 vector and transformed into *E. coli* Trans1-T1 competent cells according to the manufacturer's instructions. A positive colony harboring the recombinant plasmid was identified by colony PCR[20,21]. The recombinant plasmid was isolated and its sequence was confirmed by DNA sequencing. The resulting clone was designated as pEASY-E1-catA, which was subsequently transformed into *E. coli* BL21 (DE3) competent cells.

Expression and purification of C12O: The recombinant *E. coli* BL21 (DE3) strain harboring *pEASY-E1-catA* was

cultured in 100 mL LB broth and induced with 0.5 mM IPTG (Solarbio, China). A control strain carrying the empty vector (pEASY-E1) was cultured in paralled. After cultivation, the cells were broken by ultrasonication using an Ultrasonic Cell Disruptor (SONICS, USA), following centrifugation and washing. The lysate was clarified by centrifugation at 10000×g for 20 min and the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant C12O was purified using Ni-NTA resin (Sagon, China) and analyzed by SDS-PAGE.

Colorimetric reaction of catechol with metal ions: A colorimetric reaction system containing 1 mM catechol and right amount of purified protein in 0.1 M sodium phosphate buffer (pH 7.4) was used to determine the presence of catechol by reaction with Cu^{2+} and Fe^{3+} ions, which were added to the reaction systems at a final concentration of 1 mM. The reaction was carried at 25°C and cells containing the empty vector were used as a control.

Resting cells harvesting and biotransformation of catechol: In order to harvest the resting cells, the recombinants were collected by centrifugation after induction. Next, the pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.4) and cultured at 37°C for 6 h then stored at 4°C[22]. A resting cells reaction system containing 1 mM catechol and proper resting cells was incubated in a water bath at 25°C for 2 h. The control experiments were performed using cells carrying the empty vector. After the reaction, the sample was acidified to pH 2.0 with HCl and extracted with ethyl acetate. The extract was dried over anhydrous Na_2SO_4 followed by drying at 40°C using a rotary vacuum evaporator, then dissolved in 1 mL methanol.

The sample was assayed by High Performance Liquid Chromatography (HPLC) using a reversed-phase C18 column (5 μm, 4.6×150 mm), HPLC pump system (Waters 600 Controller, USA) and Photodiode Array Detector (Waters 2996 Photodiode Array Detector, USA). Eluent A (1% acetic acid in water) and eluent B (1% acetic acid in methanol) were used as the mobile phases. The detection wavelength was 260 nm and the elution conditions are indicated in Table 1.

Enzyme activity assay of C12O: The enzyme activity of C12O was assayed at 25°C by measuring an increase in the absorbance of cis,cis-muconic acid at 260 nm[14,17]. The assay system contained 1 mM catechol and right amount of purified protein in 0.1 M sodium phosphate buffer (pH 7.4). The reaction was started by addition a suitable amount of enzyme.

Table 1: HPLC gradient elution conditions used in the detection of resting cells reaction product

Time (min)	Flow rate (mL min^{-1})	Eluent A (%)	Eluent B (%)
0	0.8	90	10
30	0.8	50	50
35	1.0	50	50
55	1.0	10	90
60	0.8	90	10
65	0.8	90	10

HPLC: High performance liquid chromatography

Total protein concentration was determined using the Bradford assay[23]. One unit of enzyme activity is defined as the amount of enzyme that produced 1 nmol of cis,cis-muconic acid per minute at 25°C. The specific C12O activity was reported as units per milligram of protein[24].

Optimal pH and temperature of C12O: The optimal pH of the enzyme was determined by measuring the activity of C12O at 25°C over the pH range of 4.0-9.0. The buffers used in this experiment were as follows: 0.1 M HAc-NaAc buffer (pH 4.0-5.5), 0.1 M sodium phosphate buffer (pH 6.0-8.0), 0.1 M Tris-HCl buffer (pH 8.5 to 9.0). The effect of temperature was determined by measuring the activity of C12O over the temperature range 5-45°C in 0.1 M sodium phosphate buffer (pH 7.4).

Ions effect on the activity of C12O: The effect of ions (K^+, Mg^{2+}, Mn^{2+}, NH_4^+ and Zn^{2+}) was determined by assaying the enzyme activity at 25°C in 0.1 M sodium phosphate buffer (pH 7.4). The final concentrations of ions in the enzymatic reaction mixture were 1 mM.

Nucleotide sequence accession number: The nucleotide sequence determined in this study was deposited in the GenBank database under the accession number KY619084.

RESULTS

Cloning and sequence analysis of *catA* A DNA fragment was obtained by PCR of approximately 1 kb in size (Fig. 1). Subsequent purification and sequencing revealed the fragment was 852 nucleotides long and encoded a polypeptide of 283 amino acids, with a predicted pI and molecular weight of 4.7 and 31.7 kDa, respectively. Nucleotide sequence alignment (Fig. 2) indicated that the DNA fragment was identical to the *catA* target gene in strain OA1 and was highly similar to *catA* from other bacteria. The polypeptide encoded by the DNA fragment had 68% amino acid sequence identity with C12O of *R. opacus* 1CP (CAA67941.1). Therefore, the DNA fragment represented *catA* from *R. ruber* OA1.

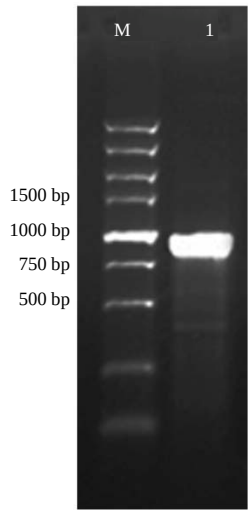

Fig. 1: PCR-amplification of OA1-*catA*
Lane M: D5000 DNA ladder, lane 1: OA1-*catA* fragment

Based on a phylogenetic analysis of *catA* (Fig. 3), it was found that OA1-*catA* clustered with similar genes from other Gram-positive bacteria, including *Nocardia* sp. C-14-1, *Streptomyces ghanaensis* ATCC 14672 and *Amycolatopsis orientalis* HCCB10007. Among these, OA1-*catA* was most closely related to the *catA* from *Nocardia* sp. C-14-1.

Expression and purification of the C12O: The expression of C12O in *E. coli* BL21 (DE3) harboring pEASY-E1-catA was analyzed by SDS-PAGE. By comparing the SDS-PAGE image of the recombinant and control strains, a highly expressed band between the 27 and 35 kDa marker bands was found in cells containing pEASY-E1-catA (Fig. 4a, lane 2). Following purification, the purified protein was analyzed by SDS-PAGE and the results showed that the recombinant protein appeared as a single band at the predicted molecular weight (Fig. 4b, lane 1).

Color reaction of catechol with metal irons: Phenol moieties reversibly react with Fe^{3+} and Cu^{2+} to produce violet and brown materials, respectively[25,26]. In this study, Fe^{3+} and Cu^{2+} were added to the reaction mixture respectively and both were colorless before the start of the reaction. By comparing the reaction color before and after the addition of recombinant OA1-C12O, there was no significant color change in the experimental group but there was a color change in the control group (Fig. 5). The color of the control reaction changed from colorless to brown (Fig. 5a) and violet (Fig. 5b), meaning that the occurrence of the coloration can be prevented by OA1-C12O. Therefore, it was proposed that OA1-C12O may be able to degrade catechol into other substances in the presence of Fe^{3+} and Cu^{2+}.

Resting cells reaction and HPLC analysis: To explore the enzymatic transformation products of C12O in the presence of catechol, resting cell reaction system was established and analyzed by HPLC (Fig. 6). By comparing the chromatograms of the control (Fig. 6b) and the experimental group (Fig. 6c), a differential peak at around 11 min was found in the chromatogram of the experimental group, which was consistent with the standard peak. Therefore, the results revealed that C12O can catalyze the degradation of catechol to form cis, cis-muconic acid.

Enzyme activity and catalytic characteristics: The specific enzyme activity of OA1-C12O was assayed under the conditions of pH 7.4 at 25°C and was found to be 231.4 U $mg^{-}1$. The optimum temperature and pH experiments (Fig. 7a, b) showed that the optimum reaction temperature and pH of OA1-C12O were 25°C and pH 7.0, respectively. Furthermore, the relative enzyme activities in the presence of different ions are shown in Fig. 7c. This indicated that Mn^{2+} enhanced enzyme activity while K^{+}, Mg^{2+} and NH_4^{+} inhibited enzyme activity. At the same time, the enzyme activity was not significantly changed in the presence of Zn^{2+}.

DISCUSSION

From the nucleotide sequence alignment result of *catA* genes (Fig. 2), it could be seen some regions of *catA* genes shared identities over than 80% (area marked with red boarder in Fig. 2), which implied that these highly conserved DNA sequences might encoding the conserved domain of C12O and control the main functions of the enzyme.

The optimal reaction pH of OA1-C12O was pH 7.0 (Fig. 7a). It was similar to that of C12O from *Rhodococcus* sp. NCIM 2891[27], *P. putida* N6[13] and *Pseudomonas aeruginosa* TKU002[28] but a little lower than others such as *S. xenophaga* QYY[15], *Acinetobacter* sp. Y64[17] and *Acinetobacter radioresistens* S13[29]. The optimal reaction pH of C12O from these strains are all around pH 7.5. Based on the study of C12O from *Candida tropicalis* JH8[30], it was supposed that the recombinant protein was unstable under acidic or alkaline conditions and stable in the neutral conditions.

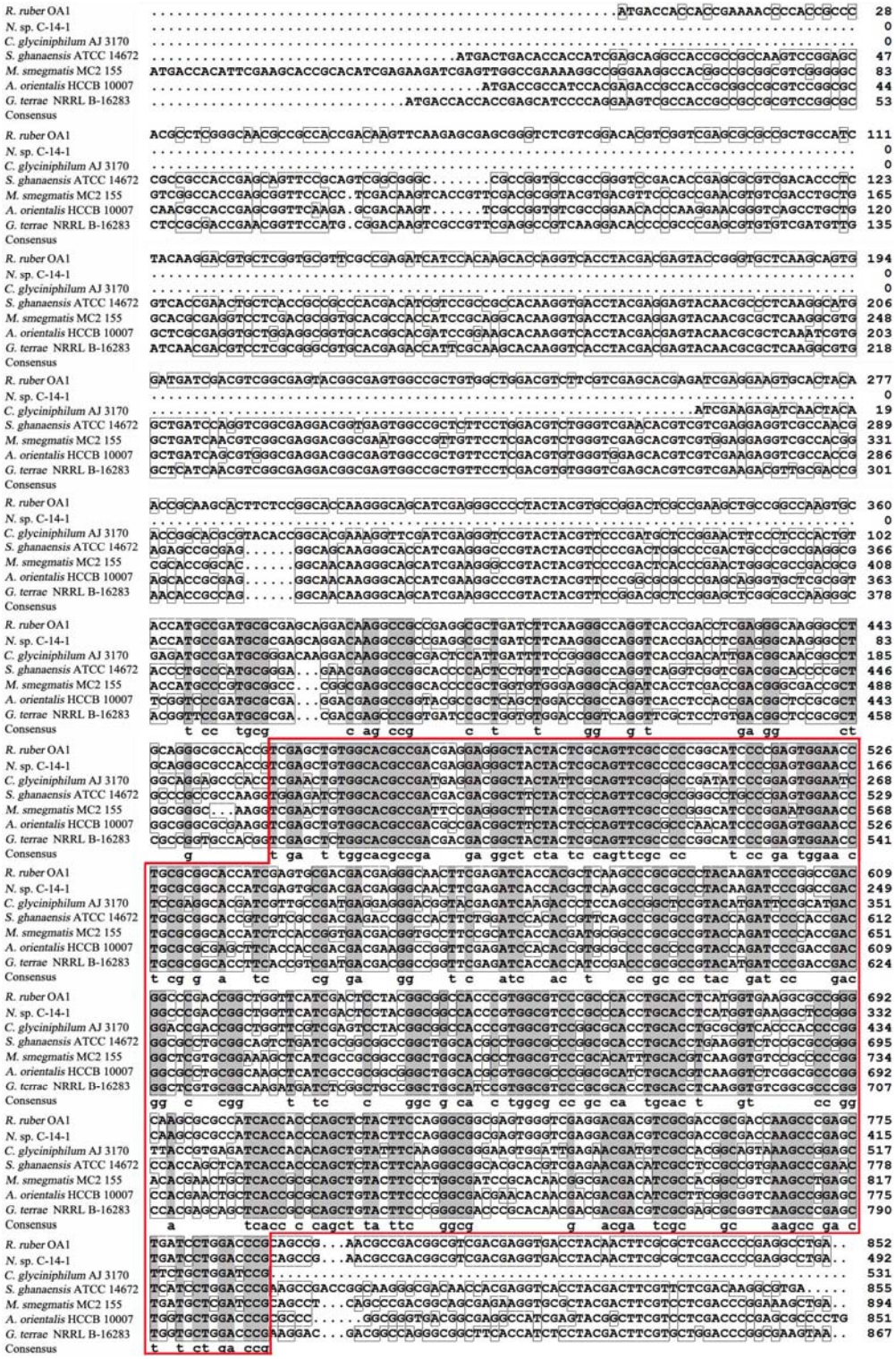

Fig. 2: Alignment of nucleotide sequences of *catA*

The alignment was performed by software of DNAMAN. The GenBank accession numbers of these sequences are as follows: KY619084 for *Rhodococcus ruber* OA1; DQ267826.1 for *Nocardia* sp. C-14-1; CP006842.1 for *Corynebacterium glyciniphilum* AJ 3170; NZ_DS999641.1 for *Streptomyces ghanaensis* ATCC 14672; NC_008596.1 for *Mycobacterium smegmatis* MC2 155; NC_021252.1 for *Amycolatopsis orientalis* HCCB10007; NZ_JNXA01000009.1 for *Gordonia terrae* NRRL B-16283. The highly similar area has been marked with red boarder

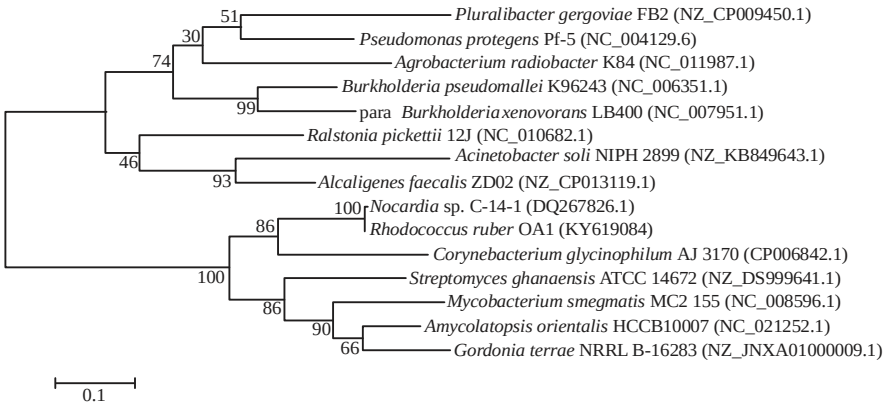

Fig. 3: Phylogenetic analysis of *catA* from *R. ruber* OA1 and other related species based on their nucleotide sequences
The numbers at the nodes are bootstrap confidence levels (%) from 1000 replicates; the scale bars represent 0.1 substitution/site

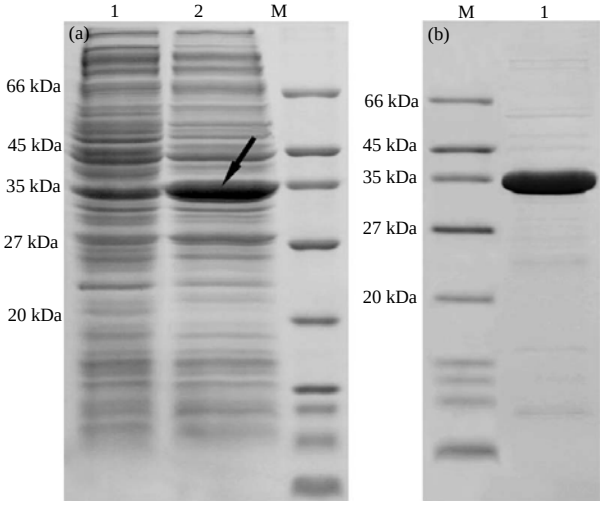

Fig. 4(a-b): SDS-PAGE analysis of heterologous expressed C12O, (a) Analysis of crude protein, Lane M: Protein molecular weight marker, lane 1: Soluble protein from cells containing the empty vector, lane 2: Soluble protein from cells containing pEASY-E1-catA, (b) Analysis of purified protein, lane M: Protein molecular weight marker, lane 1: The purified C12O protein

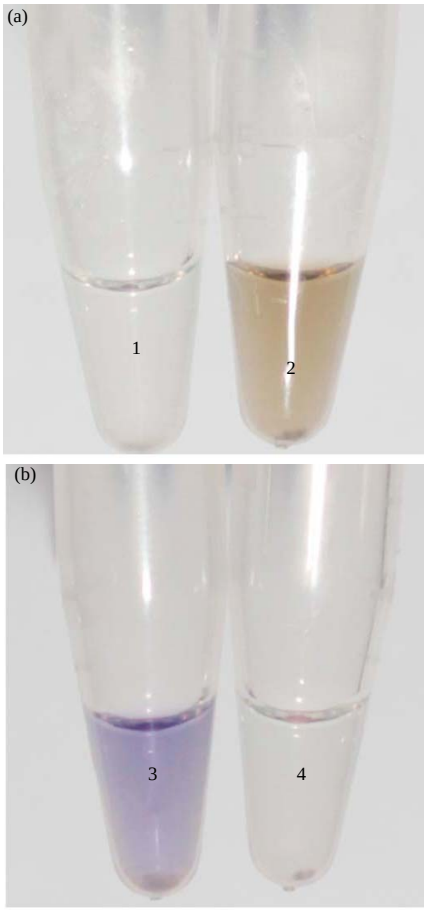

Fig. 5(a-b): Color of the reaction mixture of the control and experimental groups, (a) The effect of Fe^{3+}. 1: Experimental group and 2: Control group, (b) The effect of Cu^{2+}. 3: Control group, 4: Experimental group

Until now, two major categories of C12O enzymes have been described according to their optimal reaction temperatures: Thermophilic dioxygenases and mesophilic dioxygenases. For instance, the optimal temperature of thermophilic dioxygenases from *Geobacillus* sp. G27[14] and *S. xenophaga* QYY[15] were 55 and 50°C, respectively. However, the optimal temperature of C12O in some strains was

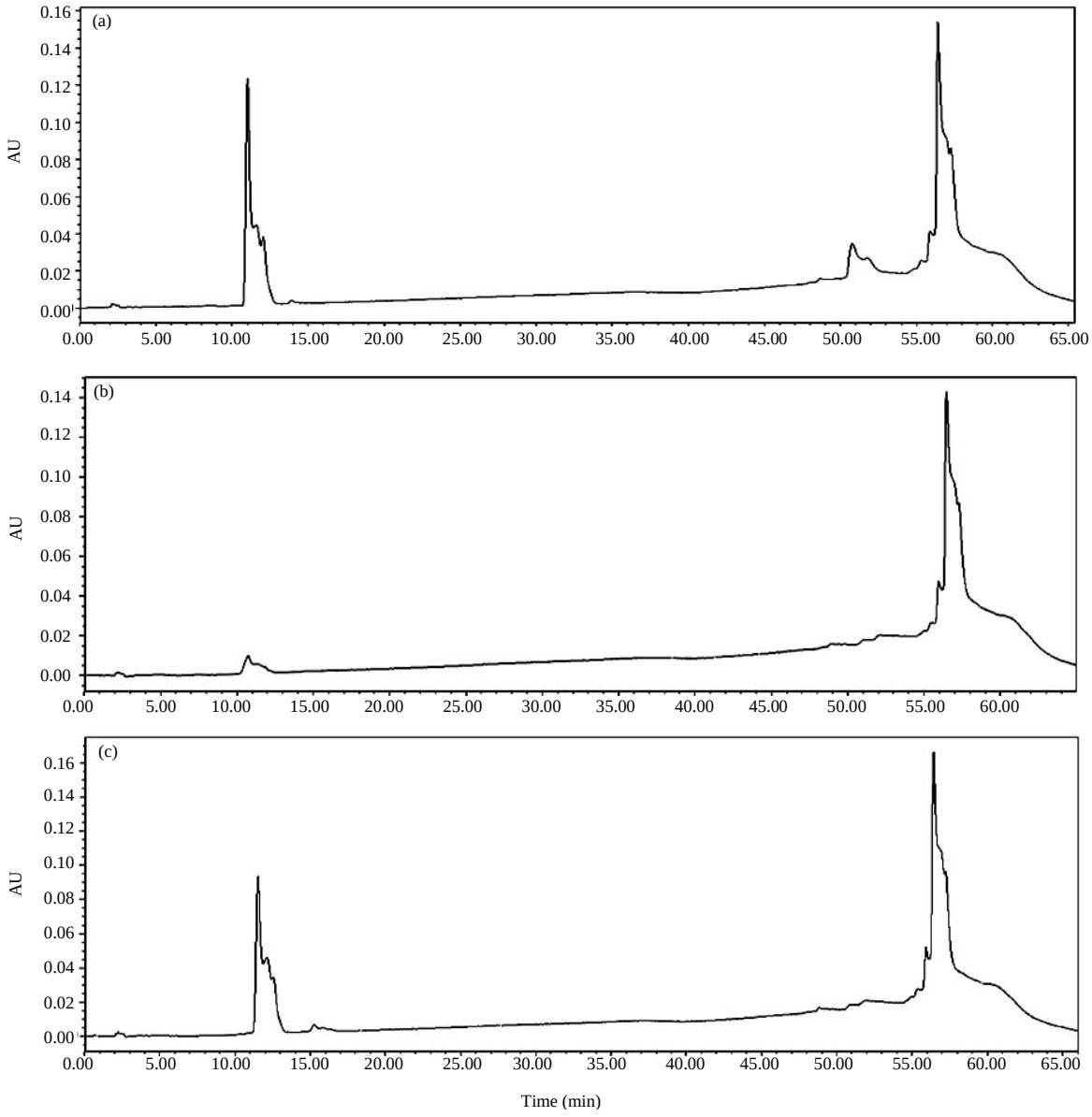

Fig. 6(a-c): HPLC chromatograms for the resting cells reactions, (a) Chromatogram of cis, cis-muconic acid standard dissolved in methanol and the peak appeared at around 11 min, (b) Chromatogram of the control group and (c) Chromatogram of the experimental group, the product peak appeared at a retention time that was consistent with the standard

found to be approximately 35°C[13,17,31], making them mesophilic dioxygenases. The C12O of *R. ruber* OA1 is a mesophilic dioxygenase but its optimal temperature is lower than that of other known C12O enzymes and this may be due to the relatively low temperature of the habitat where strain OA1 was isolated. Low optimum temperature is conducive to the application of the enzyme and its host cell in the decontamination of the environment in middle and high-latitude areas.

By studying the effects on relative enzyme activities of different ions, it was shown that Mn^{2+} enhanced the relative enzyme activity of OA1-C12O by 4 times, while Mn^{2+} slightly inhibited the activity of C12O from *S. xenophaga* QYY[15] and *P. aeruginosa* TKU002[28]. Besides, Mg^{2+} and K^{+} inhibited the relative activity of OA1-C12O by 50% while they enhanced the activity of C12O from *S. xenophaga* QYY[15]. Interestingly, Zn^{2+} had no significance influence on the activity of OA1-C12O, while the activity of C12O from *S. xenophaga* QYY[15] was

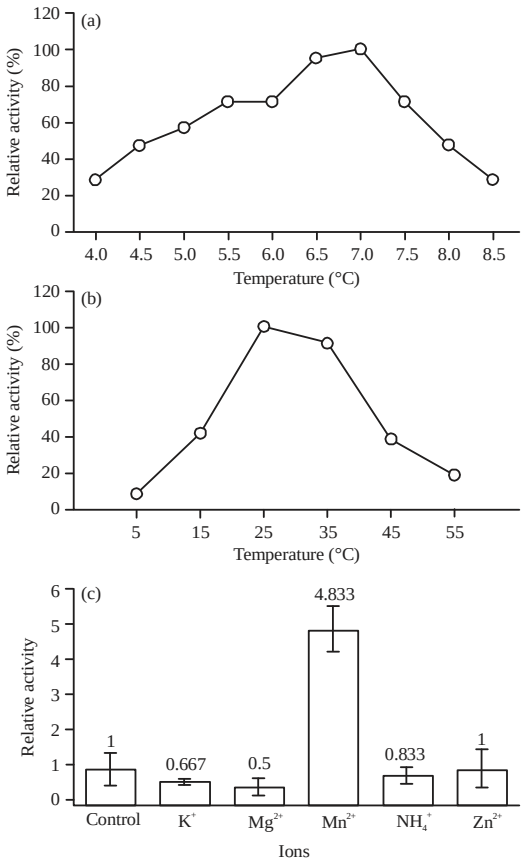

Fig. 7(a-c): Effect of environmental factors on enzyme activity, (a) Effect of pH values on the relative activity of C12O, (b) Effect of reaction temperature on the relative activity C12O and (c) Effect of positive ions on the relative activity of C12O

severely inhibited by Zn^{2+}. These indicated that although C12O from different genera could cleavage catechol ring at the 1,2-(ortho) position but the physic-chemical characteristics of the enzyme were different in some aspects.

CONCLUSION

In conclusion, a mesophilic catechol 1,2-dioxygenase from *R. ruber* OA1 was cloned and expressed in *E. coli* BL21 (DE3), which had an activity of 231.4 U mg^{-1} protein at pH 7.4 and 25°C. The catalytic characteristics of the enzyme were further investigated.

SIGNIFICANCE STATEMENTS

Catechol is a phenolic compound that is harmful to animals and human beings. Enzyme catalyzing catechol oxidation is worthy to be studied. In this study, a mesophilic catechol 1,2-dioxygenase from *Rhodococcus ruber* OA1 was expressed, then the physical and biochemical characteristics of the enzyme were investigated in detail. The study was helpful for potential application of the enzyme and its host strain.

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