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Short Communication

Cloning and expression of a novel *trans*-anethole oxygenase gene from *Paraburkholderia* sp. MR185

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***trans*-Anethole oxygenase (TAO) is the key enzyme responsible for the oxidation of *trans*-anethole to *p*-anisaldehyde. A strain, *Paraburkholderia* sp. MR185, was isolated from soil in Yulin star anise-planting regions using *trans*-anethole as a sole carbon source and a gene which encodes a protein with high similarities to a hypothetical protein of *Paraburkholderia* sp. MM5384-R2 which shows 61.27% identies with TAO from *Pseudomonas putida* JYR-1 was cloned and sequenced. The gene, *tao*, was expressed in *E. coli* cells and its protein product was purified by affinity chromatography through regenerated amorphous cellulose (RAC). SDS-PAGE analysis indicated a clear band of recombinant protein TAO, and its molecular weight, 38.3 kDa, was consistent with the theoretical value. Its enzyme activity of producing *p*-anisaldehyde from *trans*-anethole was detected by DNPH (2,4-dinitrophenylhydrazine) chromogenic reaction and HPLC, and the specific activity of TAO reached 3.93 U/mg protein. Immobilized TAO on RAC was used to catalyze the production of *p*-anisaldehyde from *trans*-anethole, and the enzyme retained more than 60% of its initial activity after 10 uses. This is the first report on *Paraburkholderia* TAO.**

Key Words: *E. coli*; *Paraburkholderia*; *trans*-anethole; *p*-anisaldehyde; *trans*-anethole oxygenase

Star anise oil is a kind of essential oil distilled from star anise (*Illicium verum*) fruits or branches. The major component of star anise oil is *trans*-anethole (*p*-methoxypropenylbenzene), which can be converted into a value-added flavor substance, *p*-anisaldehyde. Currently, chemical synthesis dominates production of *p*-anisaldehyde, which has brought about some problems, such as difficulty of product separation, strict operation condition, and serious environmental pollution. Furthermore, use of *p*-anisaldehyde produced by chemical method is restricted in food, beverages, and cosmetics by European law. Production of *p*-anisaldehyde by biotechnological method can avoid such problems, and more importantly, *p*-anisaldehyde obtained in this way, according to FDA and European legislation, can be considered natural flavor (Serra et al., 2005), which would be, despite considerably higher prices, more preferred than chemical counterparts.

There have been some attempts to synthesize *p*-anisaldehyde from *trans*-anethole using biotechnological methods, usually referred to as microbial transformation. Some microorganisms capable of utilizing *trans*-anethole as a sole source of carbon have been isolated, including *Arthrobacter* sp. TA13 (Shimoni et al., 2002), *Pseudomonas putida* JYR-1 (Ryu et al., 2005), *Burkholderia* sp. WGB31 (Shen et al., 2014). Among these strains, only one gene, *tao* coding for *trans*-anethole oxygenase catalyzing the oxidation of *trans*-anethole to *p*-anisaldehyde from *Pseudomonas putida* JYR-1, has been cloned (Han et al., 2012a; Han et al., 2012b; Han et al., 2013), and other microbial genes involved in *p*-anisaldehyde formation are still unknown.

To explore novel microbial enzymes involved in synthesis of *p*-anisaldehyde, we screened microorganisms using *trans*-anethole as a sole carbon source from soil in Yulin star anise-planting regions. Strain MR185 was isolated and identified as *Paraburkholderia* according to its 16S rRNA sequence (DDBJ accession number: LC639197). By BLASTP searching, we found a hypothetical protein (WP_184048347) from *Paraburkholderia* sp. MM5384-R2

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which has 61.27% identity with *trans*-anethole oxygenase from *Pseudomonas putida* JYR-1. Based on the coding sequence from *Paraburkholderia* sp. MM5384-R2, two primers P1 and P2 (Table 1) were used to amplify the gene encoding a corresponding protein with genomic DNA of *Paraburkholderia* sp. MR185 as template. PCR product was cloned into pMD19-T (Takara Biomedical Technology, Beijing, China) and sequenced by Sangon Biotech (Shanghai, China). To confirm the 5'- and 3'-end sequences of the cloned ORF, genome walking was carried out by SiteFinding-PCR (Tan et al., 2005), and the sequences of P1, P2 were shown to be identical to the original gene of *Paraburkholderia* sp. MR185. Sequencing resault indicated its deduced protein sequence has 99.71% identity with the hypothetical protein WP_184048347 from *Paraburkholderia* sp. MM5384-R2 (Fig. 1), 61.59% identity with TAO from *Pseudomonas putida* JYR-1, then the gene from strain MR185 was designated as *tao* (DDBJ accession number: LC639198). Other proteins with high identities were all annotated as hypothetical proteins or DUF2236 domain-containing proteins with unknown function.

To obtain pure protein encoded by *tao*, an expression vector, pRPOCDN (Yang et al., 2021) containing stress-induced *rpoS* promoter (Kang et al., 2008), *cbm3* coding for family 3 carbohydrate-binding module from *Clostridium thermocellum* (Hong et al., 2008a), and intein gene *dnaB* (Hong et al., 2008b) was utilized. Using In-Fusion HD cloning kit (Takara Biomedical Technology, Beijing,

Table 1. Oligonucleotides used in this study

Name	Sequence(5'→3')
P1	atgacagaccaagttgagacccac
P2	ctaggacttttgccttttgacgtgc
P3	gccatgggcggccgcatgacagaccaagttgagacccacg
P4	gccctcgaggaattcttaggacttttgccttttgacgtgcc
P5	gcggccgcccatggctcttc
P6	gaattcctcgagggctcttccagatctc

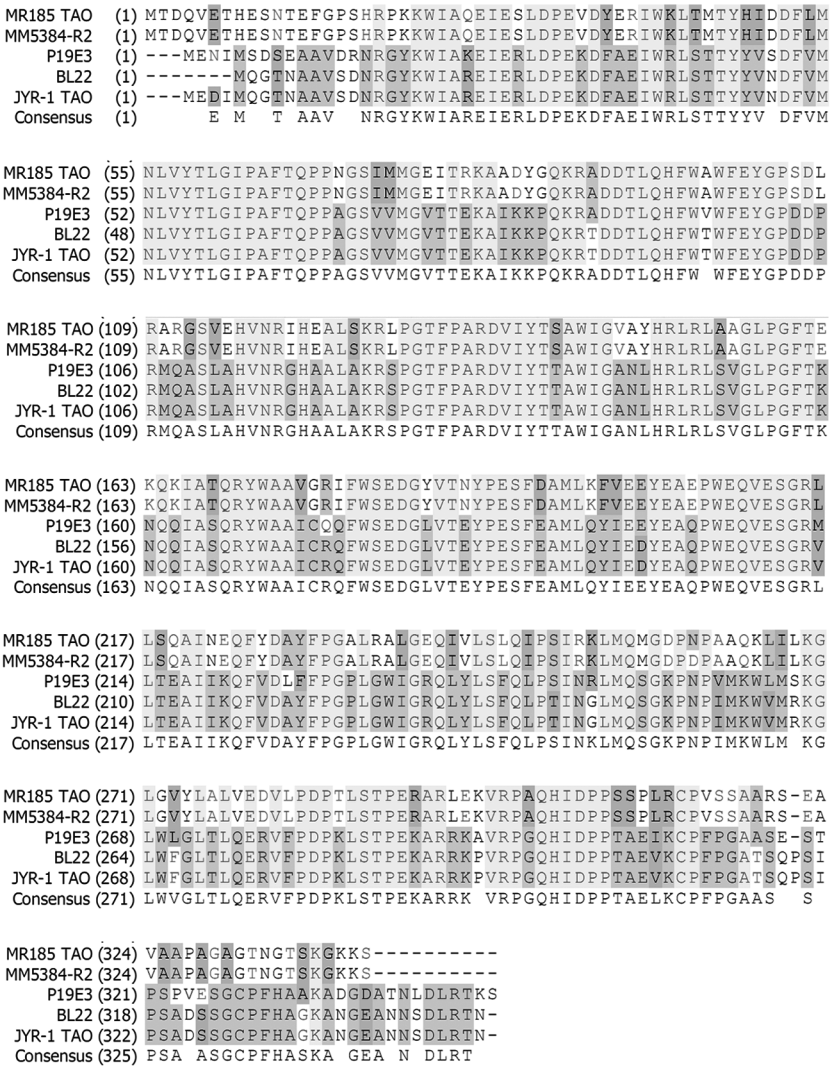

Fig. 1. Protein alignment.

MR185 TAO: TAO from *Paraburkholderia* sp. MR185; MM5384-R2: hypothetical protein from *Paraburkholderia* sp. MM5384-R2; P19E3: DUF2236 domain-containing protein from *Pseudomonas koreensis* P19E3; BL22: hypothetical protein from *Pseudomonas aeruginosa* BL22; JYR-1 TAO: TAO from *Pseudomonas putida* JYR-1.

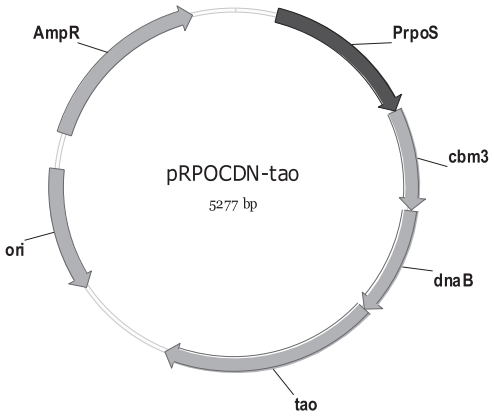

Fig. 2. pRPOCDN-*tao*.

P_{rpoS}: *rpoS* promoter; *cbm3*: coding sequence of family 3 carbohydrate-binding module from *Clostridium thermocellum*; dnaB: coding sequence of Ssp DnaB intein; tao: gene *tao* encoding *trans*-anethole oxygenase from *Paraburkholderia* sp. MR185; *ori*: replication origin; AmpR: ampicillin resistance gene.

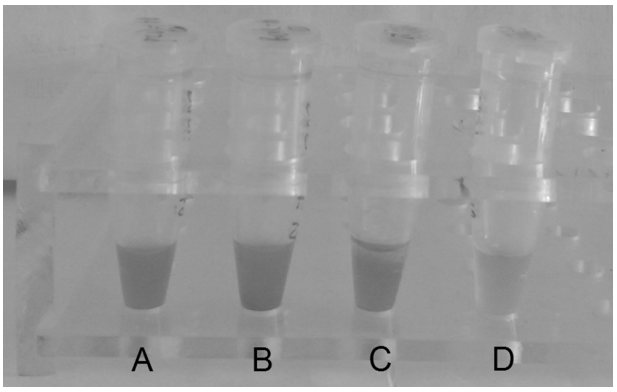

Fig. 4. DNPH color assay.

A. Product solution using purified TAO. B. Product solution using *E. coli* DH5α cells carrying pRPOCDN-*tao*. C. *p*-anisaldehyde solution. D. Product solution using *E. coli* DH5α cells. The chromogenic reaction system composed of 100 µl of sample solution, 100 µl of DNPH solution (2.5 mM 2,4-dinitrophenylhydrazine dissolved in 95% ethanol containing 3% sulfuric acid) was incubated at 30 °C for 30 min.

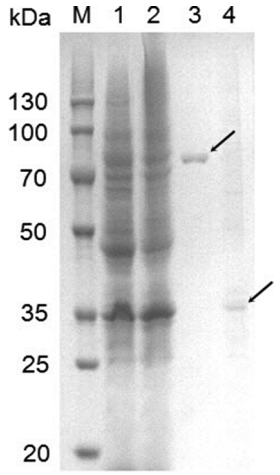

Fig. 3. SDS-PAGE analysis of TAO.

M: protein molecular weight marker; Lane 1: crude cell extract of *E. coli* DH5α harboring pRPOCDN; Lane 2: crude cell extract of *E. coli* DH5α harboring pRPOCDN-*tao*; Lane 3: fusion protein CBM3-DnaB-TAO eluted by ethylene glycol; Lane 4: purified TAO (indicated by an arrow).

China), PCR product of *tao* gene amplified with primers P3, P4 (Table 1) and linearized pRPOCDN amplified with P5, P6 (Table 1) were mixed and transformed into competent cells of *E. coli* DH5α. Recombinant plasmid was extracted form transformant cells, and sequenced to confirm *tao* gene insertion (Fig. 2). *E. coli* DH5α cells carrying the recombinant plasmid pRPOCDN-*tao* were grown in LB medium overnight and harvested by centrifuation and washed. The plasmid pRPOCDN-*tao* contained a stress-induced *rpoS* promoter, which was responsible for sigma factor RpoS expression in *E. coli* cells and was induced by stress conditions, such as osmotic shock, ethanol, nutrient limitation, low pH, and high cell density(Kang et al., 2008). So *E. coli* cells spontaneously expressed recombinant enzyme TAO when growth conditions changed into above mentioned stress, without any human interference. After disruption by ultra-sonication (SCIENTZ, China) at 4 °C, cell lysate was centrifuged at 12000 × *g*, 4 °C for 30 min. Then 15 ml of supernatant was added to a centrifuge tube containing 0.9 g of wet regenerated amorphous cellulose (RAC) prepared as previously described elsewhere(Hong et al., 2008a; Hong et al., 2008b). The mixture was incubated at room temperature for 30 min, and centrifuged at 6000 × *g*, for 20 min to obtain the RAC pellet with the adsorbed fusion protein CBM3-DnaB-TAO. 15 ml of 0.05 M Tris-HCl buffer (pH8.5) was added to remove unadsorbed proteins, flowed out and repeated three times. Finally, the RAC slurry was resuspended in 5 ml of self-cleavage buffer (0.05 M Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH6.5). After incubation at 30 °C for 12 h, intein-mediated cleavage occurred and TAO was released to the supernatant. After centrifugation with Amicon Ultra-4 10K fiter (Millipore), TAO was re-dissolved in 3 ml of Tris-HCl buffer (0.05 M, pH 8.0). SDS-PAGE analysis indicated a clear major band of 38.3 kDa (Fig. 3), which was equivalent to the theoretical molecular weight of TAO. The fusion protein (CBM3-DnaB-TAO) eluted by ethylene glycol from RAC was also shown by SDS-PAGE, which was consistent with its theoretical molecular weight (77 kDa). SDS-PAGE analysis did not suggest obvious differences between crude cell extracts from *E. coli* carrying pRPOCDN and pRPOCDN-*tao*, probably because the strength of *rpoS* promoter was not high enough, which was observed in our previous study (Yang et al., 2021). Biotransformation of *trans*-anethole was conducted in 300 µl of reaction solution containing 100 µl of TAO solution or harvested *E. coli* cells, 5 mM *trans*-anethole (TCI Shanghai, China), 10 mM NADH, 15 µM FAD, 150 mM sodium formate, and 0.15 U formate dehydrogenase from *Candida boidinii* (Macklin, Shanghai, China) at 30 °C for 6 h, and the product *p*-anisaldehyde was detected by a preliminary test method of DNPH (2,4-dinitrophenylhydrazine) color reaction as previouly described elsewhere with minor modification (Liu et al., 2016; Gong et al., 2021). DNPH reacted with *p*-anisaldehyde produced by TAO solution or *E. coli* cells expressing TAO and formed red precipitation, whereas the product solution of *E. coli* cells without TAO still remained yellow (Fig.

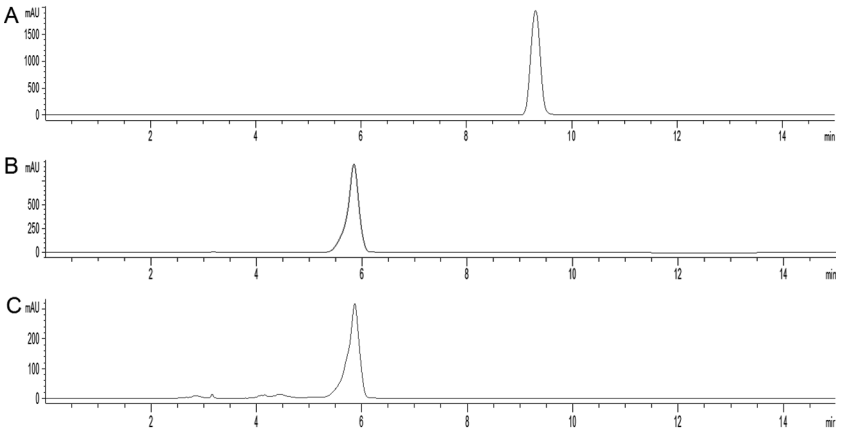

Fig. 5. HPLC analysis of reaction samples.

A. Standard sample of *trans*-anethole. B. Standard sample of *p*-anisaldehyde. C. TAO-catalyzed reaction product.

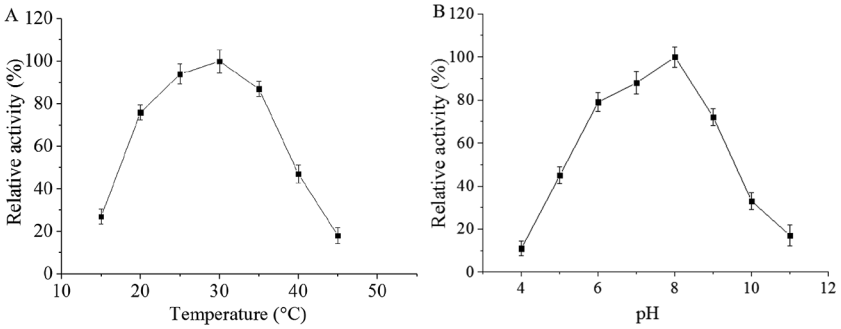

Fig. 6. Effects of temperature and pH on TAO activity.

4). To detect the product by HPLC, 600 μl of methanol was added to terminate the reaction. After centrifugation, the supernatant was filtered through 0.22 μm filters and the concentration of substrate and product were determined by using an Agilent 1260 Infinity LC System equipped with a diode array detector and a reversed phase C18 column (GreatSmart RP18, 5μm particle size, 250 mm × 4.6 mm, Dr. Maisch, Germany). The mobile phase was composed of acetonitrile:water (70:30). The flow rate with an injection volume of 10 μL was 1 ml/min, and the UV wave length was 260 nm with the single sample run time of 15 min. HPLC elution profile indicated that the rentention time of *trans*-anethole and *p*-anisaldehyde were 9.314 min, 5.851 min, respectively. The retention time of TAO-catalyzed reaction product was 5.867 min, which was highly consistent with that of *p*-anisaldehyde, and HPLC analysis implied that *trans*-anethole was almost completely converted to *p*-anisaldehyde by TAO (Fig. 5). Enzyme reaction mixture was incubated at 30 °C for 20 min, and analysis of product was performed as above described. One unit of TAO was defined as the amount of enzyme needed to produce 1 μmol of *p*-anisaldehyde in 1 min. The specific activity of recombinant *E. coli* cell lysate was 1.15 U/mg, and that of purified TAO reached 3.93 U/mg protein. To determine the reaction temperature optimum of TAO, enzyme reaction mixture was incubated at various temperatures for 20 min. At 30 °C the enzyme exhibited highest activity (Fig. 6A). Within a range of pH from 3.0 to 11.0, the maximum enzyme activity was observed at pH8.0 (Fig. 6B). Each measurement was performed in triplicate. When *trans*-anethole in enzyme reaction mixture was substituted with eugenol, isoeugenol, *O*-methyl isoeugenol, cinnamic acid, ferulic acid, and 4-coumaric acid, no oxidization product was detected. Compared to the relatively broad substrate range of TAO from *Pseudomonas putida* JYR-1 which metabolized the compounds containing a 1-propenylbenzene group, with methoxyfunctional groups at either the *para* or *meta* position on the benzene ring (Han et al., 2012a), TAO from *Paraburkholderia* sp. MR185 exhibited extremely high specificity towards *trans*-anethole, even though their amino acid sequences

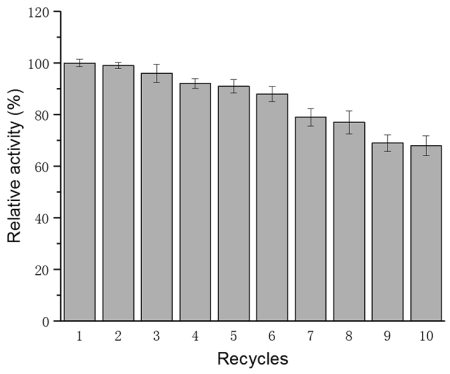

Fig. 7. Reusability of immobilized TAO on RAC.

shared 61.59% identities.

In our previous work, RAC was used as adsorbent for one step purification and immobilization of recombinant protein (Lin et al., 2017). So the RAC with adorbed fusion protein CBM3-DnaB-TAO was tested for its activity of immobilized enzyme. The matrix was resuspended in 5 ml of reaction solution, incubated at 30 °C for 2 h. The product solution was separated from the RAC with immobilized enzyme by centrifugation at 4 °C for 20 min. The RAC matrix was washed with Tris-HCl buffer (0.05 M, pH 8.0) and repeatedly mixed with reaction solution and incubated at 30 °C for 2 h. The oxidation reaction of *trans*-anethole by immobilized enzyme was repeated 10 times and the reaction product was detected by HPLC. In the 1st, 2nd, and 3rd reactions, there was almost no loss in TAO activity (Fig. 7). Nevertheless, the enzyme activity was progressively decreased after 3 recycles, and at the last time, the immobilized TAO retained more than 60% of its initial activity, which demonstrated the potential reusability of TAO immobilized on RAC.

In this study, a novel *trans*-anethole oxygenase gene from *Paraburkholderia* sp. MR185 was investigated for the first time. Since the first report on *trans*-anethole oxygenase (Han et al., 2012a), studies of characterization and directed evolution have been focused on TAOs originated from *Pseudomonas* strains (Han et al., 2013; Wen et al., 2019), and there has been no investigations on other microbial *tao* genes. Although a strain of *Burkholderia* was found to produce anisic acid from anethole, its genes involved in anethole metabolism remain unknown (Shen et al., 2014). According to BLASTp search resaults, TAO has high similarities to DUF2236 domain-containg proteins. There are thousands of proteins in NCBI database containing DUF2236 domain (pfam09995), and some of them are well-characterized rubber oxygenases (Jendrossek et al., 2019; Röther et al., 2016). Nevertheless TAO from *Paraburkholderia* sp. MR185 did not show significant similarities to those oxygenases. Discovery of a new *trans*-anethole oxygenase would facilitate the development of biosynthesis process of natural flavor *p*-anisaldehyde, and immobilization of TAO would be helpful in reducing the bioprocess cost. To further lower production cost, coexpression of TAO and a dehydrogenase for regeneration of NADH and optimization of biotransformation conditions is our future work.

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