

Isolation and Characterization of an L-Amino Acid Oxidase-Producing Marine Bacterium

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ABSTRACT

One marine bacterial strain, R3, has been newly isolated from the intertidal zone of Dinghai sea area. Measurements of α -keto acids and H_2O_2 existing in fermentation supernatant were carried out to show that R3 can produce L-amino acid oxidase (LAAO) with a broad substrate specificity. Physiological and biochemical analysis showed that it can grow great at the conditions with sodium chloride concentration of 1.5% - 3%, temperature of 15°C - 35°C and pH of 6 - 7. In addition, molecular identification of 16S rDNA was performed to show that R3 was proximal to *Pseudoalteromonas* spp. with the highest identity of 98.5% to *Pseudoalteromonas rubra*. Therefore, it was designated as *Pseudoalteromonas* sp. R3. Further studies are required to arrive at a better understanding of this LAAO and secure an application.

Keywords: LAAO; *Pseudoalteromonas*; Physiological Property; Biochemical Property; Identification

1. Introduction

L-amino acid oxidase (LAAO; EC 1.4.3.2) is dimeric flavoprotein, and each subunit contains a non-covalently bound FAD molecule as cofactor. It is able to catalyze the stereospecific oxidative deamination of L-amino acids to α -keto acids, NH_4^+ and H_2O_2 . When H_2O_2 is not degraded by catalase, it can cause a decarboxylation of the α -keto acid to the corresponding carboxylic acid.

Diverse studies have indicated that LAAOs have broad bioactivities such as inducing apoptosis [1], cytotoxicity [2,3], edema [4], hemolysis [5], hemorrhage [6], inducing or inhibiting platelet aggregation [7,8], parasite-killing activity [9], and antimicrobial activity [10]. LAAO may act as defence or attack weapons via H_2O_2 [11] or as ideal molecular mechanisms for the acquisition of nitrogen from diverse amino acid sources [12]. In addition, researches have showed that LAAO affects the relation of tumor cells with the immune system [13] and is involved in violacein synthesis [14]. So far, LAAO was found to be applied as catalysts in bio-transformation [15] and as part of biosensors in determination of the different forms (D- or L-) of free amino acids [16].

This enzyme has been widely found in nature including snake venoms, insect drugs [17], sea hare, algae [18] and terrestrial microorganisms [19]. In contrast, little is known about LAAO from marine microorganisms. The objective of this study is to isolate and characterize LAAO-

producing marine microorganism. We believe that this study lays the foundation for further investigation on enzymatic properties, structure, biological function and application of LAAOs from marine microorganisms.

2. Materials and Methods

2.1. Sample Collection and Isolation of LAAO-Producing Marine Microorganism

The intertidal zone sludge samples (30.03°N, 122.11°E) were collected from different locations at the Dinghai sea area. From each location, sample was collected at 50 to 100 cm depth under the sea surface. These samples were placed in special pre-sterilized plastic bottles and brought to the laboratory in aseptic condition. Then, 10 g of each sludge sample was subjected to 90 mL of sterilized distilled water and serially diluted (up to 10⁻⁶ dilution). After dilution, about 100 μ L of each diluted sample was plated on different agar medium including PDA (potato 200 g/L, sucrose 10 g/L, sea salt 30 g/L), Gause's Medium NO.1 (soluble starch 20 g/L, NaCl 0.5 g/L, K_2HPO_4 0.5 g/L, $FeSO_4$ 0.01 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KNO_3 1 g/L, sea salt 30 g/L; pH 7.2-7.4), and MM medium (yeast extract 3 g/L, peptone 5 g/L, sea salt 30 g/L; pH 7.2 - 7.4), and separately incubated at 28°C and 25°C for 2 - 7 days, as necessary. The isolated colonies were purified by streak-plate technique.

LAAO activity was determined by measuring its fermentation products including H_2O_2 and α -keto acids. The

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production of H_2O_2 was measured by using Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen, USA). The production of α -keto acids were spectrophotometrically measured based on hydrazine assay, according to Len Sikora's method [20].

2.2. Sodium Chloride Tolerance and Cultural Condition

Different concentrations of sodium chloride (0%, 1.5%, 3%, 4.5%, 6%, 7.5%, 9%, 10.5% and 12%) were added to the MM medium (without sea salt). The seed of isolated LAAO-producer was planted into different MM medium and incubated at 25°C, 160 rpm for 24 hours, and salt tolerance was tested. The growth of the LAAO-producer on MM medium incubated at different temperature (5°C, 15°C, 25°C, 35°C and 45°C) and at different pH (3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) was also investigated to determine its growth range of temperature and pH.

2.3. Physiological and Biochemical Characterization

The ability of the isolate to utilize various carbon and nitrogen sources, and other physiological and biochemical properties were studied by the method recommended in "The Manual of Systematic Methods of Determinative Bacterial", with minor modification by adding 3% sodium chloride to each medium.

2.4. Sequencing Analysis

The genomic DNA was isolated by bacterial genomic extraction method (EDTA treatment method). PCR reactions were performed in 50 μ L containing 37 μ L ddH₂O, 5 μ L 10 \times Easy *Taq* buffer, 4 μ L 2.5 mM dNTPs, 100 nM primer 27F (5'-GAGTTTGATCTGGCTCAG-3'), 100 nM primer 1527R (5'-AGAAAGGAGGTGATCCAGCC-3'), 1 ng genomic DNA, and 1 U *Taq* DNA polymerase with denaturation at 94°C for 5 minutes followed by 30 cycles of 1 minute at 94°C, 50 seconds at 55°C, 90 seconds at 72°C and a final 10-minutes extension at 72°C. At the end of reaction, PCR product was cooled to 4°C to await further use. After size confirmation on 1.0% agarose gel, the PCR DNA product was sent to Sangon Biotech (Shanghai) Co. Ltd for sequencing of 16 S rDNA. After sequencing, the similarity and homology of partial 16S rDNA were online analyzed using BLAST search via NCBI.

3. Results

3.1. Isolation of LAAO-Producing Strain

A total of 157 pure isolates were obtained from the intertidal zone of Dinghai sea area located in Zhoushan, Zhe-

jiang province, China. Three domains including 32 actinomycetes, 51 fungus and 74 bacteria were morphologically characterized. Out of 157 isolates subjected to screening process, one isolate (R3) showed the capability to produce LAAO based on the below results.

LAAO is able to catalyze the stereospecific oxidative deamination of L-amino acid to α -keto acid. As carbonyl derivative, α -keto acid can be easily measured by sensitive method using 2,4-dinitrophenylhydrazine (DNP) which can react with carbonyl group to generate dinitro-phenylhydrazone with a brown-red color and characteristic absorbance maxima at 520 nm. As shown in **Figure 1(c)**, R3 fermentation supernatant without L-amino acid substrate showed flaxen and OD₅₂₀ was very low. Similarly, a mixture of R3 fermentation supernatant treated at 95°C for 5 minutes and substrate L-Phe (**Figure 1(d)**) also showed flaxen with low OD₅₂₀ value. In contrast, medium with L-Phenylpyruvic acid (**Figure 1(a)**) and R3 fermentation supernatants with L-Phe (**Figure 1(b)**) both had brown-red color with high OD₅₂₀. All these results indicate that R3 with L-amino acid substrate can generate α -keto acid. Therefore, R3 can produce LAAO.

LAAO is able to catalyze L-amino acid to release H_2O_2 which can be detected using Amplex Red Hydrogen Peroxide/Peroxidase Assay kit. Our results showed that, for either R3 fermentation supernatant without substrate L-Met or R3 fermentation supernatant treated at 95°C for 10 minutes followed by addition of L-Met, no H_2O_2 was detected. In contrast, huge amount of H_2O_2 (1.84 mmol/L) was detected in R3 fermentation supernatant with L-Met, indicating that R3 can use L-Met to release H_2O_2 . Therefore, R3 can produce LAAO.

3.2. Morphological Characteristics

On MM media plate, R3 displayed a red color and round form with smooth. Based on the Gram-staining, R3 was identified as a gram-negative strain with the size of about 3.15 μ m \times 1.05 μ m (15 \times 100).

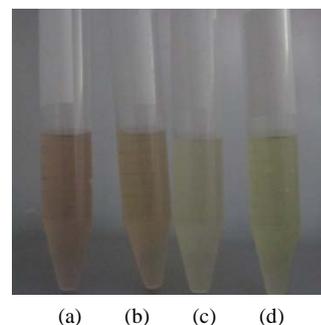


Figure 1. Detection of α -keto acids. (a) Positive control: Phenylpyruvic acid with MM medium (OD₅₂₀ 0.202); (b) L-Phe with R3 (OD₅₂₀ 0.145); (c) R3 without L-Phe (OD₅₂₀ 0.038); (d) Treatment of B3 at 95°C for 10 minutes followed by adding of L-Phe (OD₅₂₀ 0.051).

To determine its substrate specificity, 16 common L-amino acids were selected as substrates for oxidation reaction. It was found that almost 11 out of 16 substrates can be effectively used by R3 to generate H_2O_2 based on Amplex Red Hydrogen Peroxide/Peroxidase Assay kit as shown in **Figure 2**. L-Leu gave the highest activity, followed by L-Lys, L-Tyr, L-Asn, L-Gln, L-Met, L-cystine, L-Arg, L-Trp, β -Val and L-Glu. On the other hand, the other substrates showed comparatively low activity. These results indicate that LAAO of R3 has a broad substrate specificity.

3.3. Sodium Chloride Tolerance and Dependence of Temperature and pH

To assess NaCl tolerance of R3, different concentrations of NaCl from 0% to 12% were added to MM medium. **Figure 3** showed that R3 needed NaCl for growth and cannot survive in NaCl-free medium. In addition, it can grow the best with 1.5% - 3% of NaCl. Further increase of NaCl concentration inhibited its growth. All these findings totally agree with the fact that R3 was isolated from the sea area. As expected, both temperature (**Figure 4**) and pH (**Figure 5**) were very important factors for R3 growth. R3 can grow great at temperature between 15°C and 35°C and the optimal temperature was 25°C. With the either decrease or increase of temperature, the growth became worse. At either 5°C or 45°C, there was almost no growth. Regarding pH, R3 can tolerate basic condition much better than acidic one. The best pH for R3 growth was 6 - 7. At pH lower than 4, there was almost no growth.

3.4. Physiological and Biochemical Tests

In the physiological and biochemical tests (**Table 1**), R3 showed positive results (+) on starch hydrolysis test, indole test, utilization citrate test and oxidase test, and negative results (-) on lysine decarboxylation enzyme test,

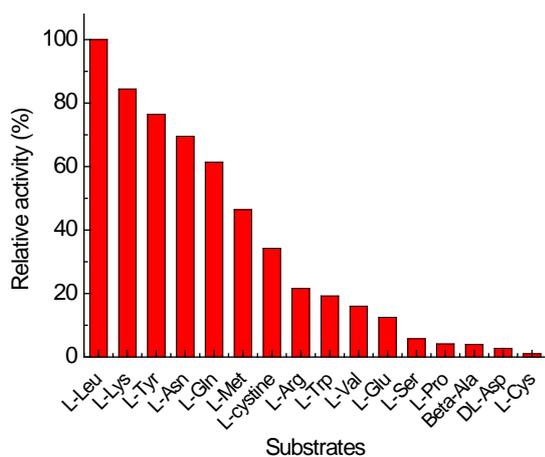


Figure 2. Substrate specificity of R3 LAAO.

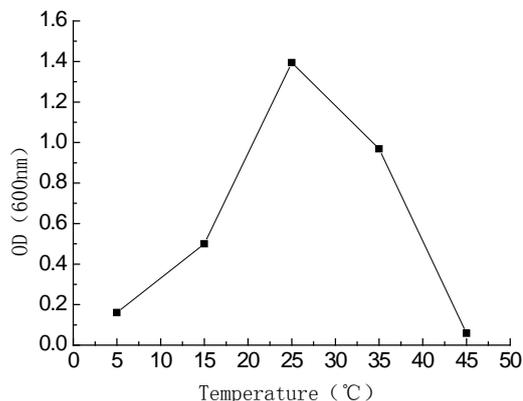


Figure 3. Effect of temperature on the R3 growth.

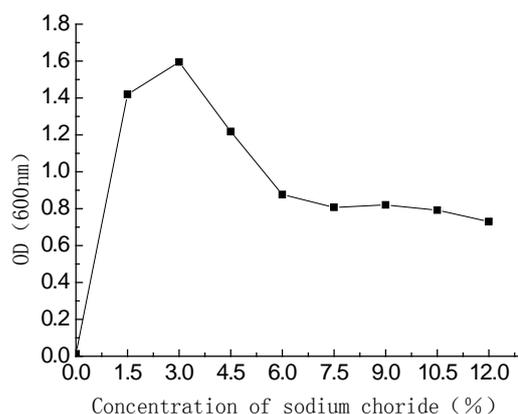


Figure 4. Effect of salt concentration on R3 growth.

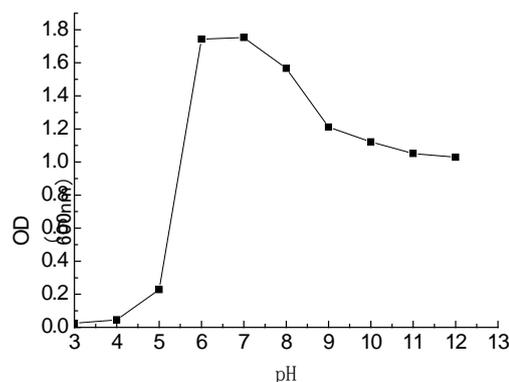


Figure 5. Effect of pH on R3 growth.

half a solid agar test, gelatin hydrolysis test, H_2S production test, acetamide test, V-P test, methyl red test and melezitose monohydrate test. It can use carbon sources including galactose, glucose, lactose, fructose, maltose and rhamnose and nitrogen sources including $(NH_4)_2HPO_4$, KNO_3 , arginine, methionine, glycine and tyrosine.

3.5. 16S rDNA Sequence Analysis

The partial 16S rDNA of R3 was amplified using universal primers 27F and 1527R. After PCR amplification,

Table 1. Physiological and biochemical characteristics of the R3 isolate.

| Items | R3 | Carbon Sources | R3 | Nitrogen Sources | R3 |
|-------------------------------|----|----------------|----|--|----|
| Lysine decarboxylation enzyme | – | Inositol | – | (NH ₄) ₂ HPO ₄ | + |
| Half a solid agar | – | Sorbitol | – | KNO ₃ | + |
| Gelatin hydrolysis | – | Mannitol | – | Arginine | + |
| Starch hydrolysis | + | Glucose | + | Methionine | + |
| H ₂ S production | – | Sucrose | – | Phenylalanine | – |
| Indole test | + | Rhamnose | + | Glycine | + |
| Methyl red test | – | Lactose | + | Tyrosine | + |
| V-P test | – | Fructose | + | | |
| Utilization citrate | + | Maltose | + | | |
| Acetamide | – | Galactose | + | | |
| Melezitose monohydrate | – | | | | |
| Oxidase test | + | | | | |

around 1.4 Kb fragment was successfully obtained (data not shown) and sequenced. Then the Blast search via NCBI was carried out to evaluate the similarity and homology of R3 with other organisms. It was found that R3 had very high homology with *Pseudoalteromonas* spp., with the highest identity of 98.5% to *Pseudoalteromonas rubra*. Therefore, we designated it as *Pseudoalteromonas* sp. R3.

4. Discussion

LAAOs form a family of proteins with various enzymatic properties, structure and biological function. Extensive studies indicate that LAAOs have promising biotechnological and medical applications. This enzyme is widely distributed in nature including snake venoms, insect drugs, sea hare, fungi, bacteria and algae. Unlike snake venom LAAOs which have been widely and deeply investigated to show broad bioactivities such as apoptosis, cytotoxicity, edema, hemolysis, hemorrhage, platelet aggregation, parasite-killing activity and antimicrobial activity, non-snake venom LAAOs need to be further studied and their functional role and application remain to be revealed. Especially, very little is known about LAAOs from marine microorganism. In this study, we successfully isolated an LAAO-producing marine bacterium from intertidal zone of Dinghai sea area. Based on physiological and biochemical tests together with molecular analysis, it was designated as *Pseudoalteromonas* sp. R3. To arrive at a better understanding and address the commercialization of LAAO from this isolate, future works are planned to clone the gene coding for this *Pseudoalteromonas* sp. R3 LAAO and to study its structure, biological and physiological roles, relationship between function and structure, and mechanism of transcription in vivo.

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