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Purification and characterization of Alanine dehydrogenase from *Streptomyces anulatus* for its application as a bioreceptor in biosensor

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ABSTRACT

Screening of different unexplored species of *Streptomyces* led to the identification of alanine dehydrogenase (AlaDH) from *Streptomyces anulatus*. This AlaDH was purified, characterized and was used as a bioreceptor for developing an ammonium biosensor that can detect ammonium ions in water samples. The AlaDH of *S. anulatus* was a dimer with each monomeric unit of Mol. Wt. 61 kDa. The optimum pH for AlaDH in oxidative deamination and reductive amination was 10 and 8.5 respectively with wide working pH range of 5–11. The optimum temperature was 40 °C in both the reactions with wide working temperature range of 20–50 °C. The enzyme retained more than 85% of its original activity after incubating at 60 °C for 30 min in the presence of DTT. Due to these properties of AlaDH, it was successfully used as a bioreceptor in the ammonium biosensor and the sensor showed linear response in the range of 0.1–300 mM NH_4^+ with the detection limit of 0.01 mM NH_4^+ and response time of 20 s. The sensor was showing good response at wide pH (5–11) and temperature range (20–50 °C) suggesting its usage at ambient and non-ambient conditions. The sensor was successfully validated with Nessler's reagent method by using real water samples.

1. Introduction

Alanine dehydrogenase (AlaDH) (E.C.1.4.1.1) belongs to an oxidoreductase family and catalyzes a reversible reaction of biological and technological importance. It catalyzes the oxidative deamination of alanine to pyruvate in the forward reaction and reductive amination of pyruvate to alanine in the backward reaction as shown below.

In microorganisms, as per the need, interconversion of alanine and pyruvate by AlaDH is central to the metabolism. The forward catabolic reaction was suggested to be responsible for the generation of energy during sporulation in the microorganisms like *Bacillus subtilis* [1]. During this reaction, catabolism of L-alanine leads to the production of pyruvate which further enters the TCA cycle. The backward reaction is essential for catabolism and synthesis of non-essential amino acid L-alanine. In the cases like *Streptomyces aureofaciens* [2] and *Rhodobacter capsulatus* [3], the NH_4^+ is assimilated from their surroundings which is converted to alanine by AlaDH and L-alanine is one of the three essential amino acids for the production of peptidoglycan layer and hence for the growth of the cells.

The gene encoding AlaDH is ald and its regulator is aldR. It is suggested that aldR is necessary for ald gene induction and aldR serves as both activator and repressor for the regulation of ald gene expression [4]. AlaDH is a member of amino-acid dehydrogenase (AADH) superfamily which includes glutamate dehydrogenase (GlDH), leucine dehydrogenase (LeDH), valine dehydrogenase (VeDH) and phenylalanine dehydrogenase (PheDH) [5]. However, AlaDH is the only AADH that do not share any evolutionary relationship with the AADH superfamily [6]. Though, both have the NAD^+/NADH binding Rossmann folds. Also, other AADHs are B-stereospecific whereas AlaDH is A-stereospecific as the hydride transfer to NAD^+ occurs at the pro(R) position of the nicotinamide ring as reported for the AlaDH of *B. subtilis* [7] and *B. Sphaericus* [8].

There are several applications of AlaDH [9] and it has been widely used in food industries. Heterologous expression of AlaDH from *Bacillus subtilis* into *Lactococcus lactis* using a lactate dehydrogenase promoter from *Streptococcus thermophilus* was used to produce L-alanine which was used in the development of healthy yogurts with sweet taste or other fermented dairy foods [10]. AlaDH from *B. subtilis* was used for the determination of L-alanine from food samples [11]. An AlaDH based assay for the measurement of L-alanine levels in serum was developed [12]. In pharmaceutical industries, AlaDH was used to produce L-β-fluoroalanine from β-fluoropyruvate [13], which is biologically active,

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Table 1
Screening of *Streptomyces* spp. for AlaDH activity at pH 8.5 and 25 °C.

S. No	*Streptomyces* spp.	MTCC number	Intra cellular AlaDH activity (Units/g dry biomass) (n = 3)
1	*S. aurantiacus*	8080	51.408 ± 1.249
2	*S. anulatus*	2528	59.9573 ± 3.225
3	*S. tendae*	3255	23.2367 ± 1.478
4	*S. lydicus*	7505	51.3607 ± 1.767
5	*S. coelicolor*	1139	25.552 ± 3.073
6	*S. aburaviensis*	7328	21.2647 ± 2.838
7	*S. albaduncus*	924	49.9887 ± 1.571
8	*S. bikiniensis*	1539	51.1893 ± 3.225
9	*S. clavifer*	4150	32.6687 ± 3.180
10	*S. peuceticus*	3000	22.894 ± 2.592
11	*S. purpeofuscus*	8377	50.0747 ± 1.723
12	*S. thermovulgaris*	1822	21.474 ± 2.154

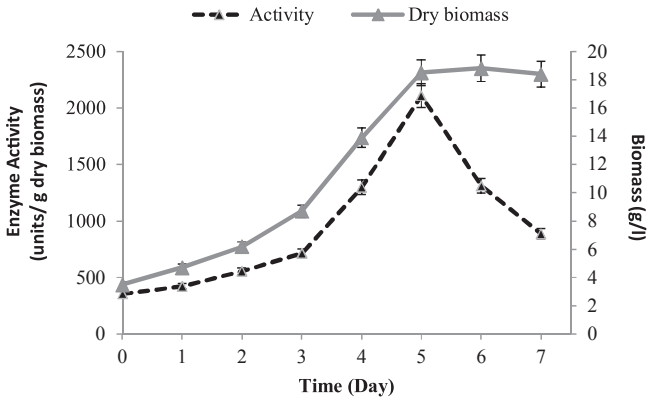

Fig. 1. Time course study on the production of AlaDH by *S. anulatus* in the submerged culture.

exhibit antihypertensive and antibacterial effects [14]. Also, fluorine derivatives of L-alanine catalyzed by AlaDH exhibit promising properties as a Positron emission tomography (PET) imaging agent [15,16]. AlaDH of *Mycobacterium tuberculosis* (MTB-AlaDH) is of special medical interest as a new class of inhibitors [17,18] were screened against MTB-AlaDH which were able to reduce both nutrient starved dormant MTB and actively replicative MTB. Thus, MTB-AlaDH was identified as a plausible drug target against *M. tuberculosis*.

AlaDH was used as a bioreceptor in the construction of amperometric biosensor for detecting ammonium levels in waste water samples due to its high specificity for ammonium [9,19–21]. Sensors that detect ammonium are useful for environmental and medical applications [22]. Sensor that can monitor ammonium levels in water samples will be helpful for taking precautionary measures since higher levels of ammonium ions are hazardous to mammals and aquatic organisms and can lead to adverse effects [23]. There are simple chemical methods based on Nessler's reagent [24] and Berthelot's indophenol reaction [25] for the detection of ammonium available, too. However, mercuric and phenolic reagents used during these colorimetric reactions are highly toxic. In addition, these methods are less selective to substrate compared to the enzyme based sensors. Hence, enzyme based amperometric biosensors will lead to specific detection and quantification of target analyte without any hazards.

In the presence of ammonium, the AlaDH operates in a backward reaction by converting pyruvate to alanine during which NADH is reduced to NAD^+ and 2 electrons are generated which can be measured by the amperometric biosensor. However, the AlaDH of *B. subtilis* used earlier [19] acts in the narrow pH range which restricts its usage at acidic pH. In the present study, we are reporting an AlaDH from *Streptomyces anulatus* which shows its activity at acidic, neutral and alkaline pH. This enzyme was purified, characterized and used as a bioreceptor in the amperometric ammonium biosensor. This biosensor will be advantageous over earlier one due to its functionality in the wide pH range.

2. Materials and methods

Twelve *Streptomyces species* used in the studies were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India (Table 1).

2.1. Cultivation of Streptomyces

A loopful culture of *Streptomyces* from a slant (stored at 4 °C) was transferred into 20 ml of liquid medium (pH 7) containing soya bean meal (1%), corn steep liquor (1%), glucose (1%) and $CaCO_3$ (0.5%). This was incubated at 30 °C and 200 rpm for 5–7 days in an orbital shaking incubator. The prepared inoculum was transferred into 180 ml of production medium (pH 7) containing glucose (0.5%), soya bean meal (1%), yeast extract (1%) and NaCl (0.3%). Culturing of the *Streptomyces* was carried out in a shaking incubator at 200 rpm and 30 °C for 5 days.

2.2. Collection of cell-free extracts

Grown cultures of *Streptomyces* were centrifuged at 5300 rcf (× g) and 4 °C for 10 min. The supernatant was discarded and pellet was processed further for collecting intracellular extract. The pellet was homogenized using pestle and mortar, freeze dried and 1 g dry biomass was re-suspended in a lysis buffer (50 mM Tris-HCl, pH 8.5) containing 0.5 mM EDTA and 0.1 mM PMSF. Further, it was sonicated using a tip sonicator (250 W, 30 kHz) for 100 cycles (15 s each) and then centrifuged at 9400 rcf (× g) for 15 min. The pellet was discarded and the supernatant was used for measuring the activity of AlaDH present in the intracellular extract.

2.3. Enzyme assay

The assay mixture (1.2 ml) for reductive amination contained 0.125 mM NADH, 2 mM pyruvate, 100 mM NH_4Cl and appropriately diluted enzyme in the 50 mM Tris-HCl (pH 8.5) buffer. The regular reaction mixture (1.2 ml) for the oxidative deamination consisted of 0.4 mM NAD^+, 80 mM L-alanine and appropriately diluted enzyme in the 50 mM sodium carbonate-bicarbonate (pH 10) buffer. The assay was carried out at room temperature (25 °C) by recording a change in the absorbance of NADH at 340 nm [26] using a spectrophotometer (Shimadzu). One unit of the enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 µmol NAD^+ or NADH in

Table 2
Purification of AlaDH from crude extracts of *Streptomyces anulatus* (Approx. 1.5 kg wet weight of washed cells used) at pH 8.5 and 25 °C.

S. No	Step	Total Activity (units)	Specific Activity (units/mg protein)	% recovery (n = 3, ± 1%)
1	Cell lysis by Sonification	3765906	2451.76	100
2	Precipitation by 80% ammonium sulfate	2115766	9661.03	56.18
3	Separation by anion-exchange chromatography (DEAE-Cellulose)	755635.25	12090.164	24.08
4	Gel Exclusion chromatography (Sephadex G 100)	224508.2	18709.02	5.96