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# Enzymatic production of 5′-inosinic acid by AMP deaminase from a newly isolated *Aspergillus oryzae*

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ABSTRACT

5′-adenylic acid deaminase (AMP deaminase), an important enzyme for the food industry, can catalyze the irreversible hydrolysis of adenosine monophosphate (AMP) to inosine monophosphate (IMP) and ammonia. In this study, a new strain was screened that efficiently produces 3191.6 U/g of AMP deaminase at 32 °C. After purification, the optimal temperature and pH of the AMP deaminase were found to be 40 °C and 6.0, respectively, but it was partially inhibited by $Fe^{3+}$, $Cu^{2+}$, $Al^{3+}$, and $Zn^{2+}$. With amplification of the AMP deaminase production system, 6 mL of crude enzyme could produce 2.00 mg/g of IMP from 2.04 mg/g of dried yeast with an 84.8% molar yield after 40 min. These results provide a new insight into AMP deaminase production and offer a potential platform for producing 5′-IMP.



## 1. Introduction

Nucleotides, including 5′-inosinic acid (5′-IMP) and 5′-guanylic acid (5′-GMP), have been widely used as food additives and pharmaceutical intermediates (Asano, Mihara, & Yamada, 1999), and have received much attention due to their characteristic taste and important application in various foodstuffs as flavour potentiators (Asano et al., 1999; Mihara, Ishikawa, Suzuki, & Asano, 2004). With improvements in living quality, the demand for nucleotides in the food additives market has been increasing, and the production of nucleotides has been thoroughly investigated. For 5′-IMP, there are four methods of production, including chemical synthesis, enzymatic synthesis, RNA enzymolysis, and microbial fermentation (Liu et al., 2012; Matsui, Sato, Enei, & Takinami, 1982). Compared with the other methods, enzymatic synthesis is a cost-effective approach with many advantages: less environmental pollution, mild reaction conditions, strong specificity, and lower cost of production. However, there are also some limitations that hinder industrial implementation of methods that involve enzymes (Schoemaker, Mink, & Wubbolts, 2003). For example, they may neither be stable enough under industrially relevant conditions (presence of organic solvents, high temperatures, etc.) nor have sufficient activity, selectivity, or specificity towards the target substrate (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011; Stepankova et al., 2013). Therefore, if it were possible to obtain a high catalysis rate and appropriate specificity of an enzyme, it would have potential applications in the industrial production of 5′-IMP, increasing the competitiveness of enzymatic synthesis.

At present, some enzymes have been isolated and investigated for producing 5′-IMP, such as acid phosphatase/phosphotransferase (AP/PTase) (Mori, Iida, Fujio, & Teshiba, 1997) and adenylate deaminase (5′-adenylic acid deaminase, AMPDA, EC 3.5.4.6) (Zheng, Tashiro, Wang, & Sonomoto, 2015). Compared to AP/PTase, AMP deaminase can catalyze the irreversible hydrolysis of adenosine monophosphate (AMP) to inosine monophosphate (IMP) and ammonia, a reaction that has been observed in a variety of eukaryotes including animals, plants, and yeast (Lushchak, 1996; Meyer, Kvalnes-Krick, & Schramm, 1989; Versavaud, Courcoux, Roulland, Dulau, & Hallet, 1995). Recently, various AMP deaminases have been isolated and their properties were thoroughly investigated (Hatti-Kaul, Tornvall, Gustafsson, & Borjesson, 2007). For example, three AMP deaminase isoforms were identified in mammals: isoform 1 predominates in skeletal muscle, isoform 2 predominates in liver and brain, and isoform 3 seems to be exclusively an erythrocytic enzyme (Elbein, Pan, Pastuszak, & Carroll, 2003). Meanwhile, an AMP deaminase originating from microbial sources has also been purified and studied (Merkler, Wali, Taylor, & Schramm, 1989; Sollitti, Merkler, Estupinan, & Schramm, 1993). However, data pertaining to microbial AMP deaminases were restricted, until

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**Table 1**
Production of AMP deaminase by different microorganisms.

| Microorganisms | Strain improvement | Fermentation strategy | Enzyme activity (U/g moldy bran) | References |
|---|---|---|---|---|
| *Aspergillus mellon* | UV and 5-F-Ura mutation | Fermentation ecology | 1300.0 | Pu, Ding, and Tao (1994) |
| *Mucor* sp. | Optimization of conditions | Solid fermentation | 472.3 | Mei et al. (2013) |
| *Aspergillus oryzae* | Optimization of conditions | Solid fermentation | 1543.5 | Duan et al. (2002) |
| *Aspergillus oryzae* | Screening from soil sample | Solid fermentation | 3191.6 | This study |

recently, to enzymes from *Saccharomyces cerevisiae* (M. H. Kim, Lee, Kim, & Oh, 1999; Merkler & Schramm, 1990), *Aspergillus* sp. (Duan, Shen, Mao, & Liu, 2002), and *Mucor* sp. (Mei et al., 2013) only (Table 1). For example, under optimal conditions, *Aspergillus orgzae* 3800 could produce only 1543.5 U/g moldy bran using wheat bran as the carbon source (Duan et al., 2002). Furthermore, AMP deaminases were produced in conjunction with some similar proteins (some of which had undesired catalytic activities towards the substrates or even the products) that decreased the final volumetric activity and catalytic capacity (Barbosa et al., 2015), so that they could not meet the requirements of industrial production.

Therefore, screening and improving the performance of microbes to achieve more efficient AMP deaminases have been meaningful in view of possible biotechnological applications and metabolic functions. For this reason, a new strain demonstrating high productivity of AMP deaminase was isolated and identified as *Aspergillus oryzae* (Generally Recognized As Safe, GRAS), according to its 16S rDNA sequence. After purification, the detailed characteristics of this AMP deaminase were investigated and showed that it has potential for more economically viable industrial production of 5′-IMP.

## 2. Materials and methods

### 2.1. Materials

AMP, GMP, and IMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the other reagents used were analytical grade.

### 2.2. Media and culture conditions

To screen the AMP deaminase-producing strain (Pang, Liang, Su, & Cen, 2009), different media were designed for use as follows. Medium A (used as a selective medium, g/L): potato 200, sucrose 20, and dried yeast powder 5, at pH 6.0; medium B (used for seed culture, g/L): yeast extract 3, potato 100, sucrose 20, $KH_2PO_4$ 1, $MgSO_4$ 0.5, and agar 20, at pH 6.0; medium C (used for solid-state fermentation, g): wheat bran 20, water 20, and yeast extract 5.

The strain was incubated in a plate of medium B at 32 °C for 24 h, and then transferred into the solid fermentation medium C (250 mL) at 32 °C for 48 h for static culture.

### 2.3. Isolation and identification of the AMP deaminase-producing strain

To effectively obtain a high-producing strain for AMP deaminase, the process of preparation and isolation was slightly modified (Duan et al., 2002; Pang et al., 2012), as follows: 2 g of different soil samples were separately added to tubes containing 20 mL of medium A and mixed thoroughly. After incubating at 32 °C for 48 h, the contents of AMP and AMP deaminase were measured. Soil samples in which AMP deaminase appeared were diluted to $10^{-2}$, $10^{-3}$, and $10^{-4}$, and spread onto screening plates containing medium B at 32 °C for 48 h. Colonies that appeared on medium B were selected and inoculated into medium C at 32 °C for 48 h to determine the AMP deaminase production capability. Strains that produced high levels of AMP deaminase were preserved in sand tubes for long-term storage. These AMP deaminase-producing strains were identified by morphological and physiological characteristics, and 16S rDNA sequence analysis. The results of 16S rDNA sequence analysis were compared with sequences in GenBank databases using the Basic Local Alignment Search Tool (BLAST) algorithm.

### 2.4. Analytical methods

#### 2.4.1. Preparation of crude enzyme

After fermentation for 48 h, the moldy bran was collected and extracted with a 10-fold volume of $H_2O$ for 1 h, and then the mixture was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant obtained contained crude enzyme.

#### 2.4.2. Enzyme assay

According to the comparative spectra of AMP and IMP (Fig. S1), a spectrophotometric method was used to measure AMP deaminase activity at 265 nm (Mei et al., 2013), as follows: 0.1 mL of crude enzyme (using $ddH_2O$ as the control) was added to 3 mL of AMP (0.1 mmol/L) at 40 °C. After incubation for 15 min, 3 mL of perchloric acid (10%) were added to terminate the reaction, and then the absorbance was measured by a UV–vis absorbance detector (Hewlett-Packard Co., Waldbrom, Germany). One activity unit (U) was defined as an absorbance value of 0.001 generated per 1 min, and the enzyme activity of AMP deaminase was calculated as follows:

$$\text{Enzyme activity}(\text{U/mL}) = (\Delta A_{265} \times 10 \times K)/(0.001 \times B) \quad (1)$$

where $\triangle A_{265}$ is the difference in absorbance between the enzymatic reaction and the control, K is the dilution ratio of the crude enzyme, and B is the time span of enzymatic reaction.

#### 2.4.3. Enzyme purification and SDS-PAGE analysis

AMP deaminase was purified from crude enzyme using a facile purification procedure (Duan et al., 2002; Pacheco-Aguilar et al., 2009), as follows. First, crude enzyme was successively filtered by microfiltration (0.22 μm, Millipore, Darmstadt, Germany) and ultrafiltration (50 kDa, Millipore), and then different concentrations of ammonium sulfate (35%–90%) were added to the supernatant to saturate the solution. After static incubation overnight at 4 °C, the solution was centrifuged at 10,000 × g for 10 min at 4 °C, and then the pellet was solubilized in 100 mM Tris buffer (pH 7.0) to measure enzyme activity. Subsequently, the solution was dialysed against 20 mM Tris buffer (pH 7.0) overnight at 4 °C to obtain the partially purified enzyme. Next, DEAE-cellulose (using 0.02 mol/L potassium phosphate (pH 7.0) and 0–1.0 mol/L KCl for gradient elution), and Sephacryl-S-200 chromatography (using 50 mM potassium phosphate (pH 6.5) for equilibration and elution) were used to further purify the AMP deaminase. Finally, the enzyme was dialysed against 50 mM sodium phosphate buffer containing 10 mM EDTA for 24 h at 4 °C to remove metal ions, and then dialyzed against 50 mM EDTA-free sodium phosphate buffer (pH 7.0) to remove EDTA (Green, 2011). The protein purity was determined by sodium dodecyl sulfate