Comparison of 2-acetyl-1-pyrroline production between Aspergillus awamori and Aspergillus oryzae

T Wongsadee, S Vatanyoopaisarn, B Thumthanaruk, C Puttanlek, D Uttapap and V Rungsardthong

1Department of Agro-Industrial, Food, and Environmental Technology, King Mongkut’s University of Technology North Bangkok, Bangkok, Thailand
2Department of Biotechnology, Silpakorn University, Nakhon Pathom, Thailand
3Division of Biochemical Technology, School of Bioresources and Technology, King Mongkut’s University of Technology Thonburi, Bangkok, Thailand

E-mail: savitri.v@sci.kmutnb.ac.th

Abstract. 2-Acetyl-1-pyrroline (ACPY) is a key flavor compound in fragrant rice and widely exploited in food flavoring. It is produced by various microorganisms. This study focused on ACPY production by two fungi (Aspergillus awamori and A. oryzae). The volatile compounds derived from mold cultivation in synthetic medium were identified by Gas chromatograph-mass spectrometer (GC-MS). Seven volatile substances were detected in the liquid culture of A. awamori, i.e. ACPY, one ketone (1-hydroxy-2-propanone), two acids (acetic acid and 4-hydroxybutanoic acid), two alcohols (2,3-butanediol and 2,5-dimethyl-4-hydroxy-3(2H)-furanone) and one saponin glycoside (2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one). Further, 12 volatile compounds were detected in A. oryzae: ACPY, four fatty acids (tetradecanoic acid, n-hexadecanoic acid, octadecanoic acid and oleic acid), one alcohol (1-butanol), two benzenes (ethylbenzene and benzene, 1,3-bis(1,1-dimethylethyl)), three alkanes (pentadecane, heptadecane and 5-methoxy-2,2,6-trimethyl-1-(3-methyl-buta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptane) and one sesquiterpenes (1,6-dimethyl-4-(1-methylhexyl)-naphthalene). Both A. awamori and A. oryzae produced the highest amount of ACPY in the stationary phase when cultivated for 72 and 80 h, with the product yield of 0.914 and 1.323 mg/L, respectively. The supplementation with spermidine and spermine resulted in a more than fivefold increase in ACPY production by A. awamori. However, the production of ACPY by A. oryzae was lower when supplemented with spermine or spermidine than without spermine or spermidine. This indicated that the intermediates involved in ACPY production were different between these two species of fungi.

1. Introduction

The food industry has increased the demand for flavoring compounds due to the aroma of food affects consumer acceptance. The volatile components contributing to the aroma of food affect consumer acceptance. Rice flavor is one of the most favorite flavors for consumers. 2-Acetyl-1-pyrroline (C9H16NO) or ACPY is the main volatile compound that contributes to the rice fragrance and is also a key flavor compound in many other foods, such as pandan leaf (Pandanus amaryllifolius come), popcorn and bread [1]. It is a heterocyclic compound. Its odor threshold in water and air has been measured to be 0.1 ng/L and 0.02 ng/L, respectively [2]. The extraction of ACPY from plant sources is complicated and requires various instruments [3]. Several methods are used for the production of ACPY such as
chemical synthesis and microbial biotechnology. The synthesis of ACPY requires organic chemicals. Its synthesis is difficult because of the unstable nature of this compound, which degrades very rapidly [4]. Microorganisms are used to carry out fermentation to improve the taste, and texture of products that can be considered natural. ACPY is produced by various bacteria such as Bacillus cereus isolated from cocoa fermentation boxes (about 0.03-0.075 mg/kg) on plate count agar [5], B. cereus ATCC 27522 and B. cereus ATCC 14737 (about 26.2 and 6.0 µg/kg, respectively) on plate count agar [6], B. cereus ATCC 10702 (85 µg/kg) [7] and Lactobacillus hilgardii DSM 20176 in off-flavor characterization of wine (0.005 mg/L) [8]. Also, several fungal strains have been found to produce ACPY. Aspergillus awamori and Acremonium niger were producing ACPY in synthetic liquid medium 18 (Syn 18) at about 2.8 mg/L and 1.11 mg/L, respectively. The yield of ACPY was about 1,000-fold higher than the aroma of jasmine rice [9]. A previous study exploring the effects of nitrogen sources (putrescine, glutamic acid, proline, and ornithine) on ACPY production by A. awamori showed that putrescine was the best nitrogen source for ACPY production. Further, the supplementation with spermidine and spermine resulted in a significant increase in ACPY production. The results indicated that putrescine, spermidine and spermine were the major intermediates of ACPY production [10]. However, the information on the optimal cultivation time for the highest ACPY production by A. awamori and the reports on the profile of volatile compounds in the liquid culture of fungi are lacking. The purpose of the present study was to identify the volatile compounds in the culture medium of A. awamori and A. oryzae and compare the effect of the major nitrogen substrates (putrescine, spermidine and spermine) on ACPY production by these two fungal species.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

Strains of A. awamori TISTR 3123 and A. oryzae TISTR 3256 (collected from Thailand Institute of Scientific and Technological Research) were studied. The two fungi were inoculated into potato dextrose agar slant and incubated at 28°C for 7 days. Fungal spores were suspended in 10 mL of 0.1% Tween 80. Then the 5 mL of spore suspension (2 x 10⁶ spores/L) was added into 200 mL of potato dextrose broth and incubated in a shake flask (200 rpm) at 28°C for 3 days. The mycelia in the liquid medium were mixed using a sterile blender and used as a starter culture. Further, 10 mL of the starter culture was inoculated into 90 mL of synthetic medium 18 (Syn 18); the composition of Syn 18 medium is displayed in table 1 and incubated with shaking (120 rpm) at 30°C for 24 h. Spermine (C₁₀H₁₆N₄) or spermidine (C₆H₁₂N₄) was incorporated into some flasks (20 mg/L each). The Syn 18 medium without supplement was used as control. All flasks were further incubated with shaking (120 rpm) at 30°C [10]. The samples were collected and filtrated through Whatman No.1 filter paper. The amount of ACPY in the filtrate was measured using GC-MS and GC-FID.

Table 1. The composition of the synthetic medium 18 (Syn 18) for fungal cultivation.

| Composition                  | Concentration |
|------------------------------|---------------|
| Glucose                      | 15.0 g/l      |
| Putrescine                   | 2.0 g/l       |
| MgSO₄·7H₂O                   | 0.5 g/l       |
| NaCl                         | 0.1 g/l       |
| CaCl₂·2H₂O                   | 0.1 g/l       |
| Phosphate buffer, pH 7.2     | 20 mM         |
| Vitamin solution             | 1.0 ml        |
| - Biotin 50 mg/l             |               |
| - Ca-pantothenate 400 mg/l   |               |
| - Inositol 2000 mg/l         |               |
| - Pyridoxine-HCl 400 mg/l    |               |
| - Thiamine-HCl 500 mg/l      |               |
The 10 ml of liquid culture (pH~2) was adjusted to pH 8.0 with 0.1 N NaOH and then 100 µl of 2,4,6-Trimethylpyridine (Internal standard) and 2 ml of diethyl ether were added and mixed. The amounts of ACPY and 2,4,6-Trimethylpyridine in the ether layer were analyzed by GC-MS and GC-FID.

2.2. Identification of volatile compounds from liquid culture by GC-MS

The extract was injected into an HP-INNOWAX part no. 19091N-133 (length 30 m, inner diameter 0.25 mm, and film thickness 0.25 µm) and an Agilent 7000C Triple Quadrupole GC/MS (Agilent Technologies Inc., USA). The injection was conducted in a split ratio of 25:1. The injector temperatures were set at 180°C. Helium gas was used as the GC carrier gas. The column temperature was held initially at 40°C for 3 min, and then the temperature was increased at a rate of 10°C/min to 120°C and was further increased to 250°C at a rate of 25°C/min. Finally, the column temperature was maintained isothermally at 250°C for 10 min. The ion source temperature was set at 250°C, the GC-MS transfer line was set to 230°C and the ionization energy was 70 eV. The compounds were identified mainly by comparing their mass spectra with the mass spectral data of the NIST2011 library [11].

2.3. Quantification of ACPY from the liquid culture using GC-FID

Gas chromatography – flame ionization detector (GC2010; Shimadzu, Japan) analysis of ACPY was carried out. The injection was conducted in the splitless mode for 5 min at 155°C. The GC was equipped with a D-WAX capillary column (length 30 mm, inner diameter 0.32 mm and film thickness 0.50 µm). The oven temperature was held initially at 40°C for 1 min and then programmed from 40°C to 120°C at a rate of 9°C/min and 120°C to 230°C at a rate of 5°C/min. Helium was used as the carrier gas, at a flow rate of 1.1 mL/min [12]. The concentration of ACPY in the liquid culture (mg/L) was calculated as follows [9]:

\[
\text{ACPY in liquid culture (mg/L)} = \left( \frac{\text{Area of ACPY}}{\text{Area of collidine}} \right) \times \left( \frac{1,000}{10} \right) \times \left( \frac{\text{added internal standard (µg)}}{1,000} \right) \times \text{RRF}
\]

where RRF is the relative recovery between 125 ppm collidine and ACPY = 1.3.

2.4. Biomass determination

The mycelium of each treatment was harvested on a filter paper (Whatman No.1), washed three times with distilled water, and dried for 24 h at 80°C. The dry weight of mycelium was subjected to the following analysis.

\[
\text{Cell dry weight (g/L)} = \left( \frac{\text{weight of filter paper} + \text{dried residue}}{\text{weight of filter paper}} \right) \times \left( \frac{1,000}{100} \right)
\]

3. Results and discussion

3.1. Profile of volatile components in the liquid culture of fungi

The ability of *A. awamori* and *A. oryzae* to produce volatile components from the Syn 18 medium was analyzed and is displayed in tables 2 and 3, respectively. As shown in table 2, the profile of volatile compounds produced by *A. awamori* revealed eight compounds: 2-acetyl-1-pyrroline, one ketone (1-hydroxy-2-propanone), two acids (acetohydroxybutanoic acid), two alcohols (2,3-butanediol and 2,5-dimethyl-4-hydroxy-3(2H)-furanone) and one saponin glycoside (2,3-dihydro-3,5-...
Dihydroxy-6-methyl-4H-pyran-4-one. Six out of seven compounds reflected the flavor of food ingredients.

Table 2. Volatile components in the liquid culture of *Aspergillus awamori*.

| Compound                          | Odor description          | Retention time | % Area |
|-----------------------------------|---------------------------|----------------|--------|
| 1. Ketones                         |                           |                |        |
| 1-Hydroxy-2-propanone              | Fragrant rice B           | 9.379          | 3.50   |
| 2-Acetyl-1-pyrroline               |                           | 9.503          | 1.40   |
| 2. Acids                           |                           |                |        |
| Acetic acid                        | Vinegar C                 | 11.252         | 33.57  |
| 4-Hydroxybutanoic acid             | Red wine D                | 12.952         | 1.40   |
| 3. Alcohols                        |                           |                |        |
| 2,3-Butanediol                     | Fruity E                  | 12.408         | 27.27  |
| 2,5-Dimethyl-4-hydroxy-3(2H)-furanone | Buckwheat F            | 15.104         | 2.10   |
| 4. Saponin glycoside               | Unknown                   | 16.143         | 12.59  |
| 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one | - | 11.479 | 18.18 |
| 5. Alkanes                         |                           |                |        |
| 1-Butanol                          | Alcohol-like, wine B      | 5.405          | 0.52   |
| 4. Benzenes                        |                           |                |        |
| Ethylbenzene                       | Benzene                   | 6.002          | 3.03   |
| Benzene, 1,3-bis(1,1-dimethylethyl) | Benzene                   | 10.795         | 1.40   |
| 5. Alkanes                         |                           |                |        |
| Pentadecane                        | Alkane                    | 7.996          | 0.81   |
| Heptadecane                        | Alkane                    | 10.916         | 1.18   |
| 5-Methoxy-2,2,6-trimethyl-1-(3-methyl-buta-1, 3-dienyl)-7-oxa-bicyclo[4.1.0]heptane | Unknown | 15.350 | 1.03   |
| 6. Sesquiterpenes                  |                           |                |        |
| 1,6-Dimethyl-4-(1-methylthyl)- naphthalene | Unknown | 19.997 | 6.95   |
| 2,4,6-Trimesitylpyridine           | -                         | 11.395         | 15.67  |

Further, 12 volatile compounds were detected in the fermentation liquid produced by *A. oryzae*: 2-acetyl-1-pyrroline, four fatty acids (tetradecanoic acid, n-hexadecanoic acid, octadecanoic acid and oleic
acid), one alcohol (1-butanol), two benzenes (ethylbenzene and benzene, 1,3-bis(1,1-dimethylethyl)), three alkanes (pentadecane, heptadecane and 5-methoxy-2,2,6-trimethyl-1-(3-methyl-buta-1,3-dienyl)-7-oxa-bicyclo[4.1.0]heptane) and one sesquiterpenes (1,6-dimethyl-4-(1-methyl ethyl)-napthalene). In a previous study, 2,3-butanediol and 1-butanol were reported in coconut water and KDML 105 jasmine rice solution, respectively [16].

ACPY was particularly detected by GC-MS which confirmed the fragrant rice aroma scent by the human perception from the cultured liquid by both fungi. The presence of ACPY assured as the major aroma compound.

3.2. Comparison of fungal growth and production of ACPY in Syn 18 medium

The comparison between the growth (dry weight of the mycelia) of the two fungi during fermentation and the amount of their ACPY are shown in figure 1. The cell dry weight of both fungi continuously increased during the first 66 h. Thereafter from 66 to 90 h the cell dry weight did not increase further as the fungi reached the stationary phase. The ACPY began to appear after 48 h and gradually rose to the maximum level after 72 and 80 h of cultivation. A. awamori produced the highest ACPY amount after 72 h (0.914 mg/L) when the cell dry weight was 6.23 g/L. In A. oryzae, the highest production of ACPY was obtained with a yield of 1.323 mg/L and 6.58 g/L of cell dry weight after 80 h of cultivation. The results indicated that the highest amount of ACPY from both fungi was obtained in the stationary phase and thus ACPY was a secondary metabolite.

![Figure 1](image_url)

**Figure 1.** Comparison of growth and production of ACPY in the fermentation liquid produced by the two fungi; (—— —) A. awamori, (--- ---) A. oryzae; ● dry weight of mycelium; ♦ ACPY in liquid culture.

3.3. Effect of polyamine supplement on ACPY production by the two fungi

Nitrogen sources were found to be essential for the production of volatile compounds. In the Syn 18 medium, putrescine (C_4H_12N_2) was a nitrogen source proved to enhance the production of ACPY in Acremonium nigricans [18]. Spermine (C_10H_26N_4) and spermidine (C_7H_19N_3) are simple aliphatic primary amines and essential constituents of eukaryotic cells [19]. These two polyamines can be converted into putrescine in plants [20]. Therefore, to see whether this conversion existed in fungi, the effect of the addition of either spermine or spermidine during the mid-log phase of growth was investigated. At each time interval, ACPY in the liquid culture of both fungi were quantified using GC-FID. Figure 2 shows the effect of polyamines on the production of ACPY by A. awamori. It was clear
that the addition of spermidine or spermine led to at least fivefold higher ACPY production compared with that in the medium with no supplement. The highest amount of ACPY obtained was up to 5.854 mg/L after 68 h, in the medium with spermine. In contrast, the result was opposite in A. oryzae (figure 3); the supplementation with spermine or spermidine did not improve the production of ACPY. The amount of ACPY was lower than that in the presence of such polyamines. Thus, for A. oryzae the highest amount of ACPY was merely 1.323 mg/L in the Syn 18 medium alone after 80 h of fermentation.

**Figure 2.** Effect of spermidine and spermine on ACPY production by A. awamori; ▲ spermidine; ● spermine; ○ without spermine/spermidine

**Figure 3.** Effect of spermidine and spermine on ACPY production by A. oryzae; ▲ spermidine; ● spermine; ○ without spermine/spermidine
The results confirmed that spermine and spermidine were major intermediates of the ACPY production by *A. awamori*. Through the conversion of spermine into spermidine and spermidine into putrescine, at each step, aminopropyl residues were removed by polyamine oxidase from spermine and spermidine, both of which were acetylated by spermidine/spermine N\(^1\)-acetyltransferase [20,21]. This result was consistent with previous findings, which explained the pathway for ACPY production by *L. hilgardii* DSM 20176, and the interaction of intermediates from two disparate pathways: 1) N-heterocyclic or 1-pyrrole derived from the catabolism of L-ornithine; and 2) acetyl side-chain accumulated from the heterolactic fermentation [6,8]. Previous studies have reported that putrescine was the decarboxylation product of ornithine; thus 1-pyrrole could be formed through putrescine [22,23].

However, the conversion of polyamine into putrescine did not occur in *A. oryzae*. Further, the volatile components, such as four fatty acids, three alkanes, two benzenes, and one sesquiterpenes were only found in liquid culture of *A. oryzae* (as shown in table 3) but not in the liquid culture of *A. awamori*, thereby suggesting that *A. oryzae* might form these volatile components from spermine and spermidine. Therefore, the differences in the pathway of ACPY production or enzymes involved need to be elucidated in the future.

4. Conclusion
The highest production of ACPY by *A. awamori* and *A. oryzae* was 0.914 mg/L (72 h of cultivation) and 1.323 mg/L (80 h of cultivation) in Syn 18 medium, respectively. The amount of ACPY reached its maximum value in the stationary growth phase of both fungi. The supplementation with spermidine and spermine was shown to increase ACPY production by *A. awamori*. While the effect was reverse in *A. oryzae*. Spermidine and spermine were the major intermediates of ACPY production by only *A. awamori*.

Acknowledgment
This research was financially supported by the National Research Council of Thailand (grant number KMUTNB-61-GOV-B-21) and the authors wish to acknowledge King Mongkut’s University of Technology North Bangkok, Bangkok, Thailand and Bansomdejchaopraya Rajabhat University, Thailand for laboratory support.

References
[1] Paule C M and Power J J 1989 *J Food Sci Technol* 54 343-5
[2] Schieberle P 1991 *J Agric Food Chem* 39 1141-4
[3] Yahya F, Lu T, Santos R C D, Fryer P J and Bakalis S 2010 *J Supercrit Fluids* 55 200-7
[4] Wei X, Handoko D D, Pather L, Methven L and Elmore J S 2017 *Food Chem* 232 531-44
[5] Romanczyk L J Jr., McClelland C J, Post L S and Aitken W M 1995 *J Agric Food Chem* 43 469-75
[6] Adams A and Kimpe D N 2006 *Food Chem* 101 1230-8
[7] Deshmukh Y, Khare P and Patra D D 2014 *Biotechnol Prog* 30 1356-63
[8] Costello P J and Henschke P A 2002 *J Agric Food Chem* 50 7079-87
[9] Rungsardthong V and Noomhoom A 2005 *Flavour Fragr J* 20 710-4
[10] Sillapapakdee J, Rungsardthong V and Prathumpai W 2014 Pathway of 2-Acetyl-1- Pyrroline formation by *Aspergillus awamori* TISTR 3193 (King Mongkut’s University of Technology North Bangkok, Bangkok, Thailand)
[11] Yoshihashi T, Huong N T T and Inatomi H 2002 *J Agric Food Chem* 50 2001-4
[12] Grimm C C, Champagne E T, Lloyd S W, Easson M, Condon B and McClung A 2011 *Cereal Chem* 88 271-7
[13] Nicolotti L, Cordero C, Bicchi C, Rubiolo P, Sgorbini B and Liberto E 2013 *Food Chem*. 138 1723-33
[14] Ubeda C, Callejón R M, Hidalgo C, Torija M J, Mas A, Troncoso A M and Morales M L 2011 *Food Res. Int.* 44 259-68
[15] Elliott S and Burgess V 2005 Forensic Sci. Int. 151 289-92
[16] Pho-am S, Kerchhoechuen O and Laohakunjit N 2015 Agricultural Sci J 46 393-6
[17] Janes D, Kantar D, Kref S and Prosen H 2008 Food Chem 112 120-4
[18] Rungsardthong V 1995 Production of 2-acetyl-1-pyrroline, A Major Component of Aromatic Rice Flavours, by Acremonium nigricans (Asian Institute of Technology, Bangkok, Thailand)
[19] Ahmed N 1987 Biochem Educ 15 106-10
[20] Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P and Tiburcio A F 2010 Planta 231 1237-49
[21] Jeon K W 2002 International Review of Cytology (Academic Press, U.S.A.)
[22] Fothergill J C and Guest J R 1976 J Gen Microbiol 99 139-55
[23] Jacoby W B and Fredericks J 1959 J Biol Chem 234 2145-50