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Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese

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ABSTRACT

A yeast isolate, *Candida maltosa* NP9, was obtained from a fermented food, Iranian commercial cheese. The strain grown on YPD agar inhibited spore germination of *Aspergillus brasiliensis* by vapor-agar contact method. Seven volatile compounds generated from the strain were detected by SPME–GC/MS analysis, and three of them were identified as isoamyl alcohol, isoamyl acetate, and phenethyl alcohol by GC/MS analysis. Although phenethyl alcohol did not inhibit the germination at 160 μl/dish by 48 h exposure, isoamyl alcohol fungicidally inhibited at 80 μl/dish and isoamyl acetate fungiostatically inhibited at 160 μl/dish. In antifungal spectra with 15 kinds of filamentous fungi, isoamyl alcohol inhibited the germination of all strains at 20 μl/dish, while isoamyl acetate did not inhibit the germination of three strains even at 160 μl/dish. We demonstrated that *C. maltosa* NP9 from the fermented food generated volatile isoamyl acetate and isoamyl alcohol to inhibit the germination of those fungi.

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1. Introduction

Food contamination by filamentous fungi causes food quality loss and shortens storage period (Filtenborg, Frisvad, & Thrane, 1996; Whitfield, 1998). In addition, ingesting mycotoxins produced by filamentous fungi causes harmful effects on health (Pier, 1992; Wilson, 1978). Pesticides are used to inhibit the growth of contaminating filamentous fungi. Since pesticide residues in foods raise concerns over food safety and health damage to pesticide users poses problems (Darko & Akoto, 2008; Knezevic & Serdar, 2009), we searched traditional fermented foods for microorganisms, which biologically control the growth of pathogenic and food-contaminating fungi.

Several microorganisms, which generated an antifungal substance, have been isolated from sources other than fermented foods. For example, plant endogenous fungus *Muscodor albus* (Strobel, Dirkse, Sears, & Markworth, 2001), plant powdery mildew *Oidium* sp. (Strobel et al., 2008), wheat leaf-derived filamentous fungus *Irpex lacteus* Kyu-W63 (Koitabashi, 2005), and soil bacterium *Bacillus subtilis* JA (Chen et al., 2008) generate volatile substances, such as alcohols and esters, to inhibit the growth of phytopathogenic fungi. Yeasts isolated during the processing of raw coffee beans, *Pichia anomala*, *P. kluyveri*, and *Hanseniaspora uvarum*, generate volatile alcohols and esters to inhibit the growth of *Aspergillus ochraceus* and the production of ochratoxin (Masoud, Cesar, Jespersen, & Jakobsen, 2004; Masoud & Kaltonft, 2006; Masoud, Poll, & Jakobsen, 2005).

Fermented foods are traditional and safe foods. No microorganism, which generates a volatile antifungal substance, has been isolated from fermented foods. Thus, we have searched fermented foods for microorganisms that generate volatile antifungal substances to inhibit the spore germination of food-contaminating filamentous fungi.

2. Materials and methods

2.1. Materials and strains

Isoamyl alcohol, isoamyl acetate, and phenethyl alcohol were purchased from Nacalai Tesuque Inc. (Kyoto, Japan). All of the other chemicals were of analytical grade.

Aspergillus brasiliensis (formerly *Aspergillus niger*) NBRC 9455 was used as a test microorganism. Tested spores were collected from *A. brasiliensis* cultured 4 days at 28 °C on the potato dextrose agar (PDA, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and were suspended in 0.8% saline supplemented with 0.05% (v/v) Tween 80, from which contaminating fungal filaments were removed through sterile gauze. The filtrate was adjusted to 1.0×10^6 spores/ml with

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0.8% sterile saline. The spore concentration was determined using the ten-fold serial dilution method.

2.2. Isolation of microorganisms, which generate an antifungal substance, from fermented foods

Tested samples were 15 commercial cheeses, 5 yogurts and 5 fermented vegetable kimchi (Korean pickle). The samples were static cultured in skim milk medium (100 g skim milk per liter, pH 6.0) at 28 °C for 48 h. Ten-fold serially diluted enrichment cultures were surface plated onto skim milk agar (100 g skim milk and 15 g agar per liter, pH 6.0) and cultured at 28 °C for 48 h. On the plate medium with colonies formed, 10 ml of spore-mixed malt extract soft agar medium (25 g malt extract and 4 g agar per liter, containing *A. brasiliensis* spores 1.0×10^4 spores/ml, pH 5.0) was overlaid and cultured at 28 °C for 48 h. Colonies with growth inhibition zones of *A. brasiliensis* were isolated as antifungal substance-producing microorganisms. The colonies were repeatedly purified and separated, suspended in a 10% (w/v) skim milk solution, and stored at −80 °C. Additionally, colony counts in different lots of Iranian commercial cheese (Caspian cheese, GELA AMOL DAIRY Co., Iran), which were isolation samples of antifungal substance-producing microorganisms, were carried out using the serial dilution method.

2.3. Identification of selected yeast strain

Isolated yeast (designated as NP9) was cultured on yeast malt agar (YM agar, 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose, 15 g agar per liter, pH 6.2) at 25 °C for 48 h. Scanning electron microscope (SEM) images of the yeast were created at 10 kV using Hitachi S-900 Field Emission Gun SEM (Hitachi, Tokyo, Japan). The yeast was fixed with a 1% (w/v) osmium tetroxide solution in phosphate buffer solution (PBS, pH 7.4) at 4 °C for 1 h. The yeast was washed with PBS six times for 10 min. The sample was immersed in ascending concentrations of ethanol, *viz*. 50, 60, 70, 80, 90, and 99.5%, for 15 min, and was finally immersed in a *tert*-butyl alcohol solution three times for 30 min and dehydrated. The sample was freeze-dried. Subsequently, the sample was sputter-coated with platinum-palladium (10 nm) using a Hitachi E-1030 Ion Sputter (Hitachi). Then, SEM images were created. Strain NP9 was identified based on a nucleotide sequence of 26S rRNA D1/D2 gene and the physiological and biochemical properties revealed by the methods of Barnett, Payne, and Yarrow (2000) and Kurtzman and Fell (1998).

The DNA fragment of D1/D2 domain of 26S rRNA gene was amplified by PCR using primer pairs NL-1 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′) and NL-4 (5′- GGTCCGTGTTTCAAGACGG-3′), and sequenced using NL-1, NL-2 (5′-CTCTCTTTTCAAA GTTCTTTTCATCT-3′), NL-3 (5′-AGATGAAAAGAACTTTGAAAAGA-GAG-3′), and NL-4 primers (O'Donnell, 1993). The sequence obtained was compared with 26S rRNA gene sequences using a BLAST program from GenBank/DDBJ/EMBL. The nucleotide sequence of the strain NP9 have been submitted to DDBJ database under the accession number AB568327.

2.4. Antifungal activity of strain NP9 and volatile compounds

The antifungal activity of strain NP9 was determined by the vapor-agar contact method of Nakahara, Alzoreky, Yoshihashi, Nguyen, and Trakoontivakorn (2003) with slight modifications. Yeast extract peptone dextrose agar (YPD agar, 10 g yeast extract, 20 g peptone, 20 g glucose, and 15 g agar per liter, pH 6.5) was added into a small glass vessel (30 mm in diameter). Strain NP9 was inoculated to the YPD agar medium in the vessel and the vessel was placed into a 100-ml glass dish (90 mm in diameter) to be cultured at 28 °C. After 24-h culture, another glass vessel (32 mm in diameter) with a spore suspension (32 spores/μl, 10 μl) of *A. brasiliensis* inoculated in a PDA plate was placed into the 100-ml glass dish at a center-to-center distance of 5 cm. Then, the 100-ml glass dish containing the two glass vessels was sealed with 30 cm of a parafilm membrane (Pechiney Plastic Packaging, Chicago) and was placed into a 500-ml plastic container, and the plastic container was incubated at 28 °C for 48 h. To demonstrate that an antifungal substance was a volatile one, activated charcoal was added into the empty space of the 100-ml glass dish in which two small vessels were placed. In the case of the volatile compounds, the antifungal activity was examined according to the described method, except that the spore of *A. brasiliensis* and the compound were placed into the 100-ml glass dish as the same time. Antifungal activities were compared with the growth of *A. brasiliensis*, a control to which distilled water was added instead of volatile substance. A judgment was made as plus ("+": complete inhibition) or minus ("−": weak or no detectable activity). The minimum inhibitory dose (MID) was defined as the lowest addition (μl/dish) with antifungal activity. The minimum fungicidal dose (MFD) defined as minimum concentration (μl/dish) which fungicidally inhibited spore germination was determined as follows: the ungerminated spore, despite the treatment with the test compound for 48 h, was aseptically removed and transferred into a new PDA plate to examine spore germination at 48 h. Each experiment was repeated three times.

2.5. Analyasis of volatile substances by SPME–GC/MS

To detect the volatile compounds produced by strain NP9, eleven YPD plates with strain NP9 colonies were placed into a glass bulb vessel (25 cm in diameter). Then, the glass bulb vessel was sealed with aluminum seal and incubated at 28 °C for 48 h. The volatile substance in the glass bulb vessel was analyzed by Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (SPME–GC/MS). SPME polydimethylsiloxane (PDMS, 100 μm) fiber (Supelco, Bellefonte, PA, USA) was inserted into the glass bulb vessel and exposed to the headspace of culture for 15 min at 28 °C. The volatile substance adsorbed to the fiber was desorbed in a GC injector maintained at 220 °C. The volatile substance was quantified by GC/MS analysis with an Agilent 6890N Network GC (Agilent Technologies, Santa Clara, CA) connected to a JEOL JMS-K9 mass spectrometer (JEOL Ltd., Tokyo, Japan). A DB-WAX capillary column (30 m, 0.32 mm inner diameter, 0.25 μm film thickness; Agilent Technologies) was used. Helium was used as carrier gas. The temperature of an oven was set as follows: 50 °C for 5 min, a temperature gradient of 4 °C/min up to 220 °C, and a final extension at 220 °C for 12.5 min. An injector was used in a splitless mode at 220 °C. Detection temperature was 230 °C. Mass spectrometer was used in an electron impact mode set at 70 eV electron energy. The linear retention indexes (LRI) were calculated for all substances using a homologous series of *n*-alkanes (C_8–C_{20}) analyzed under the same conditions as the sample. The volatile substances were identified by comparing their LRI and mass fragmentation patterns with the NIST library. Using the authentic compounds, molecular ions of isoamyl acetate, isoamyl alcohol, and phenethyl alcohol were *m/z* 130, *m/z* 88, and *m/z* 122, respectively. Additionally, the LRI of isoamyl acetate, isoamyl alcohol, and phenethyl alcohol were, 1111, 1207, and 1903, respectively. The time course analysis of the volatile compounds was performed according to the method described above with a 100-ml glass dish. The integral of the obtained graph was calculated as the total amount of exposure using Image J 1.44 software (National Institutes of Health, Bethesda, Md). Each experiment was repeated three times.

2.6. Antifungal spectra of isoamyl acetate and isoamyl alcohol

The antifungal activities of isoamyl acetate and isoamyl alcohol against 15 filamentous fungi were determined by the vapor-agar contact method described above. Following fungal strains were used in this experiment: *A. brasiliensis* NBRC 9455, *Aspergillus nidulans* NBRC 33017, *Aspergillus oryzae* NBRC 100959, *Aspergillus fumigatus* No.232, *Aspergillus versicolor* IAM 2027, *Penicillium crysogenum* IAM 13780, *P. citrinum* IFO 4640, *Rhizopus retlexus* IAM 6018, *Rhizopus stolonifer* IAM 6021, *Rhizopus javanicus* IAM 6028, *Mucor hiemalis* IAM 6088, *M. javanicus* IAM 6108, *M. roxianus* IAM 6131, *Botryotinia fuckeliana* IAM 5126 and *Fusarium oxyspolum* IAM 5009 (Table 3). Filamentous fungus spores (320 spores), which were determined by the serial dilution method, were inoculated onto PDA and cultured at 28 °C for 48 h. The experiment was repeated three times.

2.7. SEM observations

Plastic plates (3 × 5 mm) were coated with 0.01% (w/v) poly-L-lysine (PLL, Sigma–Aldrich) dissolved in PBS (pH 7.4). The spores exposed to isoamyl acetate or isoamyl alcohol and the unexposed spores on the PDA surface (control) were electrostatically attached to the PLL-coated plastic plates. SEM was conducted according to the above method.

3. Results

3.1. Screening of microorganisms that Inhibit the spore germination of A. brasiliensis from fermented foods

Microorganisms that inhibited the spore germination of *A. brasiliensis* were searched for by the layer method from 25 fermented foods. As a result, only Iranian commercial cheese (Caspian cheese, GELA AMOL DAIRY Co., Iran) enrichment culture medium inhibited the spore germination of *A. brasiliensis*. When the enrichment culture was plated onto a skim milk agar plate, 113 colonies were obtained and cultured on a new skim milk agar plate at 28 °C for 48 h. Spore-mixed malt extract soft agar was layered to the skim milk agar plate and cultured at 28 °C for 48 h. A strain NP9 with the largest growth inhibition zone of *A. brasiliensis* was selected. Additionally, colonies with the same morphology as strain NP9 and showing inhibitory activity against the spore germination of *A. brasiliensis* were detected at $(5.8 \pm 0.8) \times 10^3$ cfu/g from eight Iranian Caspian cheeses of different lots.

3.2. Morphological characteristics of strain NP9

Strain NP9 was formed white to cream, entire margin in the peripheral shape, flat protrusion, smooth surface, and wet weak luster colonies on a YM plate at 25 °C for 96 h. Strain NP9, cultured at 25 °C for 48 h on YM agar plate, had an elliptical or cylindrical shape and grew through multipolar budding (Fig. 1). Pseudohyphae were formed. No sexual reproductive organ was formed from fungus body after 1-month culture.

Table 1
Volatile compounds generated by *C. maltosa* NP9.

	LRI[a]	Relative amount (%)[b]
Unidentified compound	1045	12.2
Isoamyl acetate	1114	11.1
Isoamyl alcohol	1212	50.5
Unidentified compound	1462	6.3
Unidentified compound	1591	0.9
Unidentified compound	1691	12.2
Phenethyl alcohol	1905	6.8
Total		100

[a] Linear retention indexes were calculated for all volatile substances using a homologous series of *n*-alkanes (C_8–C_{16}) on DB-WAX column.
[b] Relative amounts were calculated from peak area of GC/MS analysis.

Table 2
Inhibition of the spore germination of *A .brasiliensis* with volatile compounds from *C. maltosa* NP9[a]

	Concentration (μl/dish)						
	0	5	10	20	40	80	160
Isoamyl acetate	−	−	−	+	+	+	+
Isoamyl alcohol	−	−	−	+	+	+	+
Phenethyl alcohol	−	−	−	−	−	−	−

[a] The spore germination of tested samples were compared with water (control).as follows: +, completely inhibition;-, weak or no detectable activity.

3.3. Identification of strain NP9

In the Blast search using an international DNA database, the nucleotide sequence of 26S rRNA D1/D2 gene (accession no. AB568327) was completely matched with *Candida maltosa* NRRL Y-17677[T] (accession no. U45745). Although reference strain, *C. maltosa* CBS5611[T], fermented trehalose, strain NP9 did not. Strain NP9 showed a property unseen for *C. maltosa*. However, the other physiological and biochemical characteristics of strain NP9 were the same as those of *C. maltosa* (Kurtzman & Fell, 1998). Accordingly, strain NP9 was identified as *C. maltosa*.

3.4. Spore germination inhibitors generated by C. maltosa NP9

Although a growth inhibition zone of *A. brasiliensis* was formed when spore-mixed soft agar was layered onto the plate with *C. maltosa* NP9 colony, no inhibitory effects on the spore germination were detected when a skim milk medium supernatant was tested by the broth microdilution method (National Committee for Clinical Laboratory Standards, 1998) or the agar spot method (Reenen, Dicks, & Chikindas, 1998). The inhibitory effect on the spore germination of *A. brasiliensis* were detected by the vapor-agar contact method, whereas no antifungal activity was detected by the vapor-agar contact method when tested with activated charcoal added into a glass dish. The results indicated that the spore germination inhibitor generated by *C. maltosa* NP9 may be a volatile substance.

3.5. Volatile compounds generated by C. maltosa NP9 and its inhibitory effects on spore germination

Volatile substances generated by *C. maltosa* NP9 on YPD plate were analyzed by SPME–GC/MS. Seven peaks, except for the volatile compounds derived from the YPD plate, were detected (Table 1). The LRI and mass spectrum of three peaks of them were consistent with those of authentic compounds, namely, isoamyl acetate (1114, m/z 55, 70, and 87 as fragment ions), isoamyl alcohol (1212, m/z 42, 55, and 70 as fragment ions), and phenethyl alcohol (1905, m/z 122 as a molecular ion and m/z 65 and 91 as fragment ions), respectively. Molecular ions of authentic compounds, isoamyl acetate (m/z 130) and isoamyl alcohol (m/z 88), were not detected. Inhibitory effects on the spore germination after 48 h exposure to isoamyl acetate, isoamyl alcohol, or phenethyl alcohol were examined by the vapor-agar contact method. Isoamyl acetate and isoamyl alcohol inhibited spore germination at 20 μl/dish. However, phenethyl alcohol did not even at 160 μl/dish (Table 2). Additionally, it was observed that isoamyl acetate fungiostatically inhibited

Table 3
Antimicrobial spectrum of volatile compounds against fungi.

Fungi	Isoamyl acetate			Isoamyl alcohol		
	Activity[a]	MID[b] (μl/dish)	MFD[c] (μl/dish)	Activity	MID (μl/dish)	MFD (μl/dish)
Aspergillus brasiliensis NBRC 9455	+	20	>160	+	20	80
Aspergillus nidulans NBRC 33017	+	80	>160	+	20	40
Aspergillus oryzae NBRC 100959	+	40	>160	+	20	40
Aspergillus fumigatus No.232	+	80	>160	+	20	80
Aspergillus versicolor IAM 2027	+	160	>160	+	20	40
Penicillium crysogenum IAM 13780	+	40	160	+	20	40
Penicillium citrinum IFO 4640	+	160	>160	+	20	40
Rhizopus retlexus IAM 6018	−	>160	>160	+	20	20
Rhizopus stolonifer IAM 6021	+	40	40	+	20	40
Rhizopus javanicus IAM 6028	+	80	>160	+	20	40
Mucor hiemalis IAM 6088	−	>160	>160	+	20	40
Mucor javanicus IAM 6108	−	>160	>160	+	20	40
Mucor roxianus IAM 6131	+	160	160	+	20	20
Botryotinia fuckeliana IAM 5126	+	80	>160	+	20	80
Fusarium oxyspolum IAM 5009	+	40	>160	+	20	40

[a] The spore germination of tested samples were compared with water (control).as follows: +, completely inhibition;-, weak or no detectable activity.
[b] The minimum inhibitory dose (MID) was defined as the lowest addition (μl/dish) with antifungal activity.
[c] The minimum fungicidal dose (MFD) was defined as minimum concentration (μl/dish) which fungicidally inhibited spore germination.

spore germination at 160 μl/dish and isoamyl alcohol fungicidally did at 80 μl/dish. When these volatile compounds were mixed according to the relative amount which identified in the gas-phase where *C. maltosa* NP9 grew on YPD plate, the mixture inhibited spore germination at 20 μl/dish. Consequently, it was shown that isoamyl acetate, isoamyl alcohol, and phenethyl alcohol were generated by *C. maltosa* NP9, and 160 μl/dish isoamyl acetate and 80 μl/dish isoamyl alcohol fungiostatically and fungicidally inhibited spore germination, respectively.

3.6. The amounts of gas-phase isoamyl acetate and isoamyl alcohol

Using 10, 20 and 160 μl of isoamyl acetate and isoamyl alcohol in the 100-ml glass dish, the vapor-agar contact method was carried out at 28 °C for 48 h. The amounts of these compounds in the gas-phase was analyzed over time by SPME–GC/MS. Isoamyl acetate, when added at 10–160 μl/dish, rapidly increased in the gas-phase for 2 h after addition, showed an almost constant value for 5 h or longer when added at 160 μl/dish, and gradually decreased when added at 20 μl/dish or below (Fig. 2a). Isoamyl alcohol, when added at 10 μl/dish or above, rapidly increased in the gas-phase for 2 h after addition, showed an almost constant value when added at 20 μl/dish or above, and gradually decreased at 12 h or later when added at 10 μl/dish (Fig. 2b). It was indicated that the isoamyl acetate and isoamyl alcohol vaporized actually acted as the inhibiter to the spore germination.

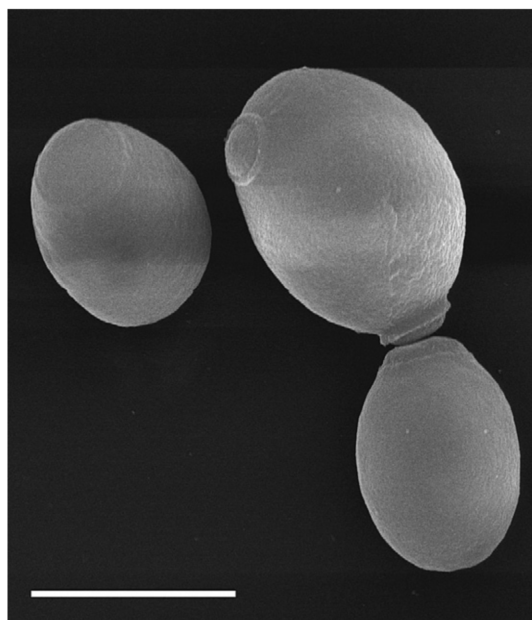

Fig. 1. Scanning electron microscope observation of strain NP9. Bar represent 3 μm.

3.7. Antifungal spectra of volatile isoamyl acetate and isoamyl alcohol

Inhibitory effects on the spore germination of 15 filamentous fungi were examined by the vapor-agar contact method. Isoamyl acetate inhibited the spore germination of 12 filamentous fungi (except for *Rhizopus retlexus*, *Mucor hiemalis*, and *M. javanicus*) at 160 μl/dish. Isoamyl acetate fungicidally inhibited the spore germination of *R. stolonifer* at 40 μl/dish and of *Penicillium chrysogenum* and *M. roxianus* at 160 μl/dish, and bacteriostatically inhibited the spore germination of all the other filamentous fungi (Table 3). Isoamyl alcohol completely inhibited the spore germination of all the filamentous fungi at 20 μl/dish, and fungicidally inhibited the spore germination of all the tested filamentous fungi at 80 μl/dish (Table 3).

3.8. SEM observations of A. brasiliensis spores exposed to volatile isoamyl acetate and isoamyl alcohol

Untreated spores cultured on PDA medium absorbed water and swelled at 5 h, formed elongated hyphae at 7 h, and formed secondary spores at 48 h (Fig. 3a). No difference was observed in the morphology and size between *A. brasiliensis* spores exposed to isoamyl acetate (160 μl/dish) from 0 h to 48 h (Fig. 3b). When the spores exposed isoamyl acetate (160 μl/dish) for 48 h were transferred into new PDA medium, the spore germination was observed at 5 h. Protrusions remained in the exposed spores although the unexposed spores had smooth surface (Fig. 3a,c). After 5-h exposure to isoamyl alcohol (20 μl/dish), the protrusions of *A. brasiliensis* spores disappeared (Fig. 3d). These spores were transferred into new PDA medium and germinated at 9 h (Fig. 3e). The spores exposed to isoamyl alcohol (160 μl/dish) lost their protrusions at 5.

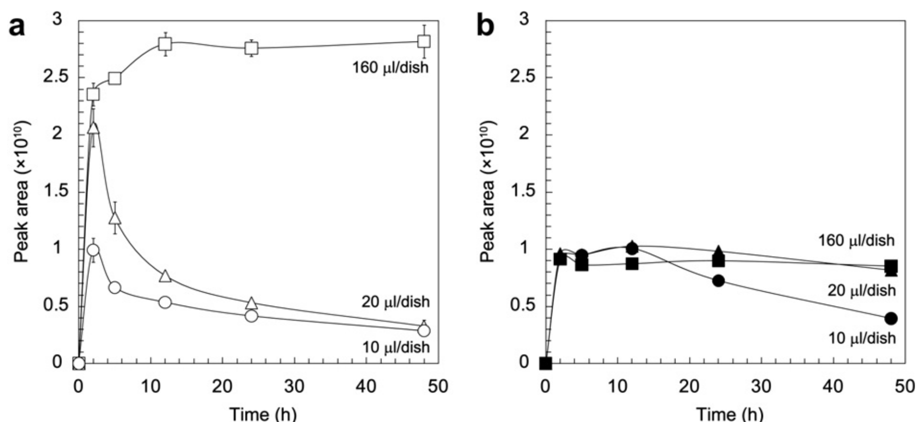

Fig. 2. Amount of volatile isoamyl acetate (a) and isoamyl alcohol (b) in gas-phase. The amounts of 10 μl (circle), 20 μl (triangle) and 160 μl (square) isoamyl acetate (open symbol) and isoamyl alcohol (closed symbol) were added in a small glass dish sealed with a parafilm membrane and incubated at 28 °C. Isoamyl acetate and isoamyl alcohol in gas-phase were assayed using SPME-GC/MS.

However, the spores did not germinate even when transferred into new PDA medium.

4. Discussion

In this study, we isolated *C. maltosa* NP9, which generates volatile antifungal substances, from an Iranian commercial cheese. As volatile antifungal substance-producing yeast, *P. anomala* and *Saccharomyces cerevisiae* CR-1 were isolated during processing of *Caffea arabica* (Masoud et al., 2005) and ethanol fermentation (Fialho, Toffano, Pedroso, Augusto, & Pascholati, 2010), respectively. A major volatility antifungal substance generated by *P. anomala* is phenethyl acetate. However, volatile antifungal substances generated by *S. cerevisiae* CR-1 remain unknown. During the preparation of Ginjo-shu (quality sake brewed from the finest rice), sake yeast generates isoamyl acetate and the compound plays an important role in the flavor of the Ginjo-shu (Arikawa, Yamada, Shimosaka, Okazaki, & Fukuzawa, 2000; Asano, Inoue, Kurose, Hiraoka, & Kawakita, 1999; Hirooka, Yamamoto, Tsutsui, & Tanaka, 2005). However, there have been no reports that the volatile substance generated by sake yeast inhibited the spore germination of filamentous fungi. We first isolated a *Candida* sp. yeast, which generated volatile antifungal substances, from a fermented food.

C. maltosa NP9 was not isolated as a contaminant of an Iranian Caspian cheese, but seems to be involved in the maturation of the cheese. Using eight Caspian cheeses of different lots, *C. maltosa* was detected from all the cheeses at $(5.8 \pm 0.8) \times 10^3$ cfu/g. The antifungal activities of several isolates were examined by the vapor-agar contact method, resulting that all the yeasts inhibited the spore germination of *A. brasiliensis*. Yeasts, *Geotrichum candidum* and *Yarrowia lipolytica*, are involved in cheese maturation (Arfi, Spinnler, Tache, & Bonnarme, 2002; Spinnler, Berger, Lapadatescu, & Bonnarme, 2001). Isoamyl acetate and phenethyl alcohol are detected as flavor ingredients in mature cheese (Lecanu, Ducruet, Jouquand, Gratadoux, & Feigenbaum, 2002). Accordingly, *C. maltosa* NP9 also generates isoamyl alcohol and isoamyl acetate, and is probably involved in the maturation of the Iranian Caspian cheese.

Yeasts generate volatile antifungal substances, such as phenethyl acetate, ethyl acetate, isobutyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl alcohol, and 2-pentanone (Bruce, Verrall, Hackett, & Wheatley, 2004; Fialho et al., 2010; Masoud et al., 2005). *C. maltosa* NP9 also generated seven substances in the gas-phase when grown on the YPD plate (Table 1). Based on peak areas, major compounds were isoamyl alcohol (50.5%) and isoamyl acetate (11.1%). Isoamyl alcohol had higher inhibitory effects on spore germination than isoamyl acetate (Table 3). It was described that the activities of volatile antifungal substances decrease in the following order: organic acid > aldehyde > alcohol > ether > ketone > ester > lactone (Maruzzella, Chiaramonte, & Garofalo, 1961). *P. anomala* and *S. cerevisiae* Y1001 generated volatile phenethyl alcohol showed high antifungal activity (Bruce et al., 2004; Masoud et al., 2005). However, phenethyl alcohol showed no inhibitory effects on the spore germination of *A. brasiliensis* by the vapor-agar contact method (Table 2). The reason for this result could be related to the volatilization volume and diffusion rate of the test volatile substance. Masoud et al. (2005) carried out an antifungal test employing the following method. Volatile substances were added to distilled water to control the concentration of volatiles in the headspace and facilitate further volatiling by heat of solution. We also carried out an additional experiment using the modified vapor-agar contact method, in which phenethyl alcohol increased to 1280 μl/dish was added to 1 ml of distilled water. As a result, phenethyl alcohol inhibited the growth of *A. brasiliensis* at 640 μl/dish containing water, but there were no inhibitory effects without water. From these results, the volatilization volume and diffusion rate of phenethyl alcohol were lower than those of isoamyl alcohol and isoamyl acetate based on the vapor-agar contact method (date not shown), which may have caused no inhibitory effects on the spore germination of *A. brasiliensis*. Additionally, *C. maltosa* NP9 produces a small amount of phenethyl alcohol (6.8%). In the case of *C. maltosa* NP9, the volatile isoamyl alcohol and isoamyl acetate probably inhibited the spore germination of filamentous fungi.

In the vapor-agar contact method, the maximum amount of gas-phase isoamyl acetate was about 2.5 times higher than the amount of isoamyl alcohol (Fig. 2). When isoamyl acetate or isoamyl alcohol was added at 20 μl/dish each, the amount of gas-phase isoamyl acetate rapidly decreased, while that of gas-phase isoamyl alcohol is not decreased for 10 h (Fig. 2). These may be caused by the fact that alcohols are less volatile because of association by hydrogen bonding via a hydroxyl group, while esters without hydroxyl group are highly volatile because there is no association. The amount of gas-phase volatile substance in the sealed container peaked at 2 h and subsequently decreased, as described by Inouye, Takizawa, and Yamaguchi (2001).

After 48-h incubation by the vapor-agar contact method, the total exposure to the spores was calculated by integrating the amounts of gas-phase isoamyl acetate and isoamyl alcohol at each time. As a result, the total exposure of the spores to isoamyl alcohol was about 37% higher than that to isoamyl acetate. Alcohols are nonselectively adsorbed and accumulated mainly in the cell membrane to inhibit membrane functions and exhibit

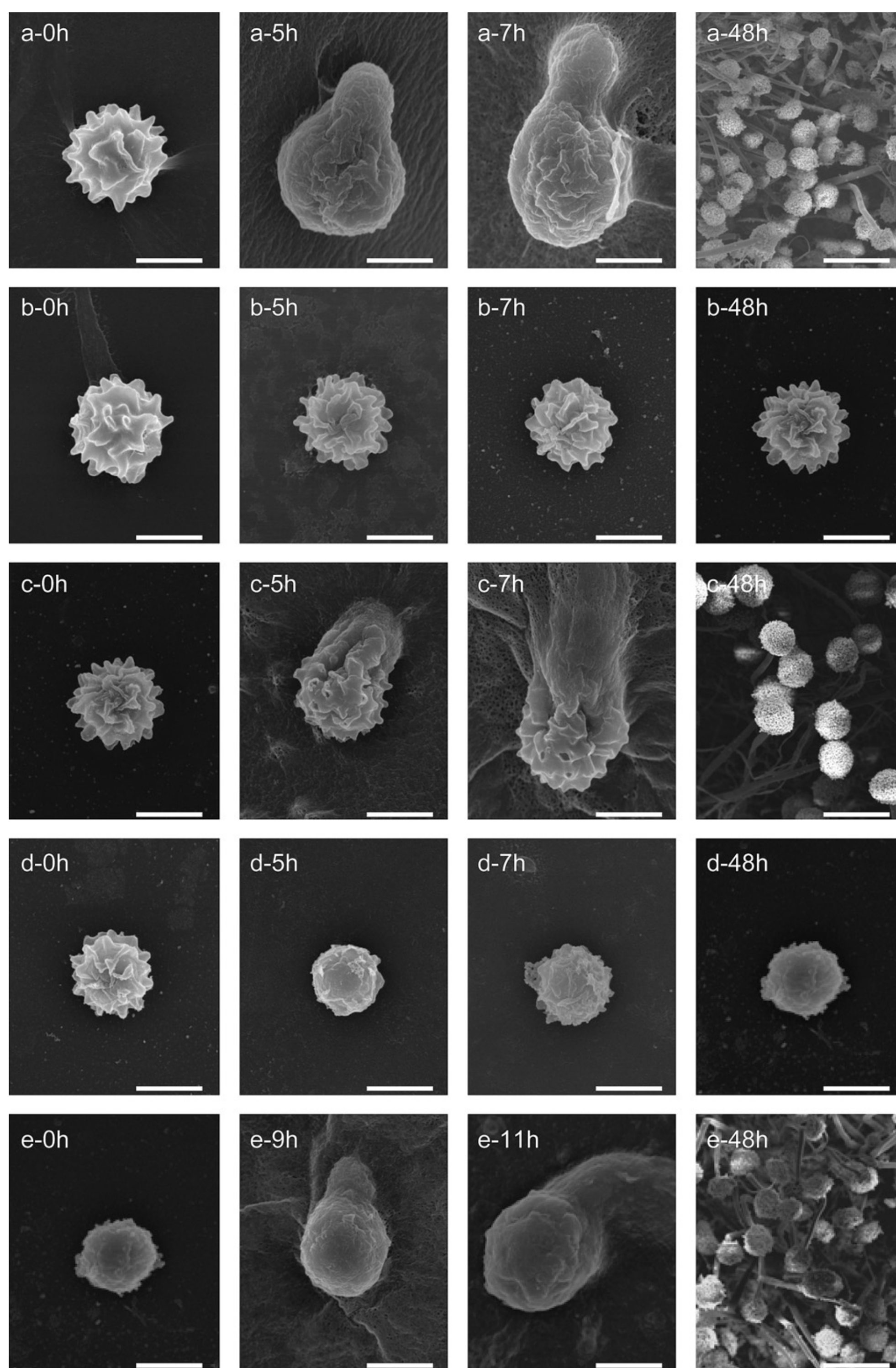

Fig. 3. Scanning electron microscope observations of *A. brasiliensis* spores incubated at 28 °C for 0, 5, 7, 9, 11 and 48 h (a) control, (b) spores exposed to 160 µl/dish isoamyl acetate, (c) spores after removing the isoamyl acetate from exposed spores, (d) spores exposed to 20 µl/dish isoamyl alcohol and (e) spores after removing the isoamyl alcohol from exposed spores. Bars represent 120 µm (a-48 h, c-48 h and e-48 h) and 3 µm (other pictures).

antimicrobial activity (Ingram & Buttke, 1984). It was seemed that isoamyl alcohol adsorbed to the spore surface is less volatile, and therefore adheres for a longer time than isoamyl acetate to act on the spores. This may have caused the high inhibitory effects of isoamyl alcohol on spore germination.

We demonstrated that volatile isoamyl acetate and isoamyl alcohol were generated by the yeast *C. maltosa* isolated from one traditional fermented food, Iranian commercial cheese, and that the compounds inhibited the spore germination of various filamentous fungi. *C. maltosa* NP9 should be applicable as a post-harvest biocontrol agent.

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