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Rahnella aquatilis Strain AY2000 Produces an Anti-Yeast Substance

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Abstract To screen for an anti-yeast substance (AYS), many bacteria were isolated from soil and a strain AY2000 was selected. The strain AY2000 was identified as *Rahnella aquatilis* by morphology, biochemical properties, and 16S r-RNA nucleotide sequence analyses. The strain AY2000 showed anti-yeast activity against *Candida albicans* and *Saccharomyces cerevisiae*, whereas *R. aquatilis* ATCC33071 as a type strain did not show the activity against the yeasts under the same condition. The growth of yeast cell was significantly inhibited by AYS produced by the strain AY2000, as shown by optical density and MTT assay. The minimum inhibitory concentration (MIC) of the AYS against *S. cerevisiae* and *C. albicans* at 28°C was 20 μg/ml and 60 μg/ml, respectively. The MIC of AYS against hyphae of *C. albicans* at 37°C was 600 μg/ml. Scanning electron microscopic analysis revealed that yeast cells treated with AYS had an irregular form with a wrinkled and rough surface.

Key words: Anti-yeast substance, *Rahnella aquatilis*, *Candida albicans*, *Saccharomyces cerevisiae*

Several *Candida* species are normally harmless commences of the gastrointestinal and genitourinary tracts. However, they can also be important pathogens that cause a range of conditions including painful superficial infections, such as virginitis on otherwise healthy women, severe surface infections of the mouth and esophagus in human immunodeficiency virus (HIV) patients, and life-threatening blood stream infections among vulnerable intensive care patients. During the last decade, this organism together with closely related *Candida* species has become one of the most common agents of hospital-aquired infection [6]. Although infections caused by other *Candida* species are increasing in prevalence, the majority of cases of candidiosis are still caused by *Candida albicans*. It has been difficult to develop effective therapies of serious *Candida* infections, because of the limited number of available antifungal agents. Some of the drugs used in the treatment of *Candida* diseases, such as amphotericin B, are very toxic, and others such as flucytosine are limited against *C. albicans* because of drug resistance [7, 18]. The incidence of *Candida* infections has risen dramatically over the past two to three decades, and this trend will inevitably continue into the 21st century. Thus, searching for alternative anti-*Candida* compounds has been a major concern in recent years [11, 13, 19]. Although a number of new anti-Candida antibiotics have been developed, there still exists a need for new agents for treating pathogenic yeast and *Candida* infections.

In this study, we isolated a new bacterium producing an anti-yeast substance (AYS) against *C. albicans*. The isolate was identified and the properties of the AYS produced by the isolate were investigated.

Materials and Methods

Isolation and Strain Used

For isolation of bacteria producing an anti-yeast substance (AYS), soil samples near plant roots were collected and serially diluted in sterile water. The dilutions were spread on PYG agar plates [glucose 1%, peptone 1%, yeast extract 0.5%, KH_2PO_4 0.1% (pH 5.4)] on which yeast cells (0.1 ml spread on surface of agar; 1×10^5 cells) were spread previously, and incubated for 2–3 days at 28°C. After incubation, bacterial colonies that formed a clear zone on PYG agar plates were selected. Yeasts used in this experiment were *C. albicans* KCTC7121 and *Saccharomyces cerevisiae* ATCC2120. A bacterium, *Rahnella aquatilis* ATCC33071, was also used as a reference strain.

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Identification of the Isolate

Cell morphology of the isolate was observed on a scanning electron microscope (SEM). Substrate utilization and other biochemical characteristics were determined with an API 20E kit (bioMerieux S.A., France) and based on Bergey's manual [5]. The 16S rRNA gene was amplified with a PCR using universal primers for the consensus region. DNA sequencing was performed by using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems), based on protocols of the manufacturer. The nucleotide sequence was aligned with 16S rRNA sequences using the electronic mail servers at the Ribosomal Database Project (RDP) [14] and BLAST search DNA sequence databank.

Preparation of AYS

The isolate AY2000 was cultured in YM broth [malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, sucrose 0.5% (pH 5.4)] at 25°C for 24 h. The cultured broth of the strain AY2000 was collected by centrifugation (12,000 rpm, 30 min) and concentrated to 1/60 volume with a rotary evaporator at 40°C. Two volumes of methanol were added to one volume of the concentrate and mixed well at room temperature. Precipitate in the mixture was discarded by centrifugation (12,000 rpm, 10 min). The methanol in the supernatant was completely removed with the rotary evaporator and was dried to make solid with a freezing-dryer. The dried substance was kept at -20°C and used as AYS sample in this experiment.

Determination of Anti-Yeast Activity and Host Spectrum of AYS

For determination of anti-yeast activity in liquid culture, the broth-dilution method [16] described by tne National Committee for Clinical Laboratory Standards (NCCLS) was used. In brief, 2-fold dilution of AYS (100 μl) was successively performed in a 96-well microplate with sterilized saline (100 μl). Then, 2-fold concentrate of liquid medium (100 μl) containing tetracycline (5 μg/ml) was added into each well of the microplate. The yeast cells (30 to 60 cells/20 μl) were also inoculated in the microplate, and they were cultured at corresponding temperatures. The yeast cells grown in the microplate were determined with a Microplate Reader at 650 nm. The anti-yeast activity was expressed as MIC (minimum inhibitory concentration) against *C. albicans* and *S. cerevisiae*. The MIC of AYS was defined as the lowest concentration that inhibits 100% of growth compared with a drug-free control. Amphotericin B (Sigma Co.) was used as the positive control in this experiment.

To investigate the effects of AYS against the growth of various microorganisms, a paper-disk method described by Cremer [3] was performed. A paper disk (Φ8 mm) soaked with AYS (20 μl, 1 mg/ml) was deposited on agar plates that had previously been inoculated with the corresponding test microorganism (1×10^5 cells, 0.1 ml spread on surface of agar). The agar plates were incubated at corresponding temperatures, and diameters of a clear zone surrounding the paper disk were measured for anti-yeast activity.

Determination of Viable Cells and Hyphae Formation of *C. albicans*

For determination of viable cells, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay was performed as described by Yang *et al.* [21]. In brief, *C. albicans* was incubated in YM broth overnight at 28°C. The yeast-form cells were collected by centrifugation (8,000 rpm, 20 min) and washed aseptically with sterilized phosphate-buffered saline (PBS). The yeast-form washed cells were aseptically adjusted to 2×10^6 cell/ml with the sterilized PBS, and a 2-fold dilution of AYS was successively performed with the sterilized PBS. The 2-fold concentrate of YM broth (100 μl) and the 2-fold dilution of AYS (100 μl) were added into each well of the microplate. The microplate was kept at 28°C for 24 h after inoculation of the adjusted yeast-form cells (10 μl). Viability of the yeast-form cells was determined by MTT assay.

For hyphae preparation of *C. albicans*, the method described by Hawser *et al.* [9] was slightly modified and performed. In brief, *C. albicans* was previously cultured overnight in YM broth at 28°C to make yeast-form cells, and the cells were collected by centrifugation (8,000 rpm, 20 min). The cells were washed and adjusted to 2×10^6 cell/ml with the sterilized PBS. The adjusted cells (10 μl) were inoculated in a 96-well microplate containing RPMI 1640 medium (200 μl), and the microplate was kept at 37°C for 24 h to induce hyphae of *C. albicans*. After that, each well of the microplate was aseptically washed 3 times with the sterilized PBS to discard the yeast-form cells of *C. albicans*. Fresh 2-fold concentrate of RPMI 1640 medium was prepared separately, and a 2-fold dilution of AYS was performed successively with sterilized PBS. The fresh 2-fold concentrate of RPMI 1640 medium (100 μl) and each 2-fold dilution of the AYS (100 μl) were introduced into each well of the microplate, which contained hyphae of *C. albicans*. The microplate was incubated at 37°C for 24 h or 48 h, and hyphae viability of *C. albicans* was determined by MTT assay. The RPMI 1640 medium in this experiment included 10% FBS, 2% glucose, and tetracycline (5 μg/ml), but did not contain Na_2CO_3.

Scanning Electron Microscopy

To observe morphological changes of yeast-form cells due to AYS, *C. albicans* was cultured in YM broth containing AYS (30 μg/ml) at 28°C for 24 h. For morphological changes of hyphae of *C. albicans*, hyphae of *C. albicans* induced as described above (hyphae formation in Materials and Methods) were incubated in RPMI medium containing AYS (300 μg/ml) at 37°C for 24 h or 48 h. After incubation, the yeasts were harvested by centrifugation (8,000 rpm,

20 min) and observed under a scanning electron microscope (Hitachi Co, Japan, Model S-2400) following fixation in 2% glutaraldehyde-containing PBS for 1 h at room temperature.

RESULTS AND DISCUSSION

Isolation and Cultivation of Bacteria

In the primary screening of soil samples, we isolated several bacteria on PYG agar plate as described in Materials and Methods. Among these bacteria, a bacterium possessing anti-yeast activity against *C. albicans* was isolated. The isolate (designated as AY2000) was surrounded by a clear zone formed on PYG agar plate on which *C. albicans* was grown. However, the anti-yeast activity of the strain AY2000 against *C. albicans* was not detected in PYG broth. It is likely that the strain AY2000 is unable to produce or secrete AYS in PYG broth. To investigate the secretion of AYS by the strain AY2000, the cells were harvested by centrifugation after cultivation in PYG broth and disrupted by ultrasonication. However, anti-yeast activity of the strain AY2000 was not detected either in intra- or extra-cells. For examination of AYS production by the strain AY2000 in liquid medium, various media and components were investigated and finally, we found that the AYS was always produced when the strain AY2000 was cultured in YM broth at 25°C for 24 h (Fig. 1).

Identification of Isolate AY2000

The isolate AY2000 was a rod-shaped, Gram-negative bacterium, about 2–3 μm in size, and grew well at 25°C, but not at 37°C. As for its metabolic characteristics, the strain was Voges-Proskauer positive and produced acid with fermentation of several carbon sources, including glucose, maltose, L-rhamnose, raffinose, and L-arabinose. Based on the morphological, physiological, and biochemical properties, the strain was identified to belong to the range defined for the genus *Rahnella* [5]. In order to further characterize this strain, the 16S rRNA sequence of the strain was determined and analyzed using the GenBank database. The sequence of the 16S rRNA of the strain exhibited a high similarity to that of *R. aquatilis* (98%). Based on these results, we classified the strain AY2000 as *R. aquatilis*. The strain has a narrow antimicrobial spectrum, because it showed growth inhibition only against yeasts, *C. albicans* and *S. cerevisiae*, on agar plate (Table 1). When the *R. aquatilis* strain ATCC33071 and strain AY2000 were cultured under the same condition (YM broth, 25°C, 24 h), both strains showed different anti-yeast activities (Fig. 1).

R. aquatilis is a Gram-negative rod, rarely isolated, and belongs to the family of Enterobacteriaceae. Its natural habitat is usually water, but the organism has been infrequently isolated from human clinical specimens [2]. It has also been reported as a phosphate-solubilizer, exopolysaccharide-producer, and a nitrogen-fixing bacteria isolated from the rhizosphere of plants [1, 12, 15]. This bacteria, isolated from the phyllosphere of leaves of ramanas rose (*Rosa rugosa*), can produce decarboxylated simple phenolics from benzoic acid derivatives with better antimicrobial activities than those of the substrates [8]. In addition, two *R. aquatilis* strains isolated from soil in Egypt have been reported as an antagonistic strain that produces sideropores as an inhibitory

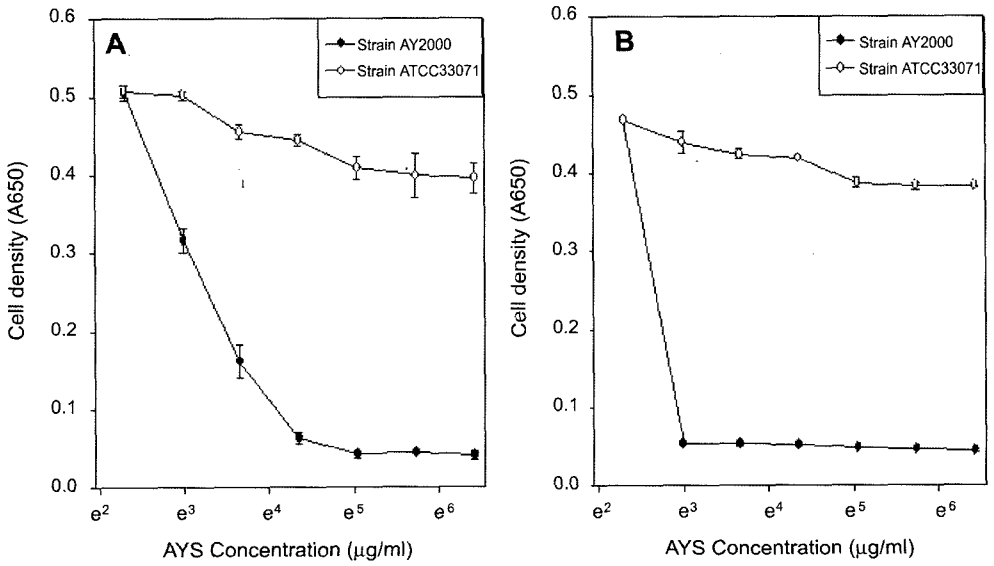

Fig. 1. Effect on yeast growth by metabolites of *R. aquatilis* strainAY2000 and strain ATCC33071.
Strain AY2000 and strain ATCC33071 were cultured in YM broth at 25°C for 24 h, respectively. *C. albicans* (**A**) and *S. cerevisiae* (**B**) were grown in YM broth containing each lyophilized metabolite of the strains, respectively. The yeast cell growth was determined by optical density.

Table 1. Host spectrum of AYS produced by strain AY2000.

Test microorganisms	Media	Antimicrobial activity (diameter in mm of the clear zone)
Saccharomyces cerevisiae ATCC 2120	YM	12.5
Candida albicans KCTC 7121	PYG	12.5
Escherichia coli KCTC1039	PYG	0
Vibrio vulnificus KCTC 2962	PYG	0
Bacillus thuringiensis HD-1	MRS	0
Lactobacillus casei KCTC 3260	Malt	0
Aspergillus oryzae KCTC 6909	Malt	0
Fusarium oxysporium KCTC 16909	Malt	0

Paper disks soaked with 60-fold concentrate (20 μl) of YM broth cultured by strain AY2000 were applied on agar plate, and diameters (mm) of a clear zone surrounding the paper disks were measured for the antimicrobial activity. Compositions of the agar plate media applied to grow test microorganism are as follows: PYG medium [glucose 1%, peptone 1%, yeast extract 0.5%, K_2HPO_4 0.1% (pH 6.8)], YM medium [malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, sucrose 0.5% (pH 5.4)], malt medium [malt extract 2.0% (pH 5.4)].

substance against bacteria [4]. However, we could not find any reports on *R. aquatilis*, which produces an anti-yeast substance, in the literature.

Characterization of the AYS

To investigate characteristics of the AYS, the AYS was further purified by a silica gel column chromatography with a solvent system of butanol:acetic acid:water (4:1:5). The eluate was concentrated with a vacuum evaporator, and the anti-yeast activity of each fraction was tested. Fractions showing the activity were pooled and applied to a Sep-pack (C_{18}) column. Subsequently, preparative HPLC (GS320 column, 3.0 ml/min, Japanese Engineering Instrument Co.) was performed with a solvent system of methanol:methyl cyanide (80:20), containing 0.1% TFA (trifluoroacetic acid). A symmetric peak with anti-yeast activity was collected by simultaneously measuring IR and UV. The collected peak material showed maximal UV absorption at 230 nm. Chemical components of the peak material showed a positive reaction to ninhydrin and anisaldehyde reagents sprayed on TLC. However, amino acids were not detected in the material (data was not shown). The preparation was composed of glucose (77.2%), mannose (16.2%), xylose (2.8%), galactose (1.5%), arabinose (1.2%), glucosamine (1.0%), and galactosamine (0.03%), but fucose was not detected by carbohydrate analysis (CarboPA1 cartridge). These results indicated that the purified AYS may be a substance with heterologous sugars and an uncharacterized 230 nm absorbed substance. However, the anti-yeast activity of the AYS tended to be rapidly decreased during purification. The purified AYS was more unstable than crude AYS preparation during storage. We are still not certain whether the unknown substance has an important role in anti-yeast activity and what the role of the heterologous sugars in AYS is. Recently,

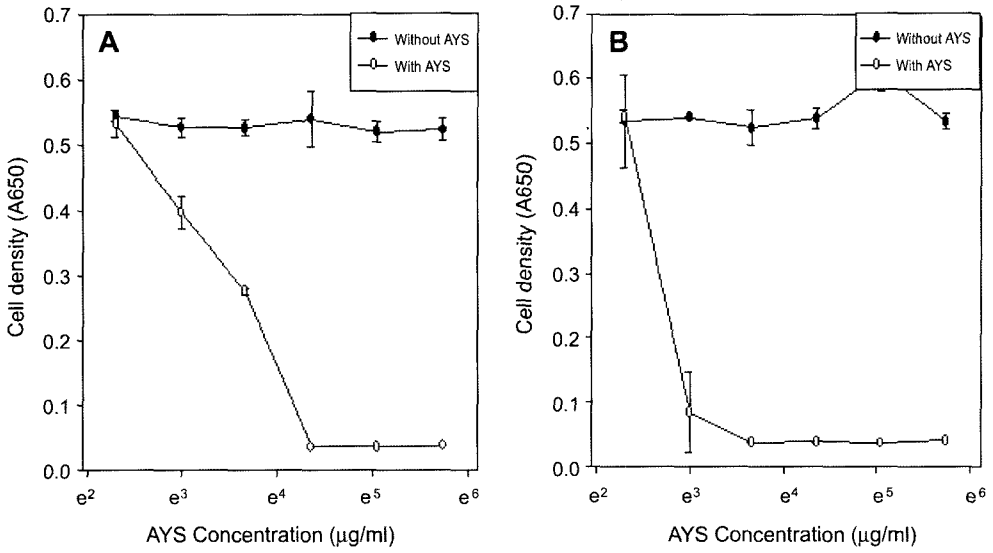

Fig. 2. Effect on growth of *C. albicans* (**A**) and *S. cerevisiae* (**B**) by AYS.
Yeast cells were cultured at 28°C in YM broth containing AYS. The cell growth was determined by optical density.

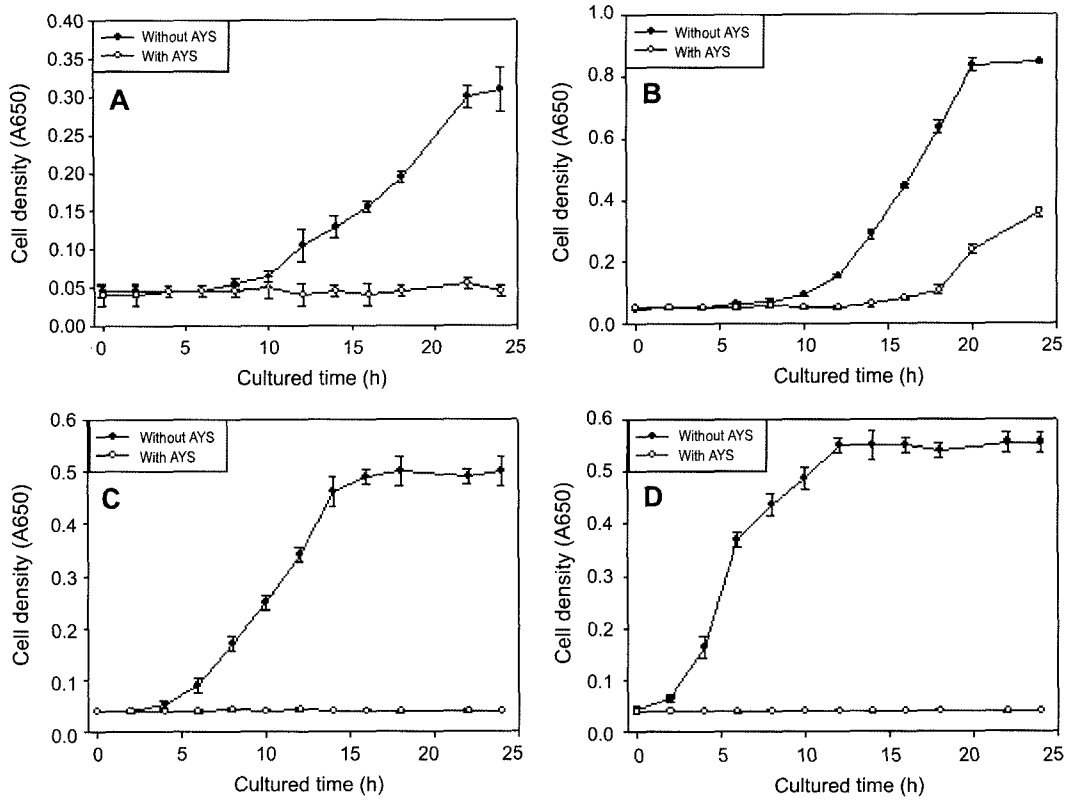

Fig. 3. Growth phenomena of *C. albicans* and *S. cerevisiae* by AYS during culture.
C. albicans was cultured at 28°C (**A**) and 37°C (**B**). *S. cerevisiae* was also cultured at 28°C (**C**) and 37°C (**D**). YM broth was used at 28°C, and RPMI 1640 broth was used at 37°C for culture of the yeasts. The cell growth was determined by optical density.

Steele *et al.* [20] described that a carbohydrate moiety of human oral epithelial cells has a potential role in anti-*Candida* activity, and Nomanbhoy *et al.* [17] also described that the anti-*Candida* activity of both oral and vaginal epithelial cells strictly requires contact to the *C. albicans* cell, but with no role for soluble factors, and that oral

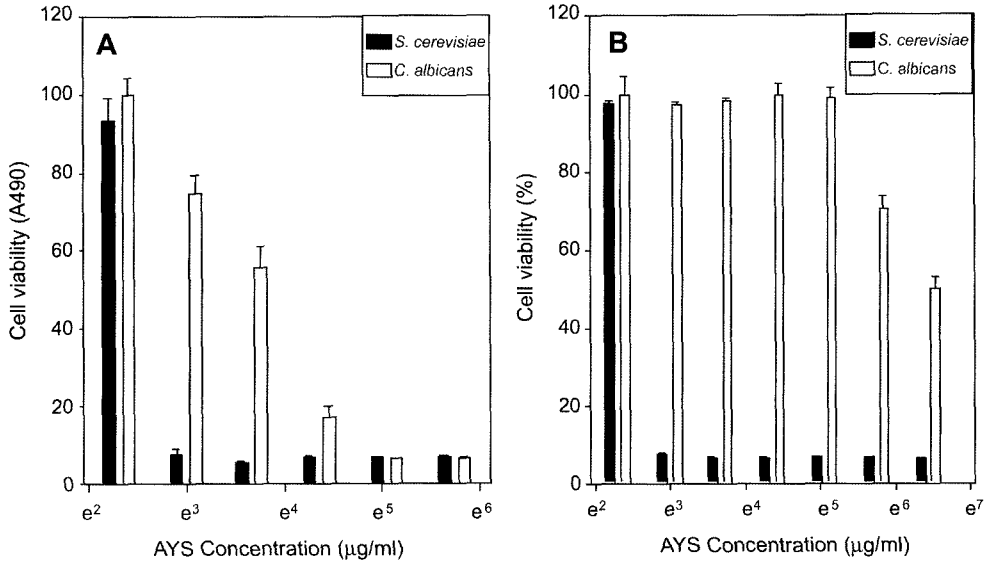

Fig. 4. Viability of yeast cells by AYS at 28°C (**A**) and 37°C (**B**).
C. albicans and *S. cerevisiae* were cultured in YM broth for 24 h, respectively. The viability of the cells was determined by MTT assay.

epithelial cells inhibit *C. albicans* through a cell-surface carbohydrate moiety. They suggested that oral and vaginal epithelial cells retard or arrest the growth rather than kill *C. albicans* through an as-yet-unidentified carbohydrate moiety in a noninflammatory manner.

The MIC of amphotericin B against *C. albicans* and *S. cerevisiae* was below 0.5 μg/ml. During various purification steps of the AYS, its MIC against *S. cerevisiae* was 18 μg/ml (Silica gel column), 15 μg/ml (Sep-pack column), and 10 μg/ml (HPLC GS320 column). However, the MICs of the purified AYS against *S. cerevisiae* and *C. albicans* were 20 μg/ml and 60 μg/ml at 28°C, respectively (Fig. 2). These results indicated that the AYS is more sensitive against *S. cerevisiae* than *C. albicans*. Moreover, the AYS inhibited the growth of *S. cerevisiae* at both 28°C and 37°C, and the growth inhibitory activity was maintained for 24 h (Figs. 3C and 3D). The AYS also inhibited the growth of *C. albicans* at both 28°C and 37°C, but *C. albicans* tended to slowly grow in RPMI medium containing the AYS after 24 h of culture at 37°C (Figs. 3A and 3B). The MIC of the AYS against *S. cerevisiae* showed the same value (20 μg/ml) at 28°C

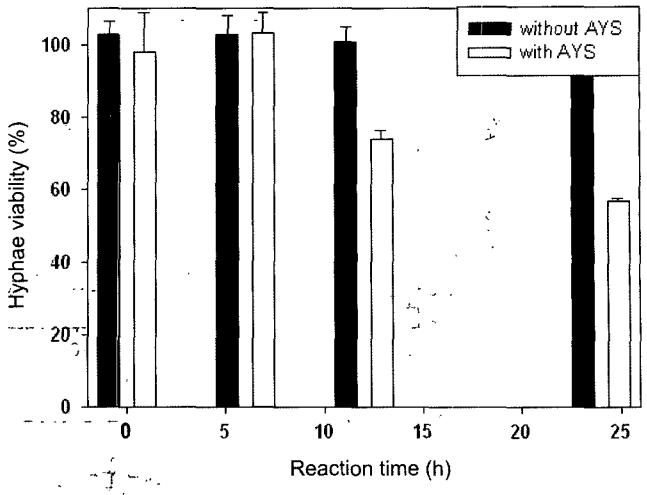

Fig. 5. Viability of hyphae of *C. albicans* by AYS during culture.

C. albicans (2×10^6 cells/ml) cultured previously at 28°C were incubated in RPMI 1640 broth at 37°C for 24 h to induce hyphae. The induced hyphae were treated with AYS in RPMI 1640 broth at 37°C for 24 h. The viability of hyphae of *C. albicans* was determined by MTT assay.

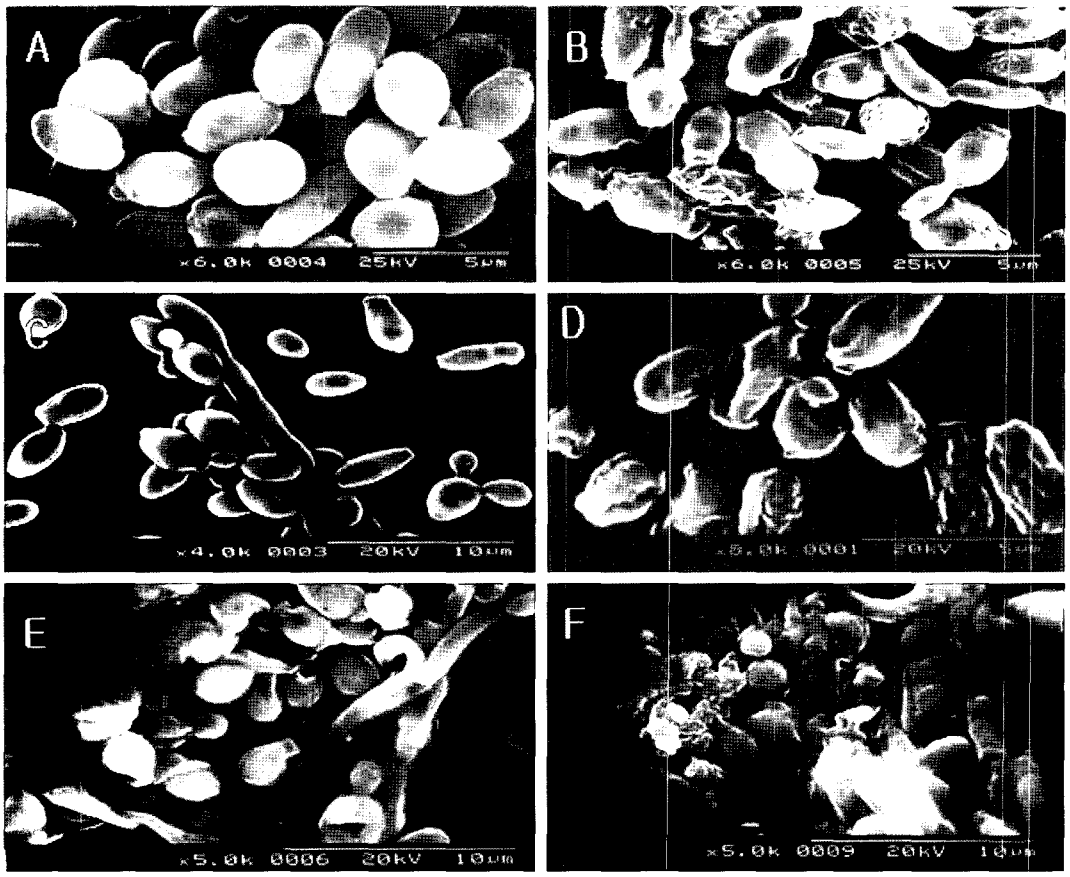

Fig. 6. Morphological changes of yeast cells by AYS on SEM.

C. albicans was cultured in YM broth without AYS (**A**) and with AYS (**B**) for 24 h at 28°C. *C. albicans* was also cultured in RPMI broth without AYS (**C**) and with AYS (**D**) for 24 h at 37°C. *C. albicans* was also incubated in RPMI broth without AYS (**E**) and with AYS (**F**) for 48 h at 37°C. The AYS was applied at 30 μg/ml in YM broth and 300 μg/ml in RPMI broth, respectively.

and 37°C; however, the MIC of the AYS against *C. albicans* showed quite different values (60 and 600 μg/ml) at both temperatures (Figs. 4 and 5). These results indicated that the AYS is more sensitive against yeast-form cells than hyphae of *C. albicans*. Hawser and Islam [10] described the morphogenic transformation/MIC ratios of antifungal agents; (i) amphotericin B, mulundocandin, and aculeacin have ratio <1 (agents that preferentially inhibit morphogenic transformation); (ii) tunicamycin has a ratio of 1–2 (those with approximately equal effects on these two processes); and (iii) azoles, terbinafine, flucytosine, and amorolfine have ratios >2 (agents with a lower ability to inhibit the hyphal form and that exert their activity by inhibiting the yeast-form cell). Therefore, according to the morphogenic transformation/MIC ratios and growth of *C. albicans* after 24 h of culture at 37°C, the AYS seems to have a fungistatic rather than a fungicidal action.

In order to investigate the effect of the AYS on the external structures of *C. albicans* and *S. cerevisiae*, the morphologies of the cells treated by the AYS were examined by scanning electron microscopy (SEM). Compared with the yeast cells without the AYS treatment (Figs. 6A and 6C), SEM revealed that the cells treated with the AYS had the surface of the cells changed to an irregular shape (Figs. 6B and 6D). Specifically, the yeast-form cells of *C. albicans* as well as cells of *S. cerevisiae* treated with the AYS (30 μg/ml) showed seriously burst and wrinkled shape (Fig. 6B). Moreover, *C. albicans* treated with the AYS (300 μg/ml) at 37°C had morphologically shortened hyphae, and a rough and wrinkled surface, compared with hyphae of *C. albicans* without the AYS treatment (Figs. 6C–6F).

In conclusion, *R aquatilis* strain AY2000 was found to produce an anti-yeast substance against *C. albicans* and *S. cerevisiae*. The AYS effectively inhibited the growth of yeast-form cells rather than hyphae of *C. albicans*. We are currently further investigating the nature of the AYS and its action mechanism, together with development of suitable applications.

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