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ASSOCIATIONS AMONG HALOTOLERANCE, OSMOTOLERANCE AND EXOPOLYSACCHARIDE PRODUCTION OF *AUREOBASIDIUM MELANOGENUM* STRAINS FROM HABITATS UNDER SALT STRESS

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Abstract

Associations among halotolerance, osmotolerance and exopolysaccharide (EPS) production of 50 *Aureobasidium melanogenum* strains isolated from various habitats along the coasts of Thailand were compared. Using Fisher's Exact Test, significant associations were found between halotolerance vs osmotolerance (P = 0.004), halotolerance vs EPS production (P = 0.049) and osmotolerance vs EPS production ($p<<0.001$). Highly to moderately halotolerant strains were found to be moderately osmotolerant, but not *vice versa*. Tolerant strains against either salt or sugar produced moderate to low EPS yield. Strains intolerant to salt and/or sugar varied widely in EPS production. The effect of osmotic stress on the growth and EPS yield were investigated with three strains different in halotolerance, osmotolerance and EPS production by comparing their cultures in media with increasing sucrose concentrations. As sucrose concentration increased, a significant reduction in conversion efficiency was observed. Both moderately halotolerant (PBUAP13) and osmotolerant (PBUAP50) strains with moderate EPS production lost their conversion efficiency more drastically than the relatively stress intolerant, high EPS producing strain (PBUAP34). The reduction in EPS production at high osmotic stress was apparently not the result of growth inhibition for both moderately tolerant strains. Cellular accumulation of mannitol was detected in all strains tested.

Key words: β-glucan, Black yeast, Pullulan, Osmolyte.

Introduction

Aureobasidium pullulans (de Bary) G. Arnuad is a ubiquitous yeast-like fungus known as black yeast due to its melanin production (Yurlova & de Hoog, 1997). Its distinctive polymorphic forms are yeast-like cells, blastospores, hyphae, swollen cells, and chlamydospores. *Aureobasidium pullulans* thrives in diverse habitats worldwide, especially in environments with fluctuating water activity including phyllosphere (Punnapayak *et al*., 2003; Prasongsuk *et al*., 2005), bathroom surfaces (Lotrakul *et al*., 2009), hypersaline habitats (Gunde-Cimerman *et al*., 2009), desert marble and Arctic glacier (Zalar *et al*., 2008). *Aureobasidium pullulans* has been considered a polyextremotolerant organism due to its tolerance against some abiotic stresses (Gostinčar *et al*., 2014). In 2008, the infraspecies classification of *A. pullulans* was redefined and four varieties were recognized including var. *pullulans*, var. *melanogenum*, var. *namibiae* and var. *subglaciale* (Zalar *et al*., 2008). These four varieties were later separated into four species: *A. pullulans* and the newly assigned *A. melanogenum*, *A. namibiae* and *A. subglaciale* based on genome comparison (Gostinčar *et al*., 2014). *Aureobasidium pullulans* is often found in mildly osmotic condition and phyllospheres of plants although it can tolerate salt stress up to 17% NaCl (w/v) (Gostinčar *et al*., 2014). Among the four species, *A. melanogenum* is characteristically melanized and forms dark conidia. It is oligotrophic, occurs in the watery habitats including marine water and can grow at 37ºC while the other three species can only grow to 35ºC. *Aureobasidium namibiae* was named for a single strain isolated from the dolomitic marble in Namibia whereas *A. subglaciale* is unique from the others with its psychrotolerance and was isolated from glacial and subglacial ice and seawater (Zalar *et al*., 2008).

Aureobasidium pullulans is of biotechnological importance and has been widely studied for potential industrial applications (Leathers, 2003; Singh *et al*., 2008; Cheng *et al*., 2011). It is most well known for its exopolysaccharide (EPS), pullulan. Pullulan is a neutral, water-soluble biopolymer that is synthesized as a cell-surface attached gel. It is colorless, tasteless, non-toxic, edible, and biodegradable, and has been used in a variety of commercial applications of which demand is still increasing (Cheng *et al*., 2011).

Pullulan production can be limited by osmotic stress caused by high sugar concentration in the culture broth (above 5% (w/v)) (Youssef *et al*., 1999; Cheng *et al*., 2011). It is more economical for pullulan production if a high concentration of sugar can be used since it would reduce the volume of solvent used during recovery. Because of this, it is of interest to improve the pullulan production yield when *A. pullulans* is cultivated in a higher sugar concentration medium. Recently, an osmotolerant strain of *A. pullulans* was studied for pullulan production from sucrose and yielded 60.7 gl^{-1}pullulan from 100 g sucrose (Cheng *et al*., 2011). Similarly, pullulan production by another osmotolerant *A. pullulans* RBF-4A3 isolated from a nectarous flower yielded 66.79 gl^{-1} of pullulan from 150 g glucose (Choudhury *et al*., 2011).

Osmotolerance can be correlated with halotolerance as shown in four food yeasts (Bubnová *et al*., 2014). The adaptation of yeasts to an environment with high osmotic pressure, either sugar or salt, is generally based on a combination of several common mechanisms, such as changes in plasma membrane composition, redox metabolism, the production and transport of glycerol and the activity of various ion transporters (Hohmann, 2002; Thome, 2007). Therefore, it was of interest to investigate whether the highly halotolerant *A. pullulans* and/or the closely related *A. melanogenum* strains would be highly osmotolerant and whether they can grow and produce enhanced pullulan at high sugar concentration.

In this study, *A. pullulans*-like colonies were isolated from habitats within the ocean salt spray zone along the Thai coastline. Strains were identified based on morphological characters and phylogenetic analysis using ribosomal DNA Internal Transcribed Spacer (ITS). Their halotolerance, osmotolerance and EPS production were also investigated and associations among these properties were determined. Three strains with different tolerance and EPS production were also selected to study the effect of high sugar concentration on growth and EPS production.

Materials and Methods

Isolation and culture maintenance: Leaf samples were collected from plants growing at various coastal habitats including mangroves and beachfront gardens in Thailand. Geographic coordinates of the collection sites were shown in Table 1. Tidal zone rock surfaces were swabbed using sterile cotton swabs. Leaves were aseptically cut and placed on half strength malt extract agar (MEA). Chloramphenicol (50 mg/L) and Rose Bengal (0.01% w/v) were added to the medium to delay bacterial and fungal contamination (Prasongsuk *et al*., 2005). Cotton swabs were streaked on the same medium. *Aureobasidium pullulans*-like colonies were transferred to new medium until pure cultures were obtained. All cultures were maintained on MEA and stored at 4ºC. For long-term storage, all cultures were kept in 20% (v/v) glycerol or freeze-dried.

Morphological identification and phylogenetic analysis: A single colony of each strain was stabbed onto potato dextrose agar (PDA), MEA and yeast malt extract agar (YMA) and incubated at 30±2°C for 7 days. Colony morphology was recorded with a CANON IXUS digital camera. For microscopic characters, a single colony of each strain growing on YMA was inoculated into YM broth and incubated at 30±2°C for 1-5 days with agitation at 150 rpm. Cell morphology was observed and photomicrographs recorded (Model Olympus BX51/DP70).

For DNA isolation, each strain was cultured in YM broth overnight at 30±2°C with agitation at 150 rpm. Cells were harvested by centrifugation (4,025xg, 5 min). DNA was extracted by the phenol-chloroform method (Sambrook *et al*., 1989). The ITS region was amplified by PCR using the primers ITS5 and ITS4 (White *et al*., 1990) with thermocycles described by Manitchotpisit *et al*. (2009). DNA sequencing was performed by dideoxy termination method at Macrogen Korea Corp. (Seoul, Korea). Multiple sequence alignment was performed by using MUSCLE (Edgar, 2004) and a phylogenetic tree was constructed by using MEGA 6 v 5.10 (Tamura *et al*., 2013). *Sydowia polyspora* (strain 10666) and *Selenophoma mahoniae* (CBS 242.64) were included as the out groups. For the neighbor-joining analysis, distances between the sequences were calculated based on Kimura's two-parameter model (Kimura, 1980), supporting the confidence limits for branching topologies with bootstrap analysis (1000 replicates). GenBank accession numbers of sequences are listed in Table 2.

Table 1. Geographic coordinates of the sample collection sites.

Collection site	*Geographic Coordinate*
Bangkok (August 2010)	13° 30′ 08.7″ N, 100° 27′05.6″ E
Chonburi (December 2010)	13° 20′ 26.7″ N, 100° 55′32.9″ E
Chonburi (February 2012)	12° 55′ 32.5″ N, 100° 46′29.5″ E
Chumphon (May 2011)	9° 57′ 12.6″ N, 99° 09′28.1″ E
Krabi (April 2011)	7° 38′ 37.4″ N, 99° 01′13.7″ E
Phetchaburi (July 2010)	12° 42′ 14.4″ N, 99° 57′28″ E
Prachuap Khiri Khan (August 2010)	12° 34′ 31.9″ N, 99° 57′29.1″ E
Samut Sakhon (May 2011)	13° 28′ 33.6″ N, 100° 06′13.9″ E
Songkhla (April 2010)	7° 09′ 23.2″ N, 100° 32′04.3″ E

Halotolerance test: Halotolerance was determined by growing the fungus on PDA containing 5% (w/v) NaCl at 30±2°C. Colony diameter was measured at day 7 and relative growth was calculated in comparison to that of the fungus growing on PDA without NaCl addition (Kurtzman *et al*., 2011). Strains with 100-60, <60-40, <40-20 and <20-0 % relative growth were considered to be highly tolerant, moderately tolerant, relatively intolerant and intolerant, respectively. The test was performed in triplicate.

Osmotolerance test: Osmotolerance was determined by growing the fungus on YMA containing 30% (w/v) glucose at 30±2°C. Colony diameter was measured at day 7 and relative growth was calculated in comparison to that of the fungus growing on YMA without glucose added. Strains with 100-60, <60-40, <40-20 and <20-0% relative growth were considered to be highly tolerant, moderately tolerant, relatively intolerant and intolerant, respectively. The test was performed in triplicate.

EPS production: For seed culture preparation, a single colony of each strain was grown overnight in 20 ml of YMB in 50 ml Erlenmeyer flasks at 30±2°C with 150-rpm agitation. Cell density was adjusted to $2.5x10^7$ cells/ml before being transferred at 1% (v/v) to 100 ml of production medium (PM) containing (all w/v) sucrose (5%), $(NH_4)_2SO_4$ (0.06%), peptone (0.06%), K_2HPO_4 (0.5%), $MgSO_4 \cdot 7H_2O$ (0.04%), NaCl (0.1%), and yeast extract (0.04%), in 200 ml Erlenmeyer flasks and grown under the same conditions for 7 days (Prasongsuk *et al*., 2007). The EPS was recovered from cell-free supernatant by ethanol precipitation as described by Choudhury *et al*. (2011). The EPS yield was measured as gram of EPS per liter of the medium, and the production efficiency (% conversion) was calculated as percentage of gram of EPS produced per gram of sugar supplied (Youssef *et al*., 1999). Strains with equal to more than 40, <40-30, <30-20, <20-10 and <10-0% conversion were considered high, relatively high, moderate, relatively low and low EPS production, respectively. The test was performed in triplicate.

Table 2. GenBank accession number of organisms used in this study.

Species	Strain*	ITS
Aureobasidium subglaciale	EXF-2481^T	FJ150895
Aureobasidium subglaciale	EXF-2479	FJ150893
Aureobasidium namibiae	CBS 147.97^T	FJ150875
Aureobasidium melanogenum	CBS 105.22^T	FJ150886
Aureobasidium melanogenum	CBS 123.37	FJ150881
Aureobasidium pullulans	CBS 109810	FJ150901
Aureobasidium pullulans	CBS 100524^T	FJ150905
Aureobasidium thailandense (NRRL 58539)	CBS 133856^T	JX462674
Aureobasidium thailandense (NRRL 58543)	CBS 133857	JX462675
Kabatiella microsticta	CBS 342.66	FJ150903
Selenophoma mahoniae	CBS 388.92	FJ150872
Sydowia polyspora	strain 10666	GQ412728
Aureobasidium melanogenum	PBUAP4	KP965436
Aureobasidium melanogenum	PBUAP5	KP965437
Aureobasidium melanogenum	PBUAP5.1	KP965438
Aureobasidium melanogenum	PBUAP7.1	KP965439
Aureobasidium melanogenum	PBUAP9	KP965440
Aureobasidium melanogenum	PBUAP13	KP965441
Aureobasidium melanogenum	PBUAP14	KP965442
Aureobasidium melanogenum	PBUAP16	KP965443
Aureobasidium thailandense	PBUAP17	KP965444
Aureobasidium melanogenum	PBUAP20	KP965445
Aureobasidium melanogenum	PBUAP22	KP965446
Aureobasidium melanogenum	PBUAP23	KP965447
Aureobasidium melanogenum	PBUAP24	KP965448
Aureobasidium melanogenum	PBUAP25	KP965449
Aureobasidium melanogenum	PBUAP26	KP965450
Aureobasidium melanogenum	PBUAP27	KP965451
Aureobasidium melanogenum	PBUAP29	KP965452
Aureobasidium melanogenum	PBUAP30	KP965453
Aureobasidium melanogenum	PBUAP31	KP965454
Aureobasidium melanogenum	PBUAP32	KP965455
Aureobasidium melanogenum	PBUAP33	KP965456
Aureobasidium melanogenum	PBUAP34	KP965457
Aureobasidium melanogenum	PBUAP35	KP965458
Aureobasidium melanogenum	PBUAP36	KP965459
Aureobasidium melanogenum	PBUAP37	KP965460
Aureobasidium melanogenum	PBUAP38	KP965461
Aureobasidium melanogenum	PBUAP39	KP965462
Aureobasidium melanogenum	PBUAP40	KP965463
Aureobasidium melanogenum	PBUAP41	KP965464
Aureobasidium melanogenum	PBUAP42	KP965465
Aureobasidium melanogenum	PBUAP43	KP965466
Aureobasidium melanogenum	PBUAP44	KP965467
Aureobasidium melanogenum	PBUAP45	KP965468
Aureobasidium melanogenum	PBUAP46	KP965469
Aureobasidium melanogenum	PBUAP47	KP965470
Aureobasidium melanogenum	PBUAP48	KP965471
Aureobasidium melanogenum	PBUAP49	KP965472
Aureobasidium melanogenum	PBUAP50	KP965473
Aureobasidium melanogenum	PBUAP51	KP965474
Aureobasidium melanogenum	PBUAP53	KP965475
Aureobasidium melanogenum	PBUAP55	KP965476
Aureobasidium melanogenum	PBUAP58	KP965477
Aureobasidium melanogenum	PBUAP59	KP965478
Aureobasidium melanogenum	PBUAP61	KP965479
Aureobasidium melanogenum	PBUAP62	KP965480
Aureobasidium melanogenum	PBUAP65	KP965481
Aureobasidium melanogenum	PBUAP67	KP965482
Aureobasidium thailandense	PBUAP70	KP965483
Aureobasidium melanogenum	PBUAP71	KP965484
Aureobasidium thailandense	PBUAP72	KP965485
Aureobasidium melanogenum	PBUAP73	KP965486
Aureobasidium melanogenum	PBUAP75	KP965487
Aureobasidium melanogenum	PBUAP76	KP965488
Aureobasidium thailandense	PBUAP77	KP965489

*^{T} indicates type specimen

Effects of sucrose concentration on growth and EPS production: Effects of sucrose concentration on growth and EPS production were investigated by growing each selected strains in PM containing concentrations of sucrose ranging from 5 to 20% (w/v) under the same conditions as previously described. Cell and EPS dry weights were measured 5 days after inoculation. Relative growth and EPS conversion were calculated in comparison to values obtained in PM containing 5% (w/v) sucrose. The test was performed in triplicate.

Detection of intracellular osmolyte: Intracellular osmolyte was extracted using the method described by Managbanag and Torzilli (2002) with minor modification. Cells grown in PM with a range of concentrations of sucrose were harvested by centrifugation (4,025xg, 5 min) and suspended in 5 mL of sterile deionized H_2O. An equal volume of sterile glass beads (0.2 mm) was added and cells were broken by 15 rounds of vortexing, each round comprised three cycles of 30 s each. The extracts were kept on ice for 15 s between cycles. Cell debris was removed by centrifugation (5 min at 1500xg) and the supernatant stored at -20°C. To detect the osmolyte, the samples were spotted onto Silica Gel 60 F524 TLC plates (Merck, Darmstadt, Germany) and separated using butanol-pyridine-water (15:30:20, v/v) as the mobile phase. Spots were developed by dipping the plates in 0.5% (w/v) $KMnO_4$ in 1 N NaOH. Mannitol (Merck, Darmstadt, Germany) and glycerol (Sigma) prepared at 2% (w/v) were used as standards.

Statistical analysis

Associations among halotolerance, osmotolerance, and EPS production were determined as paired data (halotolerance and osmotolerance, halotolerance and EPS production, osmotolerance and EPS production) using Fisher's exact test. The analysis was performed by using IBM SPSS Statistics for Windows Version 22 (IBM Corp., USA). Significant differences between relative growths among strains and at different sugar concentrations and differences between relative EPS production among strains and at different sugar concentrations were determined by one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS 17.0 software package (SPSS Inc., USA). Differences at $p<0.05$ were considered significant.

Results

Isolation of *A. melanogenum* strains from coastal habitats: Between 2010 and 2012, 54 strains of *A. pullulans*-like isolates were obtained from a variety of coastal habitats at different geographical locations, both the mainland and islands, covering both Gulf of Thailand (South China Sea) and the Andaman Sea (Indian Ocean) (Table 3). Most strains were isolated from living leaf samples including seven species of mangrove plants (*Acanthus ilicifolius* L., *Avicennia marina* (Forssk.) Vierh., *Avicennia officinalis* L., *Azima sarmentosa* (Blume) Benth. & Hook.f., *Rhizophora mucronata* Lam., *Sonneratia alba* Sm. and *Sonneratia caseolaris* (L.) Engl.), five species of sandy beach plants (*Casuarina equisetifolia* L., *Hibiscus tilliaceus* L., *Ipomoea pes-caprae* (L.) R. Br., *Thespesia populnea* (L.) Sol. ex Corrêa and *Thespesia populneoides* (Roxb.) Kostel.), one species of plant commonly found on man-made salterns (*Suaeda maritima* (L.) Dumort.) and ten species of plants that do not specifically grow in salt water habitats (*Acacia auriculiformis* Benth., *Calotropis gigantea* (L.) Dryand.,

Conocarpus erectus L., *Dimocarpus logan* Lour., *Diospyros* sp., *Ludwigia adscendens* (L.) H.Hara, *Pithecellobium dulce* (Roxb.) Benth., *Pterocarpus* sp., *Tamarindus indica* L. and *Terminalia catappa* L.). All mangrove and saltern plants were naturally exposed to brackish water directly during high tide period whereas the other plants were native to beachfronts within the salt spray zone. On leaf surfaces soaked with salt water, once the water evaporated fine salt crystals were visible. One mangrove genus, *Avicennia*, foliar salt glands are present and salt is secreted out on the surface (Tan *et al*., 2013). Four strains were isolated from rock surfaces in the intertidal zone. Despite several attempts, isolation of *A. pullulans*-like colonies directly from marine water was unsuccessful, even when an enrichment protocol was employed (data not shown).

Most of the strains obtained in this study formed dark olivaceous to black colonies when aged, which was typical of *A. pullulans* and *A. melanogenum*. Six strains (PBUAP4, PBUAP5, PBUAP5.1, PBUAP7.1, PBUAP55 and PBUAP58) were color variants that produced pink, yellow, and purple pigments instead of the usual dark melanin. Such color variants have been suggested to occur only in the tropical and subtropical habitats (Leathers *et al*., 1988). All dark and color variant strains produced polymorphic cells typical of *Aureobasidium* spp. These *Aureobasidium* strains isolated from the marine-salt water habitats were morphologically indistinguishable from their counterparts from terrestrial habitats previously reported in Thailand (Punnapayak *et al*., 2003; Prasongsuk *et al*., 2005; Prasongsuk *et al*., 2007; Lotrakul *et al*., 2009; Manitchotpisit *et al*., 2009).

To investigate the phylogenetic relationships between these *A. pullulans*-like strains, *A. pullulans* and four related species namely *A. melanogenum*, *A. namibiae*, *A. subglaciale* and *A. thailandense*, sequences from the ITS region were analyzed. Fifty strains were placed in a clade with *A. melanogenum*, whereas the other four strains seemed to be more closely related to *A. thailandense* although their placement was still inconclusive (Fig. 1).

Associations among halotolerance, osmotolerance and EPS production: When 50 strains of *A. melanogenum* and four strains of *A. thailandense*-like were tested for their halotolerance, osmotolerance and EPS production, a wide variation among the three properties was observed (Table 4). Overall, the strains tested seemed to be less tolerant to ionic osmotic (salt) stress than non-ionic osmotic (sugar) stress as severe growth inhibition (less than 20% relative growth) was observed at NaCl concentration of 10% (w/v) (~1.7 M) (data not shown) whereas most strains retained more than 30% relative growth when grown in a medium containing 30% (w/v) (~1.67 M) glucose. At 50% (w/v) sucrose, growth of all strains was strongly inhibited with less than 22% relative growth observed (data not shown). A notably halotolerant strain PBUAP48 showed 70% relative growth when grown in 5% (w/v) NaCl whereas the highly osmotolerant strains included PBUAP61, 67, 70 and 77 with more than 60% relative growth in the medium containing 30% (w/v) glucose. There was no apparent association (P = 0.249) between halotolerance and the direct exposure to salt water since some strains isolated from plant leaves and rock surfaces in the intertidal zone were relatively halointolerant. For EPS production, some strains were overproducers with more than 50% conversion rate whereas many strains did not produce detectable EPS. To determine if there were associations among these three properties of the 50 *A. melanogenum* strains, Fisher's exact test was used and significant associations were found between halotolerance vs osmotolerance (P = 0.004), halotolerance vs EPS production (P = 0.049) and osmotolerance vs EPS production ($p<<0.001$). Highly to moderately halotolerant strains were found to be moderately osmotolerant. However, highly osmotolerant strains might or might not be halotolerant. Tolerant strains against either salt or sugar stress produced low to moderate EPS yields (less than 10 % to 30 % conversion). Strains relatively intolerant to salt and intolerant to sugar varied widely in their EPS production, exhibiting % conversion in a range of undetectable to more than 60%. The four *A. thailandense*-like strains exhibited similar trend regarding associations among halotolerance, osmotolerance and EPS production. These four were too small a number to be statistically analyzed. Two of the *A. thailandense*-like strains (PBUAP17 and 77) were moderately halotolerant and highly to moderately osmotolerant (Table 4) whereas the other two strains (PBUAP70 and 72) were highly to moderately osmotolerant but relatively halointolerant (Table 4). All four strains produced EPS in very low amounts.

Effect of sucrose concentration on growth and EPS production: To investigate how *A. melanogenum* strains with different halotolerance, osmotolerance and EPS production would respond to elevating osmotic stress, i.e. sucrose concentration, three strains were selected, PBUAP13 (moderately halotolerant and moderately osmotolerant with moderate EPS production), PBUAP34 (relatively halo- and osmointolerant with high EPS production) and PBUAP50 (relatively halointolerant and moderately osmotolerant with moderate EPS production). Based on FT-IR analysis and enzyme sensitivity test (Lotrakul *et al*., 2013), the EPS produced by these three strains was pullulan (data not shown). Their EPS production was compared using the production medium and culture condition that were optimal for most Thai *A. pullulans* and *A. melanogenum* strains (Prasongsuk *et al*., 2007). The strains were grown in media containing sucrose 5 to 20% (w/v). Responses to increasing osmotic stress were observed as relative growth (% of those grown in 5% (w/v) sucrose) and relative conversion (% of those grown in 5% (w/v) sucrose). Significantly greater growth ($p<0.05$) were found in the moderately tolerant strains (PBUAP13 and 50) than the relatively intolerant strain (PBUAP34) at sucrose concentration of 15% (w/v) and greater (Fig. 2A). Similar changes in growth were found between the two moderately tolerant strains in that their cell dry weights increased when the sucrose concentration was raised from 5% to 15% (w/v). At 20% (w/v) sucrose, a slight decrease in growth was observed in both tolerant strains, but the cell dry weights were still significantly greater than those at 5% (w/v) sucrose. On the contrary, significant growth inhibition occurred in the relatively intolerant strain when the sucrose concentration reached 20% (w/v). In contrast to growth, both moderately tolerant strains lost their EPS production efficiency very quickly when the sucrose concentration was increased higher than 5% (w/v). At 20% (w/v) sucrose, the conversion efficiency of PBUAP13 and 50 were 38.3±1.2 and 38.5±3.4% of those at 5% (w/v) sucrose, respectively (Fig. 2B). The relatively intolerant strain also lost its EPS production, but not as drastically as the moderately tolerant strains. At 20% (w/v) sucrose, the conversion efficiency of PBUAP34 was 45.0±0.8% of that at 5% (w/v) sucrose (Fig. 2B). Significantly higher conversion efficiency was observed in the relatively intolerant strain than the two moderately tolerant strains at all sucrose concentrations higher than 5% (w/v).

Table 3. *Aureobasidium* strains isolated from various habitats along Thai coasts.

Strain	Source of isolation	Place and date of isolation
PBUAP4	*Thespesia populnea* (L.) Sol. ex Corrêa	Songkhla (April 2010)
PBUAP5	*Hibiscus tilliaceus* L.	Songkhla (April 2010)
PBUAP5.1	*Hibiscus tilliaceus* L.	Songkhla (April 2010)
PBUAP7.1	*Rhizophora mucronata* Lam.	Songkhla (April 2010)
PBUAP9	*Acanthus ilicifolius* L.	Songkhla (April 2010)
PBUAP13	*Calotropis gigantea* (L.) Dryand.	Songkhla (April 2010)
PBUAP14	*Ipomoea pes-caprae* (L.) R.Br.	Songkhla (April 2010)
PBUAP16	*Terminalia catappa* L.	Phetchaburi (July 2010)
PBUAP17	*Pithecellobium dulce* (Roxb.) Benth.	Phetchaburi (July 2010)
PBUAP20	*Ipomoea pes-caprae* (L.) R.Br.	Phetchaburi (July 2010)
PBUAP22	*Rhizophora mucronata* Lam.	Bangkok (August 2010)
PBUAP23	*Rhizophora mucronata* Lam.	Bangkok (August 2010)
PBUAP24	*Terminalia catappa* L.	Songkhla (April 2010)
PBUAP25	*Sonneratia caseolaris* (L.) Engl.	Bangkok (August 2010)
PBUAP26	*Avicenna officinalis* L.	Bangkok (August 2010)
PBUAP27	*Ludwigia adscendens* (L.) *H. Hara*	Prachuap Khiri Khan (August 2010)
PBUAP29	*Acacia auriculiformis* Benth.	Chonburi (December 2010)
PBUAP30	*Acacia auriculiformis* Benth.	Chonburi (December 2010)
PBUAP31	*Acacia auriculiformis* Benth.	Chonburi (December 2010)
PBUAP32	*Acacia auriculiformis* Benth.	Chonburi (December 2010)
PBUAP33	*Tamarindus indica* L.	Chonburi (December 2010)
PBUAP34	*Tamarindus indica* L.	Chonburi (December 2010)
PBUAP35	*Tamarindus indica* L.	Chonburi (December 2010)
PBUAP36	*Sonneratia alba* Sm.	Chonburi (December 2010)
PBUAP37	*Sonneratia alba* Sm.	Chonburi (December 2010)
PBUAP38	*Sonneratia alba* Sm.	Chonburi (December 2010)
PBUAP39	*Terminalia catappa* L.	Krabi (April 2011)
PBUAP40	*Casuarina equisetifolia* L.	Krabi (April 2011)
PBUAP41	*Diospyros* sp.	Krabi (April 2011)
PBUAP42	*Diospyros* sp.	Krabi (April 2011)
PBUAP43	*Diospyros* sp.	Krabi (April 2011)
PBUAP44	*Pterocarpus* sp.	Krabi (April 2011)
PBUAP45	*Pterocarpus* sp.	Krabi (April 2011)
PBUAP46	*Suaeda maritime* (L.) Dumort.	Samut Sakhon (May 2011)
PBUAP47	*Suaeda maritime* (L.) Dumort.	Samut Sakhon (May 2011)
PBUAP48	*Terminalia catappa* L.	Chumphon (May 2011)
PBUAP49	*Terminalia catappa* L.	Chumphon (May 2011)
PBUAP50	*Terminalia catappa* L.	Chumphon (May 2011)
PBUAP51	*Azima sarmentosa* (Blume) Benth. & Hook.f.	Samut Sakhon (May 2011)
PBUAP53	*Dimocarpus longan* Lour.	Chonburi (February 2012)
PBUAP55	*Conocarpus erectus* L.	Chonburi (February 2012)
PBUAP58	*Conocarpus erectus* L.	Chonburi (February 2012)
PBUAP59	*Conocarpus erectus* L.	Chonburi (February 2012)
PBUAP61	*Avicennia marina* (Forssk.) Vierh.	Chonburi (February 2012)
PBUAP62	*Avicennia marina* (Forssk.) Vierh.	Chonburi (February 2012)
PBUAP65	Rock surface	Chonburi (February 2012)
PBUAP67	Rock surface	Chonburi (February 2012)
PBUAP70	Rock surface	Chonburi (February 2012)
PBUAP71	Rock surface	Chonburi (February 2012)
PBUAP72	*Thespesia populneoides* (Roxb.) Kostel.	Chonburi (February 2012)
PBUAP73	*Diospyros* sp.	Chonburi (February 2012)
PBUAP75	*Avicennia marina* (Forssk.) Vierh.	Chonburi (February 2012)
PBUAP76	*Diospyros* sp.	Chonburi (February 2012)
PBUAP77	*Azima sarmentosa* (Blume) Benth. & Hook.f.	Chonburi (February 2012)

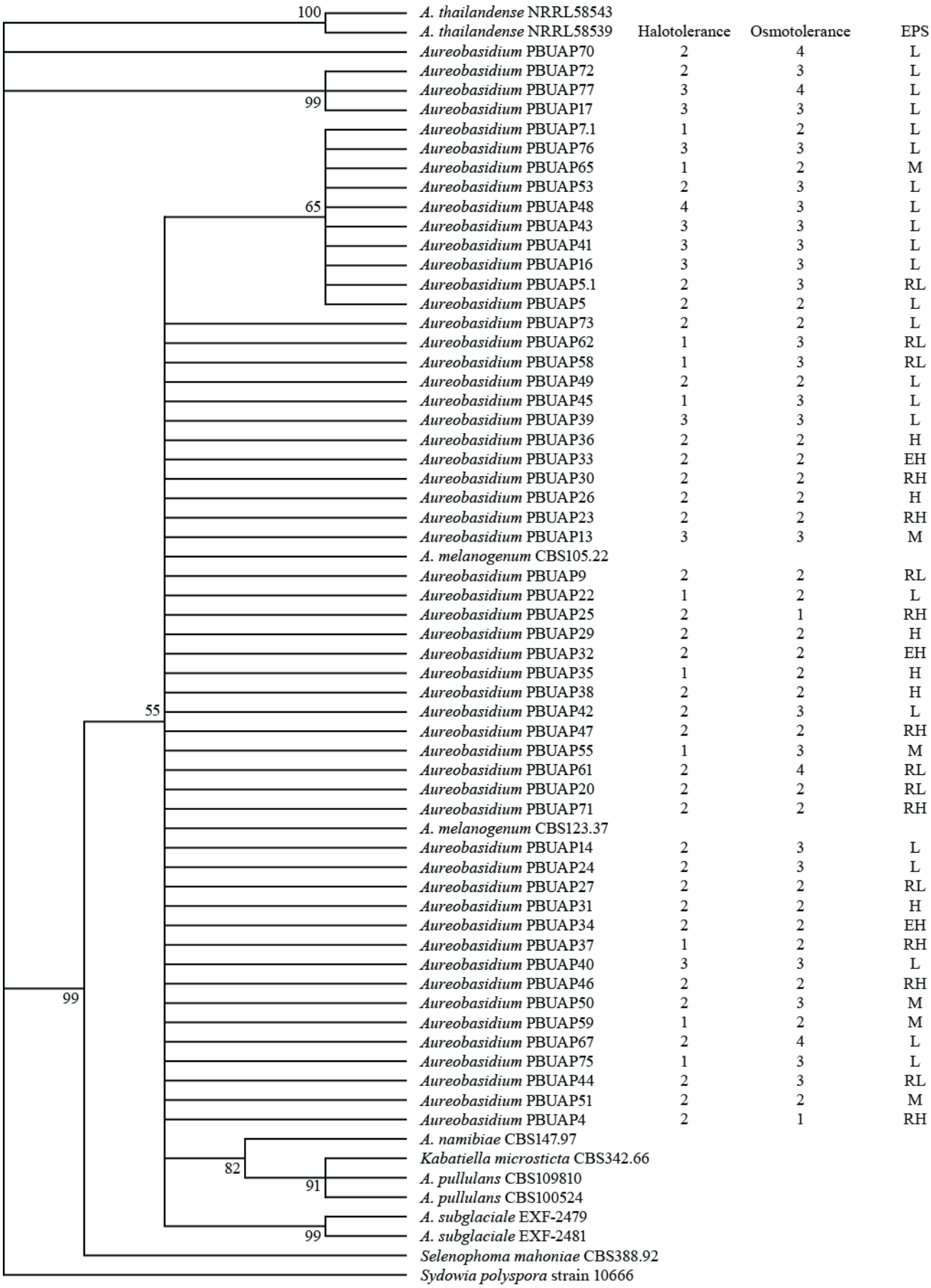

Fig. 1. Neighbor-joining tree depicting the relationships based on the partial ITS sequences between the 54 *Aureobasidium* new strains and five standard *Aureobasidium* species. Level of halotolerance, osmotolerance and EPS production are shown. Isolates are from the phyllosphere except for those with asterisks which are from rock surfaces. Numbers on the nodes indicate bootstrap supports.

Table 4. Halotolerance, osmotolerance, and EPS production of *Aureobasidium* strains. Cultures were grown on agar media containing either 5% (w/v) NaCl or 30% (w/v) glucose, and in liquid EPS production medium containing 5% (w/v) sucrose, respectively.

Strain	Halotolerance	Osmotolerance	Conversion efficiency (%)
PBUAP48	++++	+++	L (1.9)*
PBUAP13	+++	+++	M (25.4)
PBUAP16	+++	+++	L (2.0)
PBUAP17	+++	+++	L (1.6)
PBUAP39	+++	+++	L (1.9)
PBUAP40	+++	+++	L (6.9)
PBUAP41	+++	+++	L (1.7)
PBUAP43	+++	+++	L (2.8)
PBUAP76	+++	+++	L (6.9)
PBUAP77	+++	++++	L (ND)
PBUAP4	++	+	RH (31.4)
PBUAP5	++	++	L (8.1)
PBUAP5.1	++	+++	RL (11.5)
PBUAP9	++	++	RL (11.1)
PBUAP14	++	+++	L (2.1)
PBUAP20	++	++	RL (13.9)
PBUAP23	++	++	RH (30.1)
PBUAP24	++	+++	L (7.1)
PBUAP25	++	+	RH (35.3)
PBUAP26	++	++	H (40.4)
PBUAP27	++	++	RL (18.7)
PBUAP29	++	++	H (44.7)
PBUAP30	++	++	RH (35.3)
PBUAP31	++	++	H (41.4)
PBUAP32	++	++	EH (54.6)
PBUAP33	++	++	EH (59.1)
PBUAP34	++	++	EH (63.7)
PBUAP36	++	++	H (45.9)
PBUAP38	++	++	H (45.0)
PBUAP42	++	+++	L (2.7)
PBUAP44	++	+++	RL (11.4)
PBUAP46	++	++	RH (32.0)
PBUAP47	++	++	RH (31.6)
PBUAP49	++	++	L (6.9)
PBUAP50	++	+++	M (29.1)
PBUAP51	++	++	M (26.3)
PBUAP53	++	+++	L (0.8)
PBUAP61	++	++++	RL (14.9)
PBUAP67	++	++++	L (3.2)
PBUAP70	++	++++	L (5.9)
PBUAP71	++	++	RH (31.3)
PBUAP72	++	+++	L (1.4)
PBUAP73	++	++	L (ND)
PBUAP7.1	+	++	L (8.1)
PBUAP22	+	++	L (1.6)
PBUAP35	+	++	H (45.6)
PBUAP37	+	++	RH (33.1)
PBUAP45	+	+++	L (1.6)
PBUAP55	+	+++	M (24.7)
PBUAP58	+	+++	RL (11.9)
PBUAP59	+	++	M (25.5)
PBUAP62	+	+++	RL (18.4)
PBUAP65	+	++	M (24.4)
PBUAP75	+	+++	L (3.3)

Halotolerance: ++++ = relative growth ≥ 60 %, +++ = relative growth <60-40 %, ++ = relative growth <40-20%, + = relative growth <20%
Osmotolerance: ++++ = relative growth ≥ 60 %, +++ = relative growth <60-40 %, ++ = relative growth <40-20%, + = relative growth <20%
Conversion efficiency of EPS production: EH = extremely high (≥ 50 %), H = high (<50-40 %), RH = relatively high (<40-30 %), M = moderate (<30-20 %), RL = relatively low (<20-10 %), L = low (<10 %)
* Number in parentheses indicates averaged % conversion, ND = not detectable

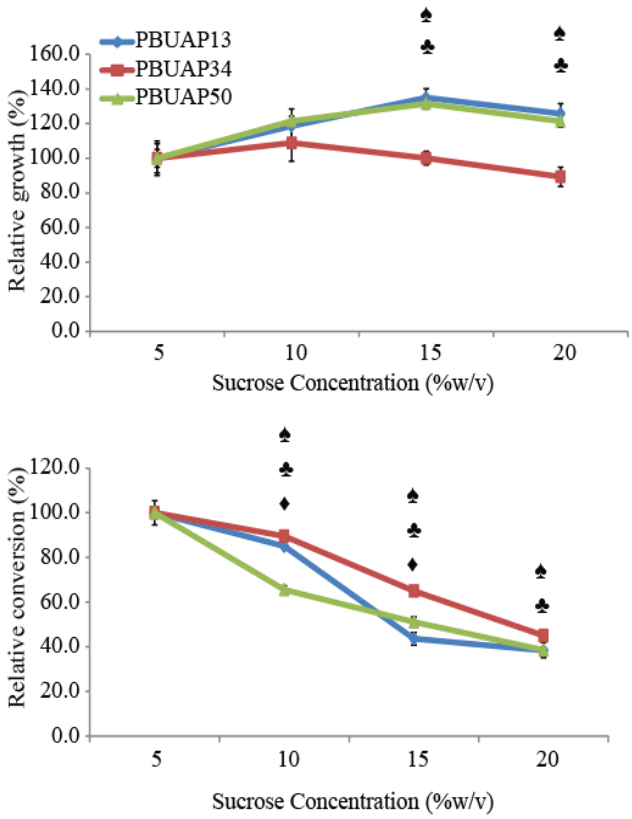

Fig. 2. Effect of sucrose concentration on growth and comparison to its efficiency of conversion. (A) Relative growth (compared with growth in the medium containing 5% (w/v) of sucrose) and (B) relative conversion (compared with conversion in the medium containing 5% (w/v) of sucrose) of *A. melanogenum* strains PBUAP13, 34 and 50. All strains were grown in production medium containing sucrose at concentrations of 5–20% (w/v) at 30±2°C with 150-rpm agitation for 7 days. The symbols: ♠ indicates significant difference between PBUAP13 and 34, ♣ indicates significant difference between PBUAP 34 and 50 and ♦ indicates significant difference between PBUAP13 and 50.

Intracellular osmolyte accumulation: Cellular accumulation of mannitol was semiquantitatively detected in all three strains tested, PBUAP13, 34 and 50 (Fig. 3). In a medium without osmotic stress (1% (w/v) sucrose), low level of mannitol accumulation was detected in all strains tested. Mannitol accumulation in the moderately halotolerant and osmotolerant PBUAP13 was not visibly changed even when sucrose concentration was raised from 5% to 20% (w/v). In the relatively halotolerant and osmointolerant PBUAP34, accumulation of mannitol increased at 15% (w/v) sucrose and higher. Accumulation of mannitol in the moderately osmotolerant but relatively halointolerant PBUAP50 was apparently a direct response to the increasing sucrose concentration. No glycerol was found in any strains and at any sucrose concentrations tested. The patterns of mannitol accumulated in all three strains were different, and it did not correlate with their tolerance properties. For example, although PBUAP13 was moderately tolerant to both salt and sugar, its mannitol accumulation did not change even when sucrose concentration reached 20% (w/v). On the other hand, in the relatively intolerant strain PBUAP34, accumulation of mannitol increased at the highest concentration of sucrose (Fig. 3). The other tolerance mechanisms must also contribute to the differences in halotolerance and osmotolerance among these strains.

Discussion

Among 54 *A. pullulans*-like strains obtained from various habitats under salt stress along Thai coasts, *A. melanogenum* was apparently the dominant species showing relatively low genetic diversity (Fig. 1) compared to their terrestrial counterparts (Manitchotpisit *et al*., 2009). Failure to isolate *A. pullulans* from the same samples was unexpected as the species has been frequently obtained from terrestrial phyllosphere and moist surfaces (Punnapayak *et al.*, 2003; Prasongsuk *et al*., 2005; Prasongsuk *et al*., 2007; Manitchotpisit *et al*., 2009) and it has been reported to be the most halotolerant among the four related species (Gostinčar *et al*., 2014).

Significant associations were found among halotolerance, osmotolerance and EPS production of the 50 *A. melanogenum* strains (Table 4). Strains tolerant to ionic stress (salt) were also tolerant to non-ionic stress (sugar). However, strains tolerant to non-ionic stress might or might not be tolerant to ionic stress. It has been suggested that highly osmotolerant food yeasts would be highly halotolerant and *vice versa* because there are common mechanisms for adaptation to environments with either ionic or non-ionic osmotic stress (Bubnová *et al*., 2014). However, this association was not found among these *A. melanogenum* strains (Table 4).

The severe growth inhibition found in most *A. melanogenum* strains when grown in a medium containing 5% (w/v) NaCl might explain why the direct isolation from marine water was unsuccessful in this study. However, a number of *A. pullulans* and *A. melanogenum* have been isolated from hypersaline water and solar salterns (Gunde-Cimerman *et al*., 2000; Liu *et al*., 2009; Wu *et al*., 2012; Gostinčar *et al*., 2014; Wang *et al*., 2014). Growth inhibition in 20 % (w/v) glucose (Fig. 2A) was likely caused by the combination of osmotic stress (Hohmann, 2002) and oxygen deprivation due to the high medium viscosity (Kumar *et al*., 2012).

Several strains of *A. pullulans* and *A. melanogenum* have been cited as halotolerant (Gunde-Cimerman *et al*., 2000; Gostinčar *et al*., 2014), but only one strain has been shown to be osmotolerant and able to grow in a medium containing high sugar concentration (Choudhury *et al*., 2011; 2012). Three isolates of *A. pullulans* and two isolates of *A. melanogenum* (identified as *A. pullulans* var. *pullulans* and *A. pullulans* var. *melanogenum*, respectively) were isolated from flowers of *Caesulia axillaris* Roxb., a freshwater plant, in India. These isolates were reportedly able to grow in glucose 60% (w/v) although the relative growth compared to those at low glucose concentration was not shown. The growth of one particular strain, *A. pullulans* RBF-4A3, was 3-fold enhanced in 15% (w/v) glucose and gradually declined at higher concentrations (Choudhury *et al*., 2011). Positive growth in 60% (w/v) sugar has been reported in a number of osmotolerant food yeasts, especially *Zygosaccharomyces* spp. (Dakal *et al*., 2014). The highest sugar concentration in which *Z. rouxii* could grow, albeit very slowly, was 90% (w/v) (5 M) glucose (Martorell *et al*., 2007). *Aureobasidium pullulans* has been considered a polyextremotolerant yeast and it can tolerate many extreme abiotic stresses (Gostinčar *et al*., 2010). However, the criteria used for considering *A. pullulans* as halotolerant and osmotolerant were not clearly drawn, especially in term of growth.

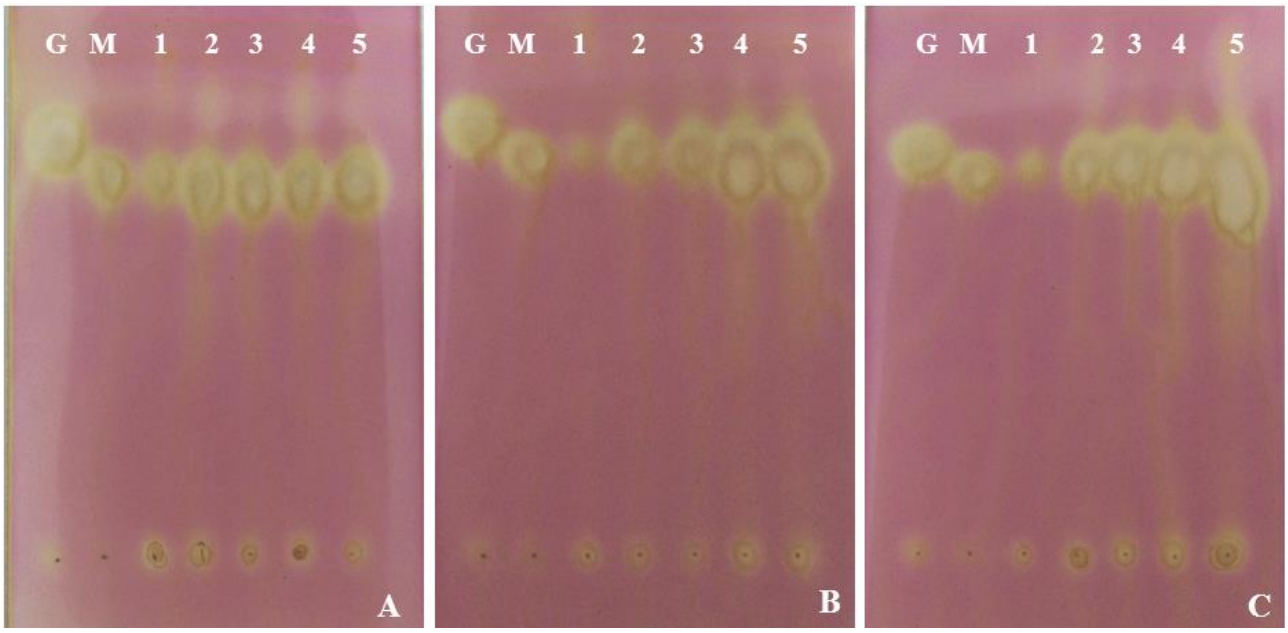

Fig. 3. Cellular extracts of *A. melanogenum* analyzed by thin layer chromatography. *A. melanogenum* strains were grown in production medium containing various concentration of sucrose at 30±2°C with 150-rpm agitation for 7 days. (A) PBUAP13, (B) PBUAP34 and (C) PBUAP50. Lane G: glycerol (0.01 mg), Lane M: mannitol (0.01 mg), Lanes 1-5: extracts of cells grown in media containing 1, 5, 10, 15 and 20% (w/v) sucrose, respectively.

There have been extensive studies on EPS, especially pullulan, production by *A. pullulans* (Cheng *et al*., 2011; Choudhury *et al*., 2012; Prjapati *et al*., 2013; Wang *et al*., 2013). However, most studies focused mainly on the EPS yield on a dry weight basis. Industrially a strict parameter is efficiency of conversion of the substrate. Though the majority of *A. melanogenum* (27 of 50) strains exhibited relatively low EPS production efficiency (less than 20 % conversion), eight were 30%, six 40%, two 50% and one over 60% efficient. This range of conversion efficiency is similar to previous reports (Prasongsuk *et al*., 2007; Manitchotpisit *et al*., 2009; Cheng *et al*., 2011; Choudhury *et al*., 2011), yet noteworthy as PBUAP 34 showed conversion rate above 60%. However, high EPS conversion efficiency was found to be associated with intolerance against either salt or sugar (Table 4).

There have been only a few reports focusing on EPS production by osmotolerant strains of *A. pullulans* at a sugar concentration above 10% (w/v). At first glance it seemed that these strains produced EPS in higher amounts when the sugar concentration was increased. However, when% conversion was considered, all reported strains lost their production efficiency drastically at sugar concentrations higher than 15% (w/v) (Wu *et al*., 2009;Cheng *et al*., 2011; Choudhury *et al*., 2011; 2012) which was similar to the results obtained in this study (Fig. 2). According to Wu *et al*. (2009), *A. pullulans* AP329 was apparently osmotolerant because its growth was not inhibited in a medium containing 15% (w/v) sweet potato hydrolysate [comprised 1% (w/v) glucose, 8.19% (w/v) maltose and 4.9% (w/v) maltotriose]. However, at this concentration, the conversion efficiency was less than 25% compared to 60% with sweet potato hydrolysate 5% (w/v). The osmotolerant *A. pullulans* RBF-4A3 optimally produced 70.4 gl^{-1} pullulan in a batch medium with 16.7% (w/v) glucose (Choudhury *et al*., 2012), only 42% efficient. In glucose concentrations of 20 and 25% (w/v), the conversion efficiency of *A. pullulans* RBF-4A3 decreased to less than 30 and 20%, respectively (Choudhury *et al*., 2011).

One of the common mechanisms that yeasts usually use to survive osmotic stress is the accumulation of intracellular osmolytes to lower their cellular water potential. Glycerol and mannitol were among the most common fungal osmolytes (Hohmann, 2002; Managbanag & Torzilli, 2002; Kogej *et al*., 2005). *A. pullulans* accumulated mannitol when it was exposed to heat and/or salt stresses whereas glycerol was accumulated only under salt stress (Managbanag & Torzilli, 2002). Similarly, *A. melanogenum* used mannitol, but not glycerol, accumulation when exposed to osmotic stress caused by high sugar concentration (Fig. 3). Therefore, mannitol is a likely universal osmolyte for all stresses involving water activity in *A. pullulans* and related species whereas glycerol is possibly a specific osmolyte for salt stress only.

Conclusion

Aureobasidium melanogenum was the dominant *Aureobasidium* species in habitats exposed to salt stress along coasts of Thailand. No association was found between the direct exposure to salt water and halotolerance. Halotolerance in *A. melanogenum* was significantly associated with osmotolerance, but not *vice versa*. Halo- and/or osmotolerant strains produced low to moderate EPS yield. This property might be one of their adaptation mechanisms for tolerance against osmotic stress as released EPS may lower the water potential of their surrounding water. The results may lead to development of a better understanding of the physiological mechanisms of tolerance against osmotic stress in the genus *Aureobasidium*. High pullulan-producing and stress tolerant strains are currently under investigation for potential in various industrial applications.

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