Morphological change, such as from yeast-like to biofilm, has been recently considered to be involved in the mode of action of some antagonistic yeasts used as postharvest biocontrol agents. In the present study, the biocontrol yeast, *Pichia kudriavzevii*, reversibly shifted from a yeast-like morphology on yeast peptone dextrose (YPD) medium with 2% agar to a biofilm morphology on YPD with 0.3% agar. The tolerance of *P. kudriavzevii* to heat and oxidative stresses, as well as the biocontrol efficacy against postharvest diseases on pear fruit, increased significantly from the yeast-like form to the biofilm form. The activity of antioxidant enzymes, including catalase and superoxidase dismutase, in the biofilm form was also significantly higher. The elevated activity of antioxidant enzymes was associated with less protein and lipid oxidation in the biofilm form, compared to the yeast-like form, under heat and oxidative stresses. These results suggest that activation of antioxidant system with the morphology shift contributes to the enhancement of abiotic stress tolerance and biocontrol performance of *P. kudriavzevii*. These findings provide new information on the biology of yeast antagonists that is essential for their potential application and development.

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the first principles and concepts of postharvest biocontrol were proposed (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992), the utilization of yeasts has been extensively studied (Droby et al., 2009; Janisiewicz and Korsten, 2002; Liu et al., 2013; Sharma et al., 2009; Spadaro and Gullino, 2004). Among the yeast antagonists, the genus *Pichia*, including *P. guilliermondii* (Wisniewski et al., 1991), *P. angusta* (Fiori et al., 2008), *P. membranaefaciens* (Liu et al., 2009), *P. anomala* (Jiakki, 2011) and *P. caribbica* (Li et al., 2014), have been reported as effective postharvest biocontrol agents.

A yeast-based biocontrol system is composed of a tritrophic interaction between a fruit host, a pathogen and a yeast antagonist, all of which are subject to environmental conditions (Liu et al., 2013). Among the environmental factors, the effects of heat and oxidative stresses on yeast viability and performance were especially emphasized in a recent review (Sui et al., 2015). Once antagonistic yeasts have been applied, especially before fruit harvest to manage latent diseases, exposure to heat stress is inevitable (Feliziani and Romanazzi, 2013; Ippolito and Nigro, 2000). During the interaction with fruit host, the biocontrol agent, which serves as an elicitor, can trigger reactive oxygen species (ROS) signaling and activate host defense system (Chan and Tian, 2006; Hershkovitz et al., 2012; Macarissin et al., 2010). In turn, antagonistic yeasts must be able to tolerate ROS-derived oxidative stress to exhibit their biocontrol performance (Castoria et al., 2003; Liu et al., 2011a, 2012). Either heat or oxidative stress could cause oxidative injury to cells, like protein carbonylation and lipid peroxidation. Thus, yeasts need to possess effective antioxidant mechanisms, mediated by complex transcriptional (Liu et al., 2012) and enzymatic (Liu et al., 2011b) changes, to cope with these cellular damages caused by abiotic stresses.

Gene expression, metabolic and physiological changes in some bacteria and fungi would occur under specific conditions, resulting in morphological shift and biofilm formation (Fanning and Mitchell, 2012; Hall-Stoodley et al., 2004). In the cases of antagonistic yeasts, this morphological change may contribute to their biocontrol ability to inhibit postharvest diseases by creating a mechanical barrier interposed between fruit host and pathogen, and interfering with pathogen spore germination signals from host (Liu et al., 2013). For example, *Saccharomyces cerevisiae* M25 strain collected at a biofilm phase exhibited greater control effect on blue mold on apple fruit, compared to the same strain at other non-biofilm phases (Schemer, 2003). More recently, *Leucosporidium scottii* AT17 isolated from Antarctic soil was identified as a good biocontrol agent for blue and gray mold on apple fruit. Further study proved that the strain had the capacity to form a biofilm when grown in apple juice, which was an important attribute to successfully colonize apple fruit tissues (Vero et al., 2013). Likewise, another yeast antagonist, *Kloeckera apiculata* 34-9 was able to form biofilm on citrus fruit and embed in an extracellular matrix, contributing to the control of blue mold on citrus (Pu et al., 2014). Based on the previous findings, biofilm formation was proposed to be one of the important modes of action in some yeast antagonists used as biocontrol agents against fruit diseases. It is worth to note that, however, not all morphological changes were positively related to biocontrol performance of antagonistic yeasts. This is especially true for the biofilm-forming strain of *Pichia fermentans* DiSAABA 726. It was initially reported that this yeast strain in a yeast-like form was capable of inhibiting brown rot on apple fruit, but formed pseudohyphae on peach fruit and caused fruit decay (Giobbe et al., 2007). Additional studies were conducted to identify genes by suppressive subtractive hybridization (Fiori et al., 2012; Sanna et al., 2014) and proteins by proteome analysis (Maseri et al., 2015) involved in the morphological change of this yeast strain. To the best of our knowledge, however, little information is available on the relationship between the morphological change and stress tolerance of antagonistic yeasts.

The objective of the present study was to evaluate the level of stress tolerance, antioxidant enzyme and biocontrol efficacy in yeast-like and biofilm forms of the antagonistic yeast, *Pichia kudriavzevii*. More specifically, this study determined this yeast in yeast-like and biofilm forms, (i) the cell viability after exposure to a range of high temperature, and a range of oxidative stress conditions, established by varying conditions of hydrogen peroxide; (ii) antioxidant enzyme activity of catalase (CAT) and superoxide dismutase (SOD), protein and lipid oxidation in response to abiotic stresses; (iii) biocontrol efficacy against two major postharvest diseases, gray mold (*Botrytis cinerea*) and anthracnose (*Colletotrichum gloeosporioides*), on pear fruit.

2. Materials and methods

2.1. Antagonistic yeast

The yeast *P. kudriavzevii* MI-1 was isolated from kiwifruit surface, and identified by its general morphology and DNA sequence of the ITS region of ribosomal DNA according to Leaw et al. (2006). *P. kudriavzevii* was cultured at 25 °C for 48 h on yeast peptone dextrose agar (YPDA, 10 g of yeast extract, 20 g of peptone, 20 g of dextrose, and 2% agar in 1 l of water).

2.2. Fungal pathogen

The fungal pathogens, *B. cinerea* and *C. gloeosporioides*, were isolated from infected pear fruit and maintained on potato dextrose agar (PDA). To reactivate the culture and verify their pathogenicity, the pathogens were inoculated into wounded pear fruit and re-isolated onto PDA once infection was established. Spore suspension of each pathogen was obtained from 2-week-old cultures at 25 °C. Spore concentration was determined using a hemocytometer and adjusted to 10^6 spores/ml with sterile distilled water prior to use.

2.3. Fruit sample

Pear (*Pyrus bretschneideri* Rehd cv. ‘Dangshansulii’) fruits were harvested at commercial maturity. Fruits without wounds or rot were selected based on uniformity of size, disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water and air-dried, prior to their use in biocontrol assays.

2.4. Morphology change

*P. kudriavzevii* was evaluated for its ability to form an elaborate pattern of biofilm formation, as described by Giobbe et al. (2007). A single colony of *P. kudriavzevii* cultured on YPD plates containing 2% agar was transferred onto the center of new YPD plates containing either 2% or 0.3% agar. The plates were then incubated at 25 °C for 2 days in the dark. Two-day-old yeast cultures obtained from 2% or 0.3% agar were evaluated for abiotic stress tolerance and biocontrol performance. Each assay utilized three replicates from three separate YPDA plates, and the assays were repeated three times. Samples were photographed prior to their use in the assays of stress tolerance and biocontrol efficacy.

2.5. Assay of yeast tolerance to abiotic stresses

Assays of abiotic stress tolerance of *P. kudriavzevii* were based on the previous study (Liu et al., 2012), with slight modification. To assay tolerance to high temperature, *P. kudriavzevii* obtained
from the 2%-agar and 0.3%-agar cultures (1-ml suspension of 1 × 10^6 CFUs/ml in 1.5-ml eppendorf tubes) were incubated for 30 min in separate water baths set at either 51, 53 or 55 °C. To simulate oxidative stress, yeast obtained from the 2%-agar and 0.3%-agar cultures (5-ml suspension of 1 × 10^7 CFUs/ml in 50-ml conical flasks) were exposed for 30 min to a final concentration of 2, 5 or 10 mM H_2O_2 at 25 °C on a shaker at 200 r.p.m. Each treatment contained three replicates, and each experiment was repeated three times.

2.6. Assay of enzyme activity

Extracts for the assay of the activity of the antioxidant enzymes, CAT, and SOD, in yeast were prepared according to Liu et al. (2005), with slight modification. The P. kudriavzevii samples (yeast-like or biofilm form) were collected from samples exposed to 53 °C or 5 mM H_2O_2 for 30 min. Yeast-like and biofilm samples before exposure to any abiotic stress were used as time 0. The samples were disintegrated in liquid nitrogen and suspended in chilled potassium phosphate buffer (0.1 M, pH 7.0). The cell homogenate was centrifuged at 10,000g for 20 min at 4 °C, and the supernatant was used for enzyme assay. For the assay of CAT activity, a reaction mixture (3 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 0.3 ml of supernatant was used to assay. One unit of CAT activity was defined as the decomposition of 1 μmol H_2O_2 per minute, and monitored in absorbance at 240 nm. For the assay of SOD activity, the reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 13 mM methionine, 75 mM nitroblue tetrazolium (NBT), 10 mM EDTA, 2 mM riboflavin, and 0.5 ml of the enzyme extract. The mixtures were illuminated using a fluorescent lamp (60 mmol/m/s) for 5 min. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate, and monitored in absorbance at 550 nm. The activity of CAT and SOD were expressed as U/mg protein, according to the previous study (Liu et al., 2011b; Reverberi et al., 2005). Protein content was measured as described by Bradford (1976), using bovine serum albumin as a standard. There were three replicates in each treatment, and the experiment was repeated three times.

2.7. Determination of protein carbonylation and lipid peroxidation

The P. kudriavzevii samples (yeast-like or biofilm form) were collected from samples exposed to 53 °C or 5 mM H_2O_2 for 30 min. Yeast-like and biofilm samples before exposure to any abiotic stress served as time 0. The samples were disintegrated in liquid nitrogen. Carbonyl content was measured and used as an indicator of oxidative damage on protein (Liu et al., 2011b). Proteins were extracted from the samples using 500 μl of 50 mM KH_2PO_4 buffer (pH 7.5) containing 10 mM Tris, 2 mM MgCl_2, 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. Aliquots of extract were reacted with 500 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 M HCl or 2.5 M HCl without DNPH (blank control) in the dark at room temperature by vortexing every 15 min for 1 h. Proteins were precipitated with 20% (v/v) trichloroacetic acid (TCA) for 10 min on ice. After centrifugation at 3000 g for 20 min, protein pellets were washed with ethanol-ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine hydrochloride with 20 mM KH_2PO_4 (pH 2.3). The absorbance was recorded at 380 nm after centrifugation at 9500 g for 10 min. The carbonyl content was calculated using the molar absorption coefficient of 22,000/M/cm and expressed as nmol per mg protein.

For assay of lipid peroxidation, the method based on the reaction of thiobarbituric acid with malondialdehyde (MDA) was used. Detection of thiobarbituric acid-reactive species was carried out as described by Garre et al. (2010). Disrupted yeast samples were resuspended in 500 μl of 50 mM KH_2PO_4 buffer (pH 6.0) containing 10% (w/v) TCA and centrifuged at 3000 g for 10 min. Supernatants were mixed with 100 μl of 0.1 M EDTA and 600 μl of 1% (w/v) thio-barbituric acid. The reaction mixture was incubated at 100 °C for 15 min and subsequently placed on ice for 10 min. After cooling down, the absorbance was monitored at 532 nm. The MDA content was calculated using the molar absorption coefficient of 153,000/M/cm and expressed as nmol per mg protein. Protein content was measured as described by Bradford (1976), using bovine serum albumin as a standard. There were three replicates in each treatment, and the experiment was repeated three times.

2.8. Biocontrol assay

Biocontrol efficacy of P. kudriavzevii was evaluated as described in a previous study (Liu et al., 2012). Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit. A 5-μl suspension (5 × 10^7 CFUs/ml) of P. kudriavzevii obtained from either 2%-agar or 0.3%-agar culture (two-day-old) was pipetted to each wound. Fruits were air-dried for 2 h and then a 5-μl spore suspension of B. cinerea or C. gloeosporioides (1 × 10^6 spores/ml) was pipetted into each wound. Treated fruits were placed in a covered plastic food tray, and each tray was enclosed in a polyethylene bag and stored at 25 °C with relative humidity (about 90% RH) in a programmable environmental chamber with temperature and humidity control system. Disease incidence and lesion diameter of pear fruits were determined after 3 days. Each treatment contained three replicates with 20 fruits per replicate, and the experiment was repeated three times.

3. Results and discussion

3.1. Change in morphology

A single colony of P. kudriavzevii Mi-1 cultured on 48-h-old YPD plates containing 2% agar was transferred onto the centre of new YPD plates containing either 2% or 0.3% agar. After incubation at 25 °C for 2 days in the dark, the P. kudriavzevii cells on 2%-agar YPD exhibited a yeast-like morphology (Fig. 1A). However, when a colony was transferred to 0.3%-agar YPD, the yeast cells transitioned to a biofilm morphology (Fig. 1B). Correspondingly, observation under a microscope revealed the single-cell morphology of individual cells on 2% agar (Fig. 1C) and the biofilm form on 0.3% agar (Fig. 1D). In addition, the biofilm cells transitioned back to a single-cell morphology with further cell division, when they were transferred back and cultured on 2% agar YPD. Similar changes in morphology transition between solid (2% agar) and soft (0.3% agar) YPD plates have been reported in other yeast species, such as S. cerevisiae L6906 (Reynolds and Fink, 2001; Sarode et al., 2014), P. fermentans DiSAAABA 726 (Giobbe et al., 2007; Sanna et al., 2014), Candida albicans SC5314 (Lotz et al., 2004) and Debaryomyces hansenii CBS767 (Gori et al., 2011). Expression of glycoprotein Flo11p, cell wall signaling protein Wsc1p, phospholipase C and glycosylphosphatidylinositol protein RBRI, and alcohol-based quorum sensing have been associated with the morphological shift. A wide range of stress tolerance greatly contributed to the competitive ability of biocontrol yeasts (Liu et al., 2013; Sui et al., 2015). Therefore, the tolerance to heat and oxidative stresses of the two different growth forms (yeast-like and biofilm) in P. kudriavzevii was evaluated in further experiments.

3.2. Tolerance to heat and oxidative stresses

Ambient temperature is one of the major environmental factors experienced by microorganisms including yeasts. Enhancement in
heat stress can markedly facilitate and extend the application of antagonistic yeasts, especially when they are applied preharvest under field conditions (Feliziani and Romanazzi, 2013; Ippolito and Nigro, 2000; Vivekananthan et al., 2006). In the present study, the viability of \( P. \) kudriavzevii at both forms decreased with temperature increase from 51, 53 to 55°C within 30 min. However, the biofilm form of \( P. \) kudriavzevii had significantly higher viability than the yeast-like form at all three temperatures. For instance, the survival of the yeast-like form was 40.7%, while that of the biofilm form was 61.3% after 30 min at 53°C (Fig. 2A).

As another major environmental stress in biocontrol system, oxidative stress plays a crucial role in regulating yeast viability and efficacy (Chen et al., 2015). The ability of antagonistic yeast to tolerate the oxidative stress in the microenvironment of fruit host during application (Castoria et al., 2003; Sui et al., 2012) or that caused during the formulation production (Liu et al., 2009), is a key attribute for a promising yeast biocontrol agent. Similar to heat stress, a pattern of \( P. \) kudriavzevii viability was observed in response to oxidative stress (Fig. 2B). While the viability of \( P. \) kudriavzevii at both the yeast-like and biofilm forms decreased with the increase of \( \text{H}_2\text{O}_2 \) concentrations from 2, 5 to 10 mM within 30 min, the biofilm form exhibited significantly higher viability under the oxidative stress at all concentrations assessed. Collectively, the stress tolerance of the biofilm form of \( P. \) kudriavzevii to heat and oxidative stresses was found to be significantly greater than that of the yeast-like form. In order to investigate the possible physiological mechanisms involved in the differences in stress tolerance of the yeast-like and biofilm forms, antioxidant enzyme activity and oxidative damage under heat/oxidative stresses were investigated.

### 3.3. Oxidative damage on protein and lipid, and antioxidant enzyme activity

Yeast viability decrease under adverse stress conditions often attributes to cellular damage (e.g., protein and lipid oxidation). Carbonyl content as an indicator of protein oxidation and MDA content as an indicator of lipid peroxidation (Saharan and Sharma, 2010) were measured in this study (Fig. 3A and B). At time 0, prior to heat and oxidative stresses, carbonyl and MDA contents in both the yeast-like and biofilm forms of \( P. \) kudriavzevii were quite low, and there were no significant differences. However, the heat (53°C, 30 min) or oxidative stress (5 mM \( \text{H}_2\text{O}_2 \), 30 min) resulted in a marked increase in carbonyl and MDA contents in yeast cells. The biofilm form had significantly lower carbonyl and MDA contents than the yeast-like form at both stresses, which corresponded well with the viability data (Fig. 2). It confirmed the premise that protein and lipid oxidation caused by heat/oxidative stress contributed to the viability decrease (Chen et al., 2015; Liu et al., 2011b, 2012).

The amelioration of oxidative damage caused by abiotic stresses is dependent on antioxidant enzymes such as CAT and SOD. The increase in the activity of these enzymes contributes to the adaptation of yeast cells to abiotic stresses (An et al., 2012; Ribeiro et al., 2015). It was found that the activity of CAT (Fig. 3C) and SOD (Fig. 3D) was significantly higher in the biofilm form than the yeast-like form at time 0, prior to exposure to any stress. This observation was consistent with the previous report that \( C. \) albicans biofilm formation was associated with increased anti-oxidative capacities (Seneviratne et al., 2008). The activity of antioxidant enzymes was elevated when exposed to heat (53°C,
30 min) or oxidative stress (5 mM H2O2, 30 min). Importantly, the biofilm form still kept higher activity than the yeast-like form even under the abiotic stresses (Fig. 3C and D), contributing to lower level of protein and lipid oxidation (Fig. 3A and B).

3.4. Biocontrol efficacy

Gray mold (B. cinerea) is one of the most significant postharvest diseases of various pear cultivars worldwide (Janisiewicz, 1988; Mari et al., 1996; Nunes et al., 2002; Yu et al., 2007), while anthracnose (C. gloeosporioides) is a major postharvest disease of Asian pear from latent infection (Chung et al., 2006; Tashiro et al., 2012). In the present study, the biocontrol efficacy of P. kudriavzevii against gray mold (Fig. 4A, C and E) and anthracnose (Fig. 4B, D and F) was evaluated on Asian pear fruit (P. bretschneideri cv. ‘Dangshansuli’). Results indicated that the biofilm form of P. kudriavzevii exhibited better control efficacy on both diseases than the yeast-like form, based on disease incidence (Fig. 4C and D) and lesion diameter (Fig. 4E and F). Based on the premise that oxidative stress resistance is necessary for antagonistic yeasts to remain viable and exhibit biocontrol performance in microenvironment of host fruit tissues (Castoria et al., 2003; Sui et al., 2012), the
higher tolerance to oxidative stress in the biofilm form may partially contribute to the greater biocontrol efficacy of *P. kudriavzevii*.

In summary, we found that *P. kudriavzevii* could exhibit two growth forms (yeast-like vs. biofilm) on YPD 2% agar vs. 0.3% agar. The biofilm form had higher tolerance to heat and oxidative stresses and greater biocontrol efficacy than the yeast-like form. Lower levels of protein and lipid damage under the abiotic stresses were observed in the biofilm form and may be because of the activation of antioxidant enzyme activity. These findings provide new information on the biology of yeast antagonists that is essential for their potential application and development. However, further study on the mechanism of morphological change at molecular level is needed.

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**References**


Candida oleophila

Candida sake

Candida albicans

Candida sake

Candida sp.

Candida tropicalis

Candida guilliermondii

Candida lusitana

Candida parapsilosis

Candida pseudotropicalis

Candida tropicalis

Candida utilis

Candida guilliermondii

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