BIODIVERSITY OF YEASTS IN NATIVE THAI BEES AND TESTING OF POTENTIAL ANTAGONISM FOR CONTROL OF SOME BACTERIA

By
MISS Nawarat CHAROENPHOL

A Thesis Submitted in Partial Fulfillment of the Requirements
for Master of Science (MICROBIOLOGY)
Department of MICROBIOLOGY
Silpakorn University
Academic Year 2023

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Field of Study	(MICROBIOLOGY)
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MISS Nawarat CHAROENPHOL : Biodiversity of Yeasts in Native Thai Bees and Testing of Potential Antagonism for Control of Some Bacteria Thesis advisor : Dr. Sujinan Meelai, Ph.D.

Insect yeasts could occur in a wide range of habitats, including bees and beetles, in which they might play important roles. However, investigation of honeybee yeasts in Thailand was scarce. Yeast communities inhabiting the digestive tracts were examined using cultivation method and compared with those inhabiting the honey. Yeasts were recovered from the hives of 4 honeybee species collected in Chiang Mai province, and 47 strains were investigated in this study. Identification based on LSU D1/D2 sequence analysis revealed a higher number of strains in the phylum Ascomycota than in the phylum Basidiomycota. The ascomycetous yeasts comprised 5 known species from 4 genera, *Aureobasidium*, *Kodamaea*, *Pichia* and *Starmerella*, and 4 candidates assumed new species. Whereas, the basidiomycetous yeasts included 1 known species from the genus *Filobasidium* and 1 candidate assumed new species. The species with the highest occurrence was a candidate assumed new species near *S. apis*. Antagonistic activity of 39 yeast strains on 14 tested bacteria was determined using agar well diffusion method. Eight strains had zones of inhibition between 10.8±0.4 to 14.6±0.5 mm for *A. calcoaceticus* TISTR 360.

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Nawarat CHAROENPHOL

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

Insects were regarded as the most species-rich organisms on Earth and play an important role in ecosystem services, such as food providing, nutrient cycling and pollination (Bosmans et al., 2018; Weisser and Siemann, 2008; Yang and Gratton, 2014). Discovery of associations among microbes and insects took place from the late nineteenth century until the present (Blackwell, 2017a). However, the role of yeasts as insect endosymbionts had drawn attention. The most promising field of study was the interaction due to yeast diversity and its presence in insect hosts (Ganter, 2006; Gonzalez, 2014; Urubschurov and Janczyk, 2011). Insect-association yeasts belonged to the genera *Candida*, *Cryptococcus*, *Metschnikowia*, *Pichia* and *Pseudozyma* that were often found in gastrointestinal tract of insects, including beetles (Gonzalez, 2014; Urubschurov and Janczyk, 2011). In addition, several new yeast species in the genus *Candida*, were also discovered in beetles (Suh and Blackwell, 2004, 2005; Suh et al., 2004, 2005). Among flower visitors, bees were considered as yeast vectors (Brysch-Herzberg, 2004) that bee activities could be influenced by the associated yeasts (Herrera et al., 2013). Not only the significant amounts of yeasts were commonly found in bee bread and flower nectar, but also more attractive bee bread and modified nectar composition were made by yeasts (Calaça et al., 2018; Gilliam, 1979; Herrera, et al., 2009; Stefanini, 2018). Brysch-Herzberg (2004), Gilliam (1979), Pozo et al. (2012) and Sandhu and Waraich (1985) studied yeast communities in bee hives, floral nectar and honey stomach. It was found that many yeast species had a wide distribution and largely depended on insect dispersion, assuming that there was a certain interdependence or an intimate relationship between yeasts and their insect vectors (Herrera and Pozo, 2010; Pozo et al., 2020). Attempts to characterize yeasts associated with bees and their food sources have previously been reported. *Candida batistae* has been isolated from solitary bee in Brazil (Rosa et al., 1999), whereas *C. lundiana* and *C. suthepensis* have been discovered in raw honey in Thailand (Saksinchai et al., 2012b). Brysch-Herzberg et al. (2019) have reported that *Schizosaccharomyces osmophilus* was obtained from bee bread of solitary bee. Two species belonging to the *Starmerella* clade, *S. meliponinorum* and *S. neotropicalis*, were found in pollen provision and adult of stingless bees (Daniel et al., 2013; Teixeira et al., 2003). Predominant yeast species recovered from bee bread and raw honey of honeybee in Hungary and Thailand were *Zygosaccharomyces favi* and *Z. siamensis*, respectively (Čadež et al., 2015; Saksinchai et al., 2012a).

Use of antagonistic bacteria to inhibit pathogens has been extensively studied, while less attention in a similar role has been given to yeasts. Therefore, potential applications of yeast antagonism are still in an early stage of development (Hatoum et al., 2012). Yeasts could antagonistically interact with other microorganisms by several mechanisms, such as competition for nutrients and space, direct parasitization effect, production of antimicrobial compounds and induction of host resistance (Ma et al., 2023). Some researchers reported antagonistic activity of yeasts, involving production of antioxidants, killer toxins and sophorolipids. Secondary metabolites identified as antioxidants of *Saccharomyces cerevisiae* had a significant antimicrobial activity against Gram-positive (*Bacillus cereus*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus* and *S. epidermidis*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella flexneri*, *Salmonella typhi*, *Vibrio cholerae*) (Fakruddin et al., 2017; Makky et al., 2021). Hipp et al. (1974) demonstrated that *C. albicans* produced a secondary metabolite known as killer toxin, inhibiting *Neisseria gonorrhoeae*. Similar to Bajaj et al. (2013), *P. kudriavzevii* toxin exhibited antimicrobial activity against *E. faecalis*, *E. coli*, *Klebsiella* sp., *P. aeruginosa*, *P. alcaligenes* and *S. aureus*. Different derivatives of sophorolipids, glycolipid biosurfactants, produced from ascomycetous (*C. tropicalis* and *S. bombicola*) and basidiomycetous yeasts (*Pseudohyphozyma bogoriensis* and *Rhodotorula bogoriensis*) had the potential to inactivate Gram-positive (*Cutibacterium acnes*, *Listeria monocytogenes* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) (Ankulkar and Chavan, 2019; Abhyankar et al., 2021; Solaiman et al., 2015, 2020; Zhang et al., 2017). The research aimed to explore culturable yeasts diversity in the digestive tract and honey samples collected in Chiang Mai province, Northern Thailand. Yeast strains were identified by sequence analyses of LSU D1/D2 domains and ITS regions. Yeasts from native Thai bees with antagonistic activity against pathogens have never previously been reported. Therefore, this study focused on antagonistic yeasts and their antimicrobial activity was investigated using agar well diffusion method.

1.2 Research Objectives

1.2.1 To survey diversity of culturable yeasts associated with native Thai bees.
1.2.2 To evaluate antagonistic activity of investigated yeasts against some bacteria.

1.3 Usefulness of the Research

1.3.1 Culturable yeasts would be identified.
1.3.2 Antagonistic interaction would be clarified.

1.4 Scop of Works

Forty-seven strains considered in this study were obtained from Native Thai bees, 4 hives of the black dwarf honeybee (*Apis andreniformis*, 4 strains), 3 hives of the Asiatic cavity-nesting honeybee (*A. cerana*, 3 strains), 1 hive of the giant honeybees (*A. dorsata*, 1 strain) and 7 hives of the red dwarf honeybee (*A. florea*, 39 strains), collected in Chiang Mai province, Northern Thailand.

1.5 Research Content

This research reviews on yeasts associated with insects and their food sources, and yeast antagonistic activity against bacteria. Thus, in Chapter 2, Part 1 discusses on yeasts. Part 2 describes in yeasts associated with bees. Part 3 comments on new yeasts species in Thailand. Part 4 focuses on antagonistic yeasts. Chapter 3 considers in the methodology for yeast isolation and maintenance, yeast grouping, rDNA sequencing and sequence analysis, and yeast antagonistic activity. Chapter 4 reports and discusses on yeast morphology and physiology, yeast identification and phylogenetic analysis, yeast diversity, and yeast antagonistic activity. The final chapter provides a conclusion.

CHAPTER 2
LITERATURE REVIEW

2.1 Yeasts
2.1.1 Definition of Yeasts

The meaning of the name "yeast" may be traced back to its Dutch counterpart "gist," which specifically denotes the froth produced during the fermentation process of beer wort. Alternative terms for yeast, such as the French term "levure," pertain to the function of yeast in facilitating the leavening process of bread dough (Hatoum et al., 2012). Yeasts, which are eukaryotic microorganisms, have a wide distribution in many natural habitats, encompassing the typical microbial flora found in people, as well as plants, airborne particles, water, food items, and several other ecological niches (Kurtzman et al., 2011a). Additionally, they demonstrate both asexual and sexual states Given yeast exists in both an asexual and a sexual stage, which are referred to as anamorph and teleomorph, respectively (Hatoum et al., 2012). Yeasts, belonging to the taxonomic groups of ascomycetes or basidiomycetes, often exhibit budding or fission as the predominant mechanisms for asexual reproduction and have sexual states that are not enclosed in fruiting bodies (Kurtzman et al., 2011a).

2.1.2 Morphological Characterization of Yeasts

2.1.2.1 Colonies

The predominant hue shown by the yeast colonies ranges from white to cream to tan. Some species, such *Phaffia*, *Rhodosporidium*, and *Sporidiobolus*, are characterized by the presence of non-diffusible red, orange, or yellow carotenoid pigments. The texture is mucoid, fluid or viscous, butyrous, friable, or membranous. The surface is glistening or dull, smooth, rough, sectored, folded, ridged, or hirsute. The elevation of the colony exhibits various elevations, including flat, depressed in the center, raised and dome-like, or conical. The edge of the streak or colony shows characteristics such as undulating, lobed, erose, or fringed with hyphae or pseudohyphae (Kurtzman et al., 2011b).

2.1.2.2 Cells

In terms of cell size, yeast cells differ greatly from one another, shape and color might vary as a result of diverse external physical and chemical growth factors, such as the temperature at which they are cultivated, presence of some chemical compounds, and composition of the growth medium or growth phase (Walker, 1998). Polysaccharides (80-90%) make up most of the structure of the yeast cell wall, mainly glucans and mannans, and a minor percentage of chitins and proteins (Kollár et al., 1997). The microfibrillar network primarily consists of $\beta(1\rightarrow6)$- and $\beta(1\rightarrow3)$-linkages, which are formed by glucans, providing strength to the cell wall. Mannans are formed by the arrangement of mannose residues in $\alpha(1\rightarrow6)$-linkage, with the presence of short oligosaccharide side chains (Engler, 1985). Chitin, a polymer composed of N-acetylglucosamine, constitutes a mere 2-4% of the cell wall's dry weight and is mostly found in bud scars. The interior portion of yeast cell walls, which is made of proteins, is what gives the cell its form. Various amounts of lipids and inorganic phosphate are also found in the cell wall. The plasma membrane, which has a thickness of around 7 nm, separates the cells from their extracellular environment. It is a thin, semi-permeable lipid bilayer formed mainly by proteins and lipids, which protects the integrity of the cell interior via its function in regulating permeability, controlling what enters and exits the cytosol, etc. (Feldmann, 2012). Between the cell wall and the plasma membrane lies a thin area known as the periplasm. It mostly comprises secreted proteins (mannoproteins) that cannot pass through the plasma membrane and cell wall (Walker, 1998).

2.1.3 Asexual Reproduction

2.1.3.1 Budding and Fission

A little evagination or protrusion forms anywhere on the cell surface to signal the beginning of budding. The dimensions of the parent (mother) cell remain relatively stable throughout the process of subsequent development, but the bud, known as a blastoconidium, experiences growth to produce a new cell. Eventually, this new cell detaches from the parent cell, often after a certain period of time. A scar remains on the parent cell through which no further budding occurs. The process of cellular budding that is confined to a single pole of the cell is referred to as monopolar, whereas the process of budding that takes place at both poles of the cell is referred to as bipolar multilateral or multipolar budding refers to the proliferation of cells at several places. (Kurtzman et al., 2011b).

Fission yeast is a rod-shaped cell that divides by medial cleavage using an actin-based contractile ring in a manner similar to animal cells. The formation of the ring occurs in the first stages of mitosis, prior to anaphase, in the central region of the cell, directly above the nucleus (Marks and Hyams, 1985). Upon completion of the mitotic process, the actin ring undergoes contraction, leading to the formation of a septum at the designated place. Subsequently, the septum was digested away, resulting in the separation of the two daughter cells.

2.1.3.2 Pseudohyphae and True Hyphae

The phenomenon of some yeasts exhibiting a proclivity to arrange themselves in chains of cells leads to the development of pseudohyphae, which may be described as a filamentous structure comprised of a series of cells that have undergone budding. Differentiating between real hyphae, pseudohyphae, and intermediate forms may provide a challenge. Wickerham (1951) used three criteria to identify different hyphae types. He used observations of the filament terminal cells to inform these standards. First, true hyphae typically feature straight, refractive septa that are thicker and more refractive than the borders of vacuoles, making them distinguishable from them. There is little or no constriction at the septum. Compared to the cells that come before them, the terminal cells are noticeably longer. Second, intercalary cells ends are curled and non-refractive, and pseudohyphae lack identifiable septa. There are usually marked constrictions where the cells join. In general the terminal cell is shorter than or almost as long as the adjacent cell. It is rare to find a pseudohypha with a terminal cell that is distinctly longer than the adjacent cell. Thirdly, only a small proportion of cells are separated by septa in intermediate forms (Kurtzman et al., 2011b).

2.1.4 Sexual Reproduction

2.1.4.1 Ascospores

The production of ascospores serves as a distinctive characteristic within the fungal phylum Ascomycota. Ascospores are typically found in clusters of four or eight within a single mother cell, the ascus (Neiman, 2005). In the presence of nutrients, they grow in budding form. According to Gancedo (2001), the initiation of mitotic development in a pseudohyphal form may be induced by the presence of a deficient nitrogen supply, such as proline. When nitrogen is completely absent and a non-fermentable carbon source like acetate is present, it induces the cessation of the mitotic cycle in cells, leading to the initiation of meiosis and subsequent sporulation. (Esposito and Klapholz, 1981). When diploid cells are deprived of nitrogen and exposed to a non-fermentable carbon source, they will initiate the process of meiosisDuring the second meiotic division, the spindle pole bodies (SPBs), which are located inside the nuclear envelope, serve as locations for the generation of prospore membranes. As meiosis II proceeds, the prospore membranes undergo expansion and enclose the developing haploid nuclei. Following the process of nuclear division, it can be seen that each prospore membrane undergoes closure, thus enclosing a haploid nucleus between two separate membranes. The process of spore wall synthesis starts inside the lumen that exists between the two membranes generated from the prospore membrane. Following the completion of spore wall production, the mother cell undergoes a collapse process, ultimately resulting in the formation of the ascus. (Neiman, 2005).

2.1.4.2 Basidiospores

The typical life cycle of basidiomycetous yeast typically involves an alternation between diplophase and haplophase stages. Both ploidies can exist as stable cultures (Choudhary and Johri, 2009). The basidiomycetous yeasts exhibit two distinct modes of sexual reproduction, namely heterothallism and homothallism. (Kurtzman et al., 2011b). In heterothallic strains, haploid cells exist in two distinct mating types, namely a and α. Mating of a and α cells results in a/α diploids that are unable to mate but can undergo meiosis. The basidium, which is the mother cell, contains the eight haploid products (known as basidiospores) that are formed by the process of meiosis from a diploid cell. The process of basidium digestion and subsequent separation of basidiospores by micromanipulation results in the production of eight haploid meiotic products (Choudhary and Johri, 2009).

2.1.5 Yeast Habitats

Yeasts have a ubiquitous distribution throughout many biomes worldwide. In a general sense, habitats may be classified into three main categories: atmospheric, aquatic, and terrestrial. (Starmer and Lachance, 2011).

2.1.5.1 Atmospheric Yeasts

Yeasts have been isolated from the atmospheric environment. The outermost layer of the Earth may be identified as a reservoir or a transitional zone, rather than a conducive environment for development and reproduction (Starmer and Lachance, 2011). Although there exists a substantial body of evidence about the presence and impact of yeasts in various terrestrial and aquatic environments, there is a relative scarcity of literature focusing on yeasts in the atmosphere (Péter et al., 2017). For example, red yeasts have been retrieved from the higher layers of the atmosphere, 18-30 km in the stratosphere (Bruch, 1967), but they are unlikely to grow there. The available records demonstrate the remarkable capacity for survival shown by some species, with a special emphasis on pigmented yeasts. (Starmer and Lachance, 2011). Microorganisms, such as yeasts, mostly disseminate into the atmosphere from sources such as soil, plants, or water (Delort et al., 2010). The quantity of airborne yeasts is much lower in comparison to that of bacteria and filamentous fungus. The vast majority of the isolated yeasts were classified within basidiomycetous genera (Péter et al., 2017). In general, ascomycetous yeasts depend on vectors, such as insects, for their movement across habitats. On the other hand, basidiomycetous yeasts have the option to use vectors, such as insects, or alternatively, they may passively distribute by the release of ballistocondia, which are carried by air currents (Starmer and Lachance, 2011). Unexpectedly, a considerable number of ascomycetous yeast strains were identified from the air in Olsztyn, Poland, subsequent to Koch sedimentation (Péter et al., 2017). Klaric and Pepeljnjak (2006) investigated the aeromycological conditions throughout the year in the city of Zagreb, Croatia. The study included two sampling locations inside the city and one sample site located in the neighboring Medvednica mountain region. Seasonal trends were seen in the abundance of culturable airborne yeasts, however the kinetics of these changes varied across the various sample locations. Unfortunately, the specific yeast strains were not identified; nonetheless, the study focused on examining the impact of various climatic conditions on the abundance of airborne yeasts. Upon entering the atmosphere, microorganisms are subjected to adverse environmental circumstances, such as exposure to solar radiation, particularly ultraviolet (UV) radiation, desiccation, low temperatures, oxidants, limited nutrition supply, acidity, and fast fluctuations in salinity. It is believed that a portion of microorganisms sent into the atmosphere may not endure the swiftly fluctuating and adverse environmental circumstances encountered within the aerial domain (Péter et al., 2017).

2.1.5.2 Aquatic Yeasts

Yeasts have been recognized for an extensive duration as inhabitants of aquatic environments, namely freshwater, marine, and estuarine settings (Péter et al., 2017). *Rhodotorula* species have been discovered in deep igneous rock aquifers located at depths ranging from 200 to 400 meters below the surface in the Baltic Sea, as reported by Ekendahl et al. (2003). Additionally, these species have been found in deep ice cores extracted from Greenland glaciers to remarkable depths of 2,000 meters below the glacial surface. The ice yeasts have shown their ability to survive in frozen water subjected to very high pressures for a period exceeding 140,000 years. (Starmer et al., 2005). *Debaryomyces hansenii* is the predominant ascomycetous yeast species found in marine environments. The broad salinity yeast tolerance (as a component of its broad basic niche) is probably crucial to its vast dispersal in the ocean (Starmer and Lachance, 2011). The presence of yeasts in aquatic environments may elicit both beneficial and detrimental effects on the surrounding flora and fauna. Yeasts have been isolated from several aquatic organisms, such as clams, mussels, shrimps, isopods, amphipods, crabs, sponges, sea urchins, polychaete worms, fish, dolphins and whales (Hagler and Ahearn, 1987). Fell (1967, 1974, 1976) and colleagues (Fell and Statzell, 1971) have conducted comprehensive research on yeasts inside distinct water masses located in the Indian and Antarctic Oceans. The study reveals the remarkable prevalence of some species, such as *D. hansenii*, throughout all marine zones, while others, like *Leucosporidium antarcticum*, have a distinct preference for certain water masses. The review articles by Hagler et al. (2017) and Libkind et al. (2017) have examined the presence of yeasts in both conventional and non-conventional aquatic environments.

2.1.5.3 Terrestrial Yeasts

Soil yeasts are often found in several soil environments, including forest ecosystems. In the past, yeasts were mostly investigated within the context of vineyard, orchard, and agricultural soils (Yurkov, 2017). In contrast to above-ground sources, the abundance of soil yeasts is rather low, with their population typically ranging from 10^3-10^4 cells g^{-1}. However, there are uncommon instances when counts may reach as high as 10^5-10^6 cells (Botha, 2006; Phaff and Starmer, 1987). The population density of yeast cells often exhibits a decline as soil depth increases (Yurkov, 2017). The distribution of yeast in soil exhibits a fragmented pattern, whereby only a limited number of species are found to be shared across different sample locations. For example, Vishniac (2006) reported that around 40% of yeasts exhibit a limited distribution, being confined to a certain geographic area. Similarly, the temperate woods in Germany, namely in three locations, shared only the presence of *Apiotrichum dulcitum* (Yurkov et al., 2012). In a particular area, three Mediterranean xerophytic woods were examined, and it was observed that there were 8 species out of a total of 57 that were present in all three studied plots (Yurkov et al., 2016). Certain species of soil yeasts, such as *Filobasidium magnum*, *Naganishia albida*, and *Lipomyces* spp., have the ability to generate extracellular polymeric compounds. These substances serve as a protective mechanism against adverse environmental factors and also play a role in the binding and formation of soil aggregates (Botha, 2006; Deng et al., 2015; Vishniac, 1995). Yeasts that have been isolated from managed soils have the ability to generate chemicals that facilitate the development of mycorrhizal fungi and plants. Additionally, these yeasts create compounds that serve to safeguard plants against fungal infections. (Azcón et al., 2010; Boby et al., 2008; Nassar et al., 2005).

Since 1955, there has been a lot of interest in the microbial communities that live on the surfaces of plants that are above ground (Aleklett et al., 2014). Numerous investigations have found new microbial species isolated from flowers, including many ascomycetous yeasts from the genera *Candida* and *Wickerhamomyces*. This suggests that flowers may be an unexplored source of microbial diversity (e.g., Groenewald et al., 2011; Jindamorakot et al., 2008; Rosa et al., 2007). Because of their high sugar content and the regular visits of pollinating insects that spread the yeast throughout the flowers of various host plants, nectars are particularly well suited for yeast growth (Phaff and Starmer, 1987). Some taxa have a continuous presence within the microbiome of flowers, spanning many plant species and including a wide geographical distribution. Two regularly found genera are *Metschnikowia* (Ascomycota) and *Cryptococcus* (Basidiomycota) (Aleklett et al., 2014). The prevalence of basidiomycetous yeast species was found to be highest on plant surfaces, whilst ascomycetous species were found to be more dominant in bees and nectar (Pozo et al., 2012). Herrera et al. (2009) conducted a quantitative survey whereby they analyzed nectar samples from a total of 130 flowering plant species. The study revealed the presence of yeasts in around 44% of the nectar samples, with variations seen across different regions. *Metschnikowia reukaufii* is a yeast species that is often found in floral nectar samples (Brysch-Herzberg, 2004). In their study, Pozo et al. (2012) conducted observations on several strains obtained from nectars of digitalis obscura and atropa baetica, as well as from associated environments such as bees, air, corolla, and pollen. The researchers found that *Metschnikowia* strains did not exhibit exceptional resistance to plant secondary chemicals or high sugar concentrations. Given the close relationship between blooming plants, nectar-dwelling yeasts, and their insect carriers, it is plausible to hypothesize that the species participating in these interactions have undergone co-evolution. (Péter et al., 2017)

The historical span ranging from the late 19th century to the present has seen a significant era of exploration and identification of connections between microorganisms and insects (Buchner, 1965). The extensive variety and plentiful presence of land-dwelling arthropods contribute to their propensity for engaging in multiple connections with yeasts, resulting in a diversified and intricate array of partnerships between the two (Starmer and Lachance, 2011) Microscopic organisms, such as yeasts with a wide range of physiological capacities, play a crucial role in facilitating the presence of arthropods, particularly insects, who represent the most abundant and varied group of species on our planet. The yeast growth form is found in the majority of the fungi and is often connected to insects. Fungi and insects have coevolved in similar environments, leading to early and informal interactions between the two groups over their geological history. As a result of this long-standing relationship, yeast attractants for insects have formed. Some insects have the ability to maintain yeasts inside specialized anatomical structures, such as mycangia and gut caeca, for extended durations (Blackwell, 2017a). There are several supplementary studies on the yeasts that are linked to a diverse range of insects. Several studies have reported the presence of lacewings and caddis flies (Nguyen et al., 2006), dung beetles (Górz and Boroń, 2016), and codling moths (Witzgall et al., 2012). Yeasts associated with insects include Ascomycota (Saccharomycotina, Pezizomycotina) and a few Basidiomycota. Beetles, homopterans, and flies play significant roles as symbiotic partners of fungi, and in turn the insects harbor yeasts inside pits, specialized exterior pouches, and modified stomach pockets. Certain species of yeasts engage in sexual reproduction inside the gastrointestinal tract of insects, therefore augmenting the genetic variety of the population. Conversely, certain other yeasts, which are well adapted to their relatively constant surroundings, may never engage in mating. (Blackwell, 2017b).

2.2 Yeasts Associations with Bees

Insects interact with microbes in a variety of contexts, from unintentional encounters to find appetizing food to the absorption of crucial nutrients lacking in the primary food supply (Stefanini, 2018). Insects make up a significant portion of the biodiversity on Earth, with about 1,000,000 recognized species and an estimated 6 million total species (Larsen et al., 2017). The insects with which we are most familiar are those that have a significant association, whether positive or negative, with our daily existence. Insects can serve various roles within ecosystems and human societies. They can be regarded as pests, such as caterpillars that cause damage to crops. Additionally, certain insects, like Anopheles spp. mosquitoes, act as vectors for human pathogens, such as malaria. Insects also have value as a food resource, both in terms of producing food, such as honey, and being consumed directly, as is the case with termites and grasshoppers. Furthermore, insects play a crucial role in maintaining natural biodiversity and contribute to plant pollination (Goulson et al., 2015). There is evidence to suggest that various species of yeasts have a significant impact on the life of insects. These yeasts fulfill important functions such as assisting in the identification of food sources, facilitating the process of food digestion, and serving as a useful reservoir of necessary nutrients for insects (Stefanini, 2018). The primary component that attracts insects to food often relies on olfactory cues (Gillott, 2005). It has been shown that beetles (Coleoptera) are specifically drawn to yeasts due to the emission of fermentative volatiles (Ganter, 2006). The initial spread of yeasts may occur by several mechanisms, including non-insect pollinators (Belisle et al., 2012), soil (Gilbert et al., 2014) or insects that rely on visual or plant-derived cues (Kulahci et al., 2008). Herrera et al. (2013) and Schaeffer et al. (2016) observed that a higher percentage of trips to flowers inhabited by yeast were made by inexperienced bumblebee foragers, including those that had not previously encountered yeast-treated flowers. Furthermore, Good et al. (2014) found that honeybees exhibit reduced nectar consumption in the presence of bacterial colonization, but yeast colonization did not have a significant impact on their nectar intake. For example, flowers that are subject to bee pollinators are more prone to hosting yeasts compared to flowers that are enclosed in bags to prevent pollinator interactions (Belisle et al., 2012; Lachance et al., 1989, 1998; Schaeffer et al., 2015). Different bee species also seem to commonly carry different yeasts (Lachance et al., 2001). The infrequent discovery of yeasts in healthy queen bees (Gilliam and Prest, 1977) and the significant yeast populations (ranging from 10^4 to 10^6 c.f.u. mL^{-1} in various specimens) found in healthy adults of the stingless bee *Tetragonisca angustula* (Teixeira et al., 2003) suggest that reactions to yeasts may vary among the various *Apis* species. Yeast and other fungi are expected to be far less prevalent in the honeybees gut than bacteria, maybe making up less than 1% of the microbiome. Interesting results and current theories from research on yeasts in bees have uncovered a number of intriguing quirks about gut-residing yeasts (Ptaszyńska et al., 2016). It seems that yeasts have advantages, such as the ability to synthesize vitamins to augment bee meals (Anderson et al., 2011). Yeasts were shown to be widely present in both recently emerged bees and nurse bees, leading to speculation that they may play a role in pollen digestion and the production of royal jelly (Yun et al., 2018).

2.2.1 Native Bees in Thailand

Honeybees are classified in the Apini tribe within the subfamily Apinae and family Apidae (Ruttner, 1988). They are a member of the large insect order Hymenoptera, which also includes ants, bees, wasps, and sawflies (Gullan and Cranston, 2000). One Western species and ten Asian species make up the only genus of real honeybees, *Apis.* The compound eyes covered in erect long hairs, the strongly convex scutellum, the pollen press on the hind leg, the greatly elongated marginal and submarginal cells of the forewing, and the jugal lobe in the hind wing are some of the most distinctive morphological characteristics of worker bees of the genus *Apis*. Each species of honeybees are highly social insects (Oldroyd and Wongsiri, 2006). This report revealed at least three criteria for defining the eusociality form in honeybees that correspond with that of Wilson (1971). There are five species of *Apis* found in Thailand: *A. andreniformis*, *A. dorsata*, *A. cerana*, *A. florea*, and *A. mellifera* (Rattanawannee et al., 2007). The first four species are indigenous to Thailand; however, *A. mellifera* was anthropogenically brought into the nation for the apiculture sector. (Wongsiri et al., 1996).

2.2.1.1 *Apis andreniformis*

The smallest species in the genus *Apis* is the black dwarf honeybee, often known as the small dwarf honeybee (*A. andreniformis*). One of the distinctive traits of *A. andreniformis*, as described by Smith (1858), is the presence of black hairs on the hind tibia and dorsolateral surface of the hind basitarsus in worker bees. This distinguishes *A. andreniformis* from *A. florea*, which has white hairs in same areas. The nests of *A. andreniformis* are mostly located inside undisturbed, mixed deciduous to evergreen forests. Their preferred nesting environment is often dim and shaded (20-35% sun), frequently next to or above streams. In northern Thailand, they frequently build their nests on the thin branches of small trees such as bamboos, bananas, or bushes, as well as coffee and tea trees. (Wongsiri et al., 1996).

2.2.1.2 *Apis cerana*

The Asian honeybee species, *A. cerana*, is widely distributed throughout many parts of Asia and has a considerable presence in Thailand. *A. cerana* is a bee of moderate size, and its accurate assessment may be achieved by measuring the length of the forewing. The measurement range for *A. cerana* falls between 8.89 mm and 7.47 mm. (Limbipichai, 1990; Sylvester et al., 1998). It is a cavity-nesting honeybee that nests in cavities and may be found in all ecosystems, including rainforests and highly disturbed areas such as human settlements.

2.2.1.3 *Apis dorsata*

There are three species in the subgenus Megapis, one of which being the common gigantic honeybee, *A. dorsata*. The external morphology of *A. dorsata* is different from that of *A. florea and A. andreniformis.* Individuals that are workers of *A. dorsata* are relatively large, measuring around 17 mm in length. The giant honeybees in Thailand differed from the other four honeybee species based on their much larger body size and their fuscous, and quite hairy. (Oldroyd and Wongsiri, 2006). *A. dorsata* has a yellow body color and is reddish-brown at tergites 2 and 3 (Crane, 1990). Compared with the other species, *A. dorsata* builds much larger combs. When the nest is full of brood, honey, and adult bees, this species needs strong support for their heavy comb.They, therefore, nest on large, strong branches of larger trees. Their nests may sometimes be discovered on mountain cliffs or man-made structures like water towers or tall buildings (Wongsiri et al., 2000).

2.2.1.4 *Apis florea*

The red dwarf honeybee, *A. florea*, is quite common across Asia. It may be found from Vietnam and southeastern China to continental Asia along and below the southern Himalayas, west to the Iranian Plateau, and south into Oman (Hepburn and Hepburn, 2005). The workers of *A. florea contain* less black pigment, which is consistent with the perception that they are mostly yellow bees, while *A. andreniformis* is mostly a black bee. A notable exception to this rule is the pigmentation of the scutellum. With a few exceptions, the scutellum color of *A. andreniformis* workers trends toward yellow, whereas that of *A. florea* workers trends toward black. Additionally, they support their nest, which is often in a shaded area, using a short branch. For instance, nests of the *A. florea* have been seen in Thailand on the roofs of buildings, the walls of buildings, and tall trees (Wongsiri et al., 1990).

2.3 New Yeast Species in Thailand

Thailand has been widely reported for its abundant microbial diversity, including a variety of yeasts (Jindamorakot et al., 2004). It has been reported that novel yeasts found in Thailand are rich in species diversity and they have been described so far (Table 1)

Table 1 Investigated yeast community in the 2010s and new yeast species found in Thailand

Species	Source	References
Candida asiatica, C. bambusicola, C. berkhoutiae, C. chanthaburiensis, C. chumphonensis, C. inulinophila, C. konsanensis, C. kungkrabaensis, C. loeiensis, C. lundiana, C. maleeae, C. mattranensis, C. namnaoensis, C. nongkhaiensis, C. phyllophila, C. potacharoeniae, C. sakaeoensis, C. saraburiensis, C. sirachaensis, C. spenceri, C. succicola, C. suratensis, C. suthepensis, C. tanticharoeniae, C. thasaenensis, C. uthaithanina, C. vitiphila, C. wangnamkhiaoensis,	decaying corncobs, detached branch and leaf submerged, estuarine water, exudate, flowers, insect frass, leaves, moss, mushroom, raw honey, soil, stingless bee	Boonmak et al. (2011); Kaewwichian et al. (2019); Koowadjanakul et al. (2011); Limtong and Kaewwichian (2013); Limtong and Yongmanitchai (2010); Limtong et al. (2010, 2011, 2012a, 2012b); Nitiyon et al. (2011); Poomtien et al. (2013); Saksinchai et al. (2012b); Sarawan et al. (2013)

Table 1 (continued)

Species	Source	References
C. xylosifermentans, C. xylanilytica, C. xylosifermentans		
Cryptotrichosporon siamense	peat	Kaewwichian et al. (2018)
Cyberlindnera samutprakarnensis	wastewater	Poomtien et al. (2013)
Geotrichum siamensis, G. phurueaensis	forest soil, water	Kaewwichian et al. (2010)
Goffeauzyma siamensis	pineapple Leaves	Nutaratat et al. (2022)
Hannaella phyllophila	leaves	Surussawadee et al. (2015)
Heterocephalacria mucosa	decaying tree bark	Kunthiphun et al. (2019)
Kazachstania surinensis	traditional Thai fermented foods	Punyauppa-path et al. (2022)
Kodamaea samutsakhonensis	mushroom	Nualthaisong et al. (2023)
Limtongozyma siamensis	grease	Boontham et al. (2020)
Metschnikowia lopburiensis, M. saccharicola	leaves	Kaewwichian et al. (2012)
Millerozyma phetchabunensis	soil	Tammawong et al. (2010)
Nakazawaea todaengensis	peat swamp forest	Polburee et al. (2017)
Ogataea kanchanaburiensis, O. phyllophila, O. wangdongensis	leave	Koowadjanakul et al. (2011); Limtong et al. (2013)
Papiliotrema phichitensis	leaves	Khunnamwong et al. (2018)
Pseudozyma vetiver	phylloplane	Chamnanpa et al. (2013)
Saturnispora kantuleensis	peat	Khunnamwong and Limtong (2018)
Spencerozyma siamensis	soft coral	Kaewkrajay and Limtong (2018)
Savitreea pentosicarens	grease trap	Sakpuntoon et al. (2020)
Savitreella phatthalungensis	pineapple Leaves	Nutaratat et al. (2022)
Torulaspora nypae	nipa palm	Kaewwichian et al. (2020)
Vanderwaltozyma tropicalis	moss	Nakase et al. (2010)
Wickerhamiella nakhonpathomensis	mushroom, flower	Khunnamwong et al. (2022)
Wickerhamomyces siamensis, W. tratensis	flower, leaves	Nakase et al. (2012); Kaewwichian et al. (2013a)

Table 1 (continued)

Species	Source	References
Yamadazyma siamensis, Y. phyllophila, Y. ubonensis	corn, leaves, tree bark	Junyapate et al. (2014); Kaewwichian et al. (2013b)
Zygosaccharomyces siamensis	raw honey	Saksinchai et al. (2012a)

2.4 Antagonistic Yeasts

Throughout the years, much study has been conducted on the utilization of antagonistic bacteria as a means to inhibit the growth of harmful bacteria. However, there has been comparatively less emphasis on exploring the potential of yeasts to fulfill a similar role (Hatoum et al., 2012). The inhibitory activity of yeast was discovered first by Hayduck (1909), reported the presence of a volatile thermolabile toxic extract from yeast, which was likely an amine compound. This extract was shown to impede the development of both *Escherichia coli* and Staphylococci (Viljoen, 2006). Fatichenti et al. (1983) demonstrated that the antibacterial efficacy of *Debaryomyces hansenii* against *Clostridium tyrobutyricum* and *C. butyricum* was attributed to its capacity to generate antimicrobial chemicals both within and outside of its cellular structure. Bilink and Casey (1989) reported the growth suppression of *Bacillus megaterium* and *Lactobacillus plantarum*, which are bacteria known to ruin beer. This inhibition was attributed to the conversion of methylene blue into a pharmacologically active form by the microorganisms *Kloeckera apiculate* and *Kluyveromyces thermotoolerans*. Dieuleveux et al. (1998) showed the suppression of *Listeria* growth by the actions of a particular strain of *Geotrichum candidum* that was obtained from French red smear cheese. The stability of the two anti-listerial compounds, namely D-3-phenyllactic and D-3-indollactic acid, remains consistent throughout a broad pH range. Additionally, these compounds exhibit thermal stability, as they can withstand heating at 120 ºC for a duration of 20 min. Also, Cavalero and Cooper (2003) provided evidence of *Candida bombicola* ability to create extracellular glycolipids known as sophorosides. These sophorosides have been found to possess antibacterial properties against *Staphylococcus aureus* and also have inhibitory effects on *C. albicans*. Compared to other biocontrols, yeasts provide specific advantages because of their low nutritional requirements and capacity for large-scale cultivation on inexpensive substrate medium. Some yeast species are harmless for the environment, humans, and host plant and are not expected to become resistant (Bagy et al., 2023). Gradually, the standards for identifying antagonistic yeasts have improved (Zajc et al., 2020). An ideal antagonistic yeast should be resistant to several diseases, possess minimal nutritional needs, be effective in harsh environments and at low concentrations, and be genetically stable (Dukare et al., 2018; Nunes, 2012). Furthermore, it is vital for an antagonistic yeast to possess advantageous commercial prospects. The ideal characteristics of the organism in question include the ability to thrive on a cost-effective growing substrate, facilitate convenient storage and dispensing, and exhibit compatibility with various physical and chemical interventions such as controlled atmospheres, varying temperature ranges, chemical fungicides and pesticides, as well as phytohormones (Liu, et al., 2013). Regarding biosafety, an ideal antagonistic yeast would include characteristics that are ecologically sustainable, exhibit non-pathogenicity towards the host fruits, refrain from generating metabolites that pose risks to human health, and lack the ability to induce infections in people (Dukare et al., 2018; Liu et al., 2013). Antagonism of microorganisms by yeasts has been attributed primarily to (1) competition for nutrients, the pathogens and antagonistic yeasts need the presence of essential nutrients, such as carbohydrates and nitrogen, in order to successfully colonize and undergo development. Hence, the major mechanism by which antagonistic yeasts inhibit the growth of pathogens is widely acknowledged to be the struggle for resources and space (Liu et al., 2013; Spadaro and Droby, 2016). When antagonistic yeasts come into contact with pathogens, they have the ability to occupy and deplete nutrients at a rapid rate (Li et al., 2013; Liu et al., 2012). *Metschnikowia pulcherrima*, a strain of yeast, has the ability to synthesize iron chelators that effectively compete with pathogens for the essential iron resources. Consequently, this competitive interaction impedes the proliferation of the infections (Gore-Lloyd et al., 2019). (2) Direct parasitism effect, mycoparasitism refers to the biological phenomenon wherein antagonistic yeasts engage in the consumption of fungal pathogens, attaching themselves to the hyphae of these pathogens and then releasing enzymes that facilitate the degradation of cell walls, so resulting in the demise or lysis of the fungi. In instances of nutritional deficits, it is possible for organisms to assimilate resources from pathogenic cells, resulting in the demise of these "prey" cells. The involvement of secreted enzymes, including β-1,3-glucanase (GLU), chitinase (CHT), and proteases, is widely acknowledged to be of paramount importance in the context of biocontrol (DiFrancesco et al., 2016). Additionally, there have been reports indicating the ability of both *Pichia membranefaciens* and *C. albidus* to adhere to and break down the hyphae of *Penicillium expansum*, *Monilinia fructicola*, and *Rhizopus stolonifer* (Chan and Tian, 2005). (3) Induction of host resistance, Host resistance can be roughly described as the capacity of the host to restrict the amount of pathogens present in its system (Raberg et al., 2009) and consists of a variety of defenses including skin barriers, behavioral adjustments, or a quick immunology reaction (Restif and Koella, 2004; Roy and Kirchner, 2000). Therefore, the presence of resistance can exert selection pressure on pathogen characteristics, such as virulence and transmissibility, which has important consequences for the evolutionary dynamics between hosts and pathogens. The presence of resistance can exert selection pressure on pathogen characteristics, such as virulence and transmissibility, which has consequences for the evolutionary dynamics between hosts and pathogens (Boots and Bowers, 1999; Baalen M, 1998). Chan et al. (2007) revealed that *P. membranaefaciens*, a yeast known for its antagonistic properties, has the ability to stimulate the production of three pathogenesis-related proteins. This discovery suggests that the presence of *P. membranaefaciens* might potentially enhance the resistance of peach fruit against *P. expansum*. The potential methods by which *Wickerhamomyces anomalus* inhibits blue mold decay induced by *P. expansum* in pears include the induction of defense-related genes and the modulation of defense-related enzyme activity (Zhang et al., 2019). Multiple mechanisms may be simultaneously involved in the resistance induction by antagonistic yeasts. For example, several antagonistic yeasts, such as *Cryptococcus laurentii* (Lai et al., 2018), *P. membranaefaciens* (Chan et al., 2007), *P. guilliermondii* (Zhao et al., 2008), *Rhodotorula glutinis* (Xu et al., 2008), and *R. paludigenum* (Lu et al., 2013), elicited alterations in the activity of defense-related enzymes as well as antioxidant enzymes in the fruit. The induction of disease resistance by the use of antagonistic yeasts is subject to the influence of both pathogens and environmental circumstances. (4) Secretion of antibacterial compounds and release of antimicrobial substances. In comparison to filamentous fungus, yeasts exhibit a diminished secretory capability and generate a limited number of secondary metabolites (Zhang et al., 2020). Mycocins are extracellular (glycol) proteins that inhibit growth of fungi, bacteria and protozoans. The yeast killer phenomenon was first observed by Bevan and Makower (1963) in *Saccharomyces cerevisiae* strains. Subsequent research has demonstrated that this phenomenon is also observed in several other yeast species, including *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Wickerhamomyces*, *Ustilago*, *Williopsis* and *Zygosaccharomyces* (Schmitt and Breinig 2002; Tay et al., 2014). One plausible method by which mycocins exert their effects is through the suppression of beta-glucan production or beta-glucan hydrolysis inside the cell wall of susceptible strains (Muccilli et al., 2013). In addition to their impact on β-glucan production and breakdown, mycocins exhibit several additional actions. These substances interfere with the process of cell division and impede the production of DNA (deoxyribonucleic acid) (Klassen and Meinhardt, 2005; Marquina et al., 2002); cleave ribonucleic acid transporter (tRNA) (Klassen et al. 2008); block calcium absorption (Brown, 2010)(Brown, 2010) and development of channels in the cytoplasm might lead to ion leakage (Santos et al., 2007; Schmitt and Breinig, 2006). However, the mechanism by which bacteria are targeted remains elusive (Olstorpe et al., 2010; Passoth et al., 2011).

CHAPTER 3
MATERIALS AND METHODS

3.1 Bee Hive Collection and Yeast Isolation

Collections were made on Year 2012-2019 in Chiang Mai provinces, Northern Thailand. Collecting data are shown in Table 2, i.e. 4 hives of the black dwarf honeybee (*Apis andreniformis*), 3 hives of the Asiatic cavity-nesting honeybee (*Apis cerana*), 1 hive of the giant honeybee (*Apis dorsata*) and 17 hives of the red dwarf honeybee (*Apis florea*) were sampled. Honey was aseptically squeezed, diluted in approximately 10 volumes of sterile water and vortexed for 1 min. Adult bees were collected and employed for dissection to pick up digestive tracts from head to guts. Samples were suspended in 1 ml of sterile water and vortexed for 1 min. One hundred microliters of successive decimal dilutions were spread on yeast extract-malt extract (YM) agar (1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2% agar, w/v) supplemented with 100 mg l^{-1} chloramphenicol. Plates were incubated at 25ºC and examined periodically. Yeast colonies were counted and representatives of each morphological type were purified. Yeast strains were preserved on YM agar slants stored at 4ºC and subcultured every 2 months. Cultures were also kept at -20ºC and -80ºC in 30% (v/v) glycerol solution.

3.2 Yeast Strains and Their Maintenance

Forty-seven strains considered in this study were kindly provided by Charoenphol (2018), Dumsuwan (2016), Kulee (2018), Laksitanon (2018), Photinakae (2015), Sangprasert (2016), Silakam (2018), Thipsawek (2021) and Thongnum (2015), isolating from the digestive tracts and honey of native Thai bees (Table 3). All yeasts were restreak on yeast extract-malt extract agar (YMA, 1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2% agar) supplemented with 100 mg l^{-1} chloramphenicol. The plates were incubated at 25ºC and examined periodically. Yeast strains were preserved on YM agar slants stored at 4ºC and subcultured every 2 months. Cultures were also kept at -20ºC in glycerol solution (30% v/v).

3.3 Physiological Observation for Yeast Grouping

In order to group yeasts associated with digestive tracts and honey, yeast strains were streak (straight line) on 50% glucose (13 g of agar and 500 g of glucose in 500 g solution of 1% yeast extract) and 60% glucose (22.5 g of glucose and 600 g of glucose in 400 g solution of 1% yeast extract) and incubated at 25ºC for 1 month.

Table 2 Collecting data

Bee species	Hive no.	Locality	Collecting data
Apis andreniformis	2	Mae Raem, Mae Rim	19 Oct 2013
	3	Rim Tai, Mae Rim	27 Oct 2012
	7	Pong Yaeng, Mae Rim	4 Apr 2019
	8	Pong Yaeng, Mae Rim	4 Apr 2019
A. cerana	13	Pong Yaeng, Mae Rim	25 Jan 2014
	32	Mae Raem, Mae Rim	4 Apr 2019
	34	Pong Yaeng, Mae Rim	4 Apr 2019
A. dorsata	9	Pong Yaeng, Mae Rim	4 Apr 2019
A. florea	1	Pong Yaeng, Mae Rim	25 Jan 2014
	3	Mae Raem, Mae Rim	20 Jan 2013
	4	Pong Yaeng, Mae Rim	25 Jan 2014
	5	Pong Yaeng, Mae Rim	25 Jan 2014
	6	Pong Yaeng, Mae Rim	19 Apr 2015
	7	Pong Yaeng, Mae Rim	19 Apr 2015
	8	Pong Yaeng, Mae Rim	19 Apr 2015
	10	Pong Yaeng, Mae Rim	19 Apr 2015
	12	Pong Yaeng, Mae Rim	19 Apr 2015
	20	Samoeng Nuea, Samoeng	19 Apr 2017
	22	Huai Kaeo, Mae On	9 Apr 2017
	23	Pong Yaeng, Mae Rim	9 Apr 2017
	28	Samoeng Nuea, Samoeng	9 Apr 2017
	32	Pong Yaeng, Mae Rim	4 Apr 2019
	33	Pong Yaeng, Mae Rim	4 Apr 2019
	41	Samoeng Nuea, Samoeng	27 Jul 2019
	42	Samoeng Nuea, Samoeng	27 Jul 2019

Table 3 Summary of forty-seven yeast strains investigated in this study

Bee species	Hive no.	Sample	strain no.
A. andreniformis	2	Digestive tract	F19
	3	Honey	AN20H
	7	Honey	PLA0701H
	8	Digestive tract	PLA0801
A. cerana	13	Honey	CE41_3
	32	Digestive tract	PLC3201
	34	Digestive tract	PLC3401
A. dorsata	9	Honey	PLD0901H
A. florea	1	Honey	F0101H, F1, FL4H
	3	Honey	FL9H, FL10H
	4	Digestive tract	F18
	5	Digestive tract	F10, F15
		Honey	FL13H, FL15H
	6	Digestive tract	DO0601
	7	Digestive tract	DO0701, DO0702, DO0705_3
		Honey	F0709H, PL0702
	8	Digestive tract	DO0805
		Honey	F0810H
	10	Honey	F1016H
	12	Honey	F1222H
	20	Digestive tract	M2004
	22	Digestive tract	AM0507
		Honey	H2203H, TO2201H, TO2203H
	23	Honey	TO2301H
	28	Honey	H2802H, TO2802H, TO2803H, TO2804H
	32	Honey	PLF3201H, PLF3202H, PLF3203H, PLF3204H, PLF3205H, PLF3206H
	33	Honey	PLF3301H
	41	Digestive tract	NP4101
	42	Digestive tract	NP4201

3.4 Ribosomal RNA gene (rDNA) Sequencing and Sequence Analysis

Genomic DNA was prepared using the YeaStar Genomic DNA Kit™ (Zymo Research, California) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed according to the following profiles (Table 3.3), for the amplification of LSU D1/D2 domains (O'Donnell, 1993) and ITS regions (White et al., 1990). PCR amplification primers used in this study are listed in Table 5 Amplified DNA was analyzed on 1.5% agarose gel in electrophoretic conditions (80 V, 55 min), stained with ethidium bromide and visualized in UV transilluminator. PCR products were purified with the PureLink® PCR Purification Kit (Life Technologies, New York) and the QIAquick® Gel Extraction Kit (QIAGEN GmbH, Hidden) according to the manufacturer's instructions. Amplicons were sequenced commercially by the ATGC Co., Ltd. (ATGC, Pathum Thani) and sequence assembly was performed in the Sequencher (Gene Codes, Michigan). Sequence data were aligned by the MEGA X program (Kumar et al., 2018) and compared with those available in the GenBank database using the BLASTN program (Altschul et al., 1997). Nucleotide substitution rate was determined by the Kimura's two-parameter method (Kimura, 1980) and phylogenetic tree was constructed for selected yeast species with the neighbor-joining method (Saitou and Nei, 1987) on the CLUSTAL_W package (Thompson et al., 1994). Topology of the phylogenetic tree was tested by performing bootstrap resampling 1,000 replicates (Felsenstein, 1985).

Table 4 PCR conditions used in this study

Protocol	Step	Temperature (ºC)	Time
LSU D1/D2	Predenaturation	94	5 min
	Denaturation	94	30 sec
	Annealing	55	30 sec
	Extension	72	30 sec
	Final extension	72	7 min
ITS	Predenaturation	94	3 min
	Denaturation	94	30 sec
	Annealing	57	30 sec
	Extension	72	1.20 min
	Final extension	72	10 min

Table 5 PCR amplification primers used in this study

Primer	Nucleotide Sequence (5'-3')
NL1	GCATATCAATAAGCGGAGGAAAAG
NL4	GGTCCGTGTTTCAAGACGG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

3.5 Antagonistic Activity

Candidates assumed new yeast species were tested for their activities against four Gram-positive bacteria (*Bacillus cereus* TISTR 687, *B. subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885 and *S. epidermidis* TISTR 518) and ten Gram-negative bacteria (*Acinetobacter calcoaceticus* TISTR 360, *Escherichia coli* TISTR 887, *Klebsiella oxytoca* TISTR 556, *Proteus mirabilis* TISTR 100, *P. morganii* TISTR 098, *Pseudomonas aeruginosa* TISTR 1287, *P. fluorescens* TISTR 358, *Salmonella enterica* subsp. *enterica* ATCC 10708, *S. enterica* subsp. *enterica* serovar Typhimurium TISTR 292 and *Serratia marcescens* TISTR 1354) using the modified method of Fadahunsi and Olubodun (2021). Single yeast colony was inoculated in 20 ml of yeast extract-malt extract broth (YMB) and incubated at 25ºC for 48 h. Fifteen milliliters of cell suspension were transferred to 150 ml of YMB in the 250 ml Erlenmeyer flask and incubated at 25ºC and sampled at 2, 3, 4, 5, 6, 7, 8, 9 and 10 days. Centrifugation was carried out at 13,000 rpm for 5 min and the supernatant was further used to determine the antagonistic activity. Single bacterial colony was grown in 5 ml of nutrient broth (NB, 0.5% peptone, 0.5% sodium chloride, 0.15% HM peptone B, 0.15% yeast extract, w/v) and incubated at 37ºC for 24 h. Bacterial density was adjusted to the 0.5 McFarland turbidity standards and a small amount of bacterial culture was inoculated on the YMA plate with a swab. Wells were bored aseptically on an agar plate using the 7-mm Cork Borer and filled with 100 µl of yeast supernatant. Plates were incubated at 37ºC and inhibition zones were measured periodically in millimeters. Data obtained from the experiments of antagonistic activity were analyzed by one-way ANOVA and means were separated by Duncan test at the significant level of 0.05 (Al-Qaysi et al., 2017).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Yeast Morphology and Physiology

Forty-seven yeast strains considered in this study were kindly provided by Charoenphol (2018), Dumsuwan (2016), Kulee (2018), Laksitanon (2018), Photinakae (2015), Sangprasert (2016), Silakam (2018), Thipsawek (2021) and Thongnum (2015) including 4 hives from *Apis andreniformis*, 3 hives from *Apis cerana*, 1 hive from *Apis dorsata* and 17 hives from *Apis florea* (Table 2-3). In yeast extract-malt extract (YM) broth after 7 days at 25ºC, cells were spheroidal to ellipsoidal, 1.5-1.5×12.5-17.5 µm. Budding is monopolar, bipolar or multilateral (Table 6; Appendix A, Table 12). On YM agar after 7 days at 25ºC, colonies were white to cream, convex, smooth, with an entire margin, exception of the strains M2004, PLA0801 and PLC3201 (Table 7; Appendix A, Table 13). Growth at 50% and 60% glucose agar are positive. Most yeasts with exception of the strains M2004, PLA0701H, TO2301H and TO2804H were osmotolerant (Table 8; Appendix A, Table 14).

Table 6 Cell morphology of investigated yeasts

Strain no.	Cell morphology
AM0507	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-7.5 µm, multilateral
AN20H	Spheroidal to ellipsoidal, 3.75-3.75 ×3.75-5.0 µm, multilateral
CE41_3	Spheroidal to ellipsoidal, 5.0-6.25 × 5.0-10.0 µm, bipolar
DO0601	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral
DO0701	Ellipsoidal, 2.5-3.75 × 5.0-10.0 µm, bipolar
DO0702	Ellipsoidal, 1.5-1.5 × 5.0-10.0 µm, bipolar
DO0705_3	Ellipsoidal, 2.5-2.5 × 3.75-7.5 µm, monopolar
DO0805	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral
F0101H	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-7.5 µm, multilateral
F0709H	Spheroidal to ellipsoidal, 2.5-2.5 × 3.75-7.5 µm, multilateral
F0810H	Spheroidal to ellipsoidal, 2.5-5.0 × 7.5-7.5 µm, multilateral
F1	Spheroidal to ellipsoidal, 2.5-3.75 × 3.75-5.0 µm, multilateral
F10	Ellipsoidal, 2.5-2.5 × 5.0-7.5 µm, monopolar
F1016H	Spheroidal to ellipsoidal, 3.75-3.75 × 3.75-5.0 µm, multilateral
F1222H	Spheroidal to ellipsoidal, 3.75-3.75 × 3.75-5.0 µm, multilateral
F15	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral
F18	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-5.0 µm, multilateral
F19	Spheroidal, 2.5-7.5 × 2.5-7.5 µm, multilateral
FL4H	Spheroidal to ellipsoidal, 2.5-5.0 × 2.5-7.5 µm, multilateral
FL9H	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-5.0 µm, multilateral
FL10H	Spheroidal to ellipsoidal, 2.5-5.0 × 2.5-12.5 µm, multilateral
FL13H	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-6.25 µm, multilateral
FL15H	Spheroidal to ellipsoidal, 2.5-6.25 × 2.5-6.25 µm, multilateral
H2203H	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral
H2802H	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral
M2004	Ellipsoidal, 5.0-6.25 × 7.5-12.5 µm, bipolar

Table 6 (continued)

Strain no.	Cell morphology
NP4101	Ellipsoidal, 2.5-2.5 × 2.5-7.5 µm, monopolar
NP4201	Spheroidal, 3.75-6.25 × 3.75-6.25 µm, multilateral
PL0702	Ellipsoidal, 1.25-3.75 × 5.0-10 µm, monopolar
PLA0701H	Spheroidal to ellipsoidal, 5.0-7.5 × 13.75 µm, monopolar
PLA0801	Ellipsoidal, 2.5-5.0 × 5.0-25 µm, bipolar
PLC3201	Spheroidal to ellipsoidal, 2.5-3.75 × 7.5-15 µm, monopolar, pseudohyphae
PLC3401	Ellipsoidal, 2.5-3.75 × 5.0-15.0 µm, monopolar
PLD0901H	Spheroidal to ellipsoidal, 5.0-5.0 × 5.0-7.5 µm, multilateral
PLF3201H	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-7.5 µm, monopolar
PLF3202H	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-7.5 µm, monopolar
PLF3203H	Ellipsoidal, 2.5 -2.5 × 5.0-7.5 µm, monopolar
PLF3204H	Spheroidal to ellipsoidal, 1.25-2.5 × 3.75-6.25 µm, monopolar
PLF3205H	Ellipsoidal, 2.5-2.5 × 5.0-7.5 µm, monopolar
PLF3206H	Ellipsoidal, 2.5-2.5 × 5.0-6.25 µm, monopolar
PLF3301H	Spheroidal to ellipsoidal, 2.5-3.75 × 6.25-7.5 µm, multilateral
TO2201H	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral
TO2203H	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral
TO2301H	Spheroidal to ellipsoidal, 5.0-6.25 × 5.0-12.5 µm, monopolar
TO2802H	Spheroidal to ellipsoidal, 3.75-3.75 × 5.0-7.5 µm, multilateral
TO2803H	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-6.25 µm, multilateral
TO2804H	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral

After 7 days incubation at 25ºC in YMB

Table 7 Colony morphology of investigated yeasts

Strain no.	Colony morphology
AM0507	White, mat, convex, smooth, entire margin
AN20H	White, slightly glistening, convex, smooth, entire margin
CE41H_3	White to tannish-white, shiny, convex, smooth, entire margin
DO0601	White, slightly glistening, convex, smooth, entire margin
DO0701	Cream, slightly glistening, convex, smooth, entire margin
DO0702	White, slightly glistening, convex, smooth, entire margin
DO0705_3	White, glistening, convex, smooth, entire margin
DO0805	Cream, mat, convex, smooth, entire margin
F0101H	White, mat, convex, smooth, entire margin
F0709H	White, mat, convex, smooth, entire margin
F0810H	White, mat, convex, smooth, entire margin
F1	White, mat, convex, smooth, entire margin
F10	White, slightly glistening, convex, smooth, entire margin
F1016H	White, mat, convex, smooth, entire margin
F1222H	White, mat, convex, smooth, entire margin
F15	White, mat, convex, smooth, entire margin
F18	White, mat, convex, smooth, entire margin

Table 7 (continued)

Strain no.	Colony morphology
F19	White, mat, convex, smooth, entire margin
FL4H	White, slightly glistening, convex, smooth, entire margin
FL9H	White, mat, convex, smooth, entire margin
FL10H	White, slightly glistening, convex, smooth, entire margin
FL13H	White, mat, convex, smooth, entire margin
FL15H	White, slightly glistening, convex, smooth, entire margin
H2203H	White, slightly glistening, convex, smooth, entire margin
H2802H	White, slightly glistening, convex, smooth, entire margin
M2004	Orange, butyrous, glistening, convex, smooth, entire margin
NP4101	White, mat, convex, smooth, entire margin
NP4201	White, slightly glistening, convex, smooth, entire margin
PL0702	White, glistening, convex, smooth, entire margin
PLA0701H	Gray-cream, mucoid, shiny, convex, smooth, entire margin
PLA0801	Cream, flat, smooth, irregular margin
PLC3201	White, wrinkled, rough, irregular margin
PLC3401	White, slightly glistening, convex, smooth, entire margin
PLD0901H	White, mat, convex, smooth, entire margin
PLF3201H	White, slightly glistening, convex, smooth, entire margin
PLF3202H	White, slightly glistening, convex, smooth, entire margin
PLF3203H	White, glistening, convex, smooth, entire margin
PLF3204H	White, mat, convex, smooth, entire margin
PLF3205H	White, slightly glistening, convex, smooth, entire margin
PLF3206H	White, slightly glistening, convex, smooth, entire margin
PLF3301H	White, mat, convex, smooth, entire margin
TO2201H	White, mat, convex, smooth, entire margin
TO2203H	White, slightly glistening, convex, smooth, entire margin
TO2301H	Gray-cream, mucoid, convex, smooth, entire margin
TO2802H	White, mat, convex, smooth, entire margin
TO2803H	White, mat, convex, smooth, entire margin
TO2804H	White, mat, convex, smooth, entire margin

After 7 days incubation at 25ºC on YMA

Table 8 Growth at high sugar concentration of investigated yeasts

Strain no.	Growth at high sugar concentration	References
AM0507	Osmotolerant	Silakam (2018)
AN20H	Osmotolerant	Photinakae (2015)
CE41_3	Osmotolerant	Photinakae (2015)
DO0601	Osmotolerant	Dumsuwan (2016)
DO0701	Osmotolerant	Dumsuwan (2016)
DO0702	Osmotolerant	Dumsuwan (2016)
DO0705_3	Osmotolerant	Dumsuwan (2016)
DO0805	Osmotolerant	Dumsuwan (2016)
F0101H	Osmotolerant	Sangprasert (2016)

Table 8 (continued)

Strain no.	Growth at high sugar concentration	References
F0709H	Osmotolerant	Sangprasert (2016)
F0810H	Osmotolerant	Sangprasert (2016)
F1	Osmotolerant	Thongnum (2015)
F10	Osmophile	Thongnum (2015)
F1016H	Osmotolerant	Sangprasert (2016)
F1222H	Osmotolerant	Sangprasert (2016)
F15	Osmotolerant	Thongnum (2015)
F18	Osmotolerant	Thongnum (2015)
F19	Osmotolerant	Thongnum (2015)
FL4H	Osmophile	Photinakae (2015)
FL9H	Osmotolerant	Photinakae (2015)
FL10H	Osmotolerant	Photinakae (2015)
FL13H	Osmotolerant	Photinakae (2015)
FL15H	Osmotolerant	Photinakae (2015)
H2203H	Osmophile	Laksitanon (2018)
H2802H	Osmophile	Laksitanon (2018)
M2004	Non-osmophile	Laksitanon (2018)
NP4101	Osmophile	Thipsawek (2021)
NP4201	Osmophile	Thipsawek (2021)
PL0702	Osmotolerant	Charoenphol (2018)
PLA0701H	Non-osmophile	Sumkaew (2021)
PLA0801	Osmophile	Sumkaew (2021)
PLC3201	Osmophile	Sumkaew (2021)
PLC3401	Osmophile	Sumkaew (2021)
PLD0901H	Osmotolerant	Buddama (2021)
PLF3201H	Osmophile	Buddama (2021)
PLF3202H	Osmophile	Buddama (2021)
PLF3203H	Osmophile	Buddama (2021)
PLF3204H	Osmophile	Buddama (2021)
PLF3205H	Osmophile	Buddama (2021)
PLF3206H	Osmophile	Buddama (2021)
PLF3301H	Osmophile	Buddama (2021)
TO2201H	Osmophile	Kulee (2018)
TO2203H	Osmophile	Kulee (2018)
TO2301H	Non-osmophile	Kulee (2018)
TO2802H	Osmophile	Kulee (2018)
TO2803H	Osmotolerant	Kulee (2018)
TO2804H	Non-osmophile	Kulee (2018)

After 1 month incubation at 25ºC on 50% and 60% glucose agar

4.2 Yeast Identification and Phylogenetic Analysis

Approximately 34% (n = 16) and 66% (n = 31) of yeast strains were obtained from the digestive tract and honey samples, respectively. Studies have shown that the strains with >1% nucleotide substitutions in LSU D1/D2 domains usually represent the distinct species (Kurtzman and Robnett, 1998). According to summarized data in Table 9 (Appendix A, Table 15), the yeasts isolated from native Thai bees were divided into 2 groups. The first group contained the strains with <1% nucleotide substitutions in the LSU D1/D2 domains. The name of the most closely related species found in the BLASTn search was used for the species name. In this group, 3 (19%) and 5 (16%) yeast strains were digestive tract and honey isolates, respectively. Four out of six species, namely, *Aureobasidium thailandense*, *Filobasidium mali*, *Kodamaea ohmeri* and *Pichia kudriavzevii*, have previously been reported not only on honeybees, but also on banana, beetles, berries, caterpillar frass, muscoid fly, plam, plant leaves, stingless bee, wooden surfaces and woodrose flower (Kurtzman, 2011a; Lachance and Kurtzman, 2011; Li et al., 2020; Peterson et al., 2013). The second group included the strains with >1% nucleotide substitutions in the LSU D1/D2 domains. In this group, 13 (81%) and 26 (84%) yeast strains were digestive tract and honey isolates, respectively. They represented novel yeast taxa. Figure 1 depicts the phylogenetic placement of candidates assumed new yeasts obtained from the LSU D1/D2 domains. The ascomycetous yeasts were distributed in the genera *Starmerella* (37) and *Zygotorulaspora* (1), while the basidiomycetous yeast was member of the genus *Occultifur* (1). These clades were defined by Rosa and Lachance (1998), Kurtzman (2003) and Oberwinkler (1990), respectively (Kurtzman, 2011b; Lachance, 2011; Sampaio and Oberwinkler, 2011). The ITS regions have been used in yeast taxonomy because they show a similar amount of intraspecific variation (Kurtzman and Robnette, 2003; Scorzetii et al., 2002). A combined sequence analysis of the LSU D1/D2 domains and the ITS regions for yeast species identification has been recommended (Scorzetii et al., 2002). Figure 2 depicts the phylogenetic placement of candidates assumed new yeasts obtained from the ITS regions. The results revealed that 29 (74%) yeast strains belonged to candidates assumed new species in two genera of the order Saccharomycetales, phylum Ascomycota and one genus of the order Cystobasidiales, phylum Basidiomycota. The ITS sequencing failed for the strains AM0507, DO0601, F1, F1222H, FL9H, FL10H, FL13H, NP4201, TO2802H and TO2803H, indicating that they might have heterogeneous ITS copies (Egli and Henick-Kling 2001). Most yeast species in the *Starmerella* clade were associated with bees and other insects. Some species appeared highly specialized, whereas others had a broader distribution (Brysch-Herzberg, 2004; Daniel et al., 2013), e.g. *S. meliponinorum* mostly from *Tetragonisca angustula*, *S. apicola* species complex from *Melipona quadrifasciata* and *M. rufiventris* (Rosa et al., 2003), and *S. batistae* from *Diadasina distincta* and *Ptilotrix plumata* (Rosa et al., 1999). Similar results were observed in this study, showing that a candidate assumed new species near *S. apis* exhibited a strong association with specific bee species (*Apis florea*). The candidates assumed new species in the genus *Occultifur*, *Starmerella* and *Zygotorulaspora* were occasionally found in this study. Their closet relatives have previously been isolated from flowers, insects, silage, soil and water (Kurtzman, 2011b; Šibanc et al., 2018; Sipiczki, 2010, 2013).

Table 9 Honeybee yeast strains and their LSU D1/D2 and ITS sequence similarity to those of their relatives

Strain no.	Identification by		Identification species	References
	LSU D1D2 (identity)	ITS (identity)		
Group 1: known yeasts				
PLA0701H	*F. mali* (99.36%)	nd	*Filobasidium mali*	Sumkaew (2021)
PLA0801	*A. thailandense* (100%)	nd	*Aureobasidium thailandense*	Sumkaew (2021)
PLC3201	*K. ohmeri* (99.81%)	nd	*Kodamaea ohmeri*	Sumkaew (2021)
PLC3401	*P. kudriavzevii* (99.66%)	nd	*Pichia kudriavzevii*	Sumkaew (2021)
PLD0901H	*S. meliponinorum* (99.60%)	nd	*Starmerella meliponinorum*	Buddama (2021)
PLF3202H	*S. apicola* (100%)	nd	*Starmerella apicola*	Buddama (2021)
PLF3204H	*S. apicola* (100%)	nd	*Starmerella apicola*	Buddama (2021)
TO2301H	*F. mali* (100%)	nd	*Filobasidium mali*	
Group 2: new yeasts				
AM0507	*S. apis* (98.20%)	-	*Starmerella apis*	
AN20H	*S. apis* (98.17%)	*S. apis* (88.49%)	*Starmerella apis*	Aonwimon (2017)
CE41_3	*Z. mrakii* (94.11%)	*Z. mrakii* (82.92%)	*Zygotorulaspora mrakii*	Tangcham (2018)
DO0601	*S. stigmatis* (94.65%)	-	*Starmerella stigmatis*	Chalangsut (2017)
DO0701	*S. caucasica* (97.63%)	*S. caucasica* (89.66%)	*Starmerella caucasica*	Chalangsut (2017)
DO0702	*S. caucasica* (97.63%)	*S. caucasica* (89.32%)	*Starmerella caucasica*	Chalangsut (2017)
DO0705_3	*S. caucasica* (97.63%)	*S. caucasica* (89.37%)	*Starmerella caucasica*	Tangcham (2018)
DO0805	*S. apis* (98.20%)	*S. apis* (88.95%)	*Starmerella apis*	Aonwimon (2017)
F0101H	*S. apis* (98.20%)	*S. apis* (88.33%)	*Starmerella apis*	Aonwimon (2017)
F0709H	*S. apis* (98.20%)	*S. apis* (89.20%)	*Starmerella apis*	Aonwimon (2017)
F0810H	*S. apis* (98.20%)	*S. apis* (88.97%)	*Starmerella apis*	Aonwimon (2017)

Table 9 (continued)

Strain no.	Identification by		Identification species	References
	LSU D1D2 (identity)	ITS (identity)		
F1	*S. apis* (98.19%)	-	*Starmerella apis*	Lahwthong (2016)
F10	*S. caucasica* (97.63%)	*S. caucasica* (88.91%)	*Starmerella caucasica*	Tangcham (2018)
F1016H	*S. apis* (97.97%)	*S. apis* (87.93%)	*Starmerella apis*	Aonwimon (2017)
F1222H	*S. stigmatis* (94.65%)	-	*Starmerella stigmatis*	Chalangsut (2017)
F15	*S. apis* (98.20%)	*S. apis* (88.41%)	*Starmerella apis*	Lahwthong (2016)
F18	*S. apis* (98.20%)	*S. apis* (88.41%)	*Starmerella apis*	Lahwthong (2016)
F19	*S. apis* (98.19%)	*S. apis* (88.41%)	*Starmerella apis*	Lahwthong (2016)
FL4H	*S. apis* (98.20%)	*S. apis* (88.46%)	*Starmerella apis*	
FL9H	*S. apis* (98.17%)	-	*Starmerella apis*	Lahwthong (2016)
FL10H	*S. apis* (98.10%)	-	*Starmerella apis*	Aonwimon (2017)
FL13H	*S. apis* (98.20%)	-	*Starmerella apis*	Aonwimon (2017)
FL15H	*S. apis* (98.20%)	*S. apis* (88.49%)	*Starmerella apis*	Lahwthong (2016)
H2203H	*S. apis* (98.20%)	*S. apis* (89.02%)	*Starmerella apis*	
H2802H	*S. apis* (98.20%)	*S. apis* (94.03%)	*Starmerella apis*	
M2004	*O. mephitis* (98.27%)	*O. mephitis* (93.03%)	*Occultifur mephitis*	
NP4101	*S. caucasica* (97.47%)	*S. caucasica* (89.35%)	*Starmerella caucasica*	
NP4201	*S. apis* (98.23%)	-	*Starmerella apis*	
PL0702	*S. caucasica* (97.41%)	*S. caucasica* (89.66%)	*Starmerella caucasica*	
PLF3201H	*S. caucasica* (95.91%)	*S. caucasica* (89.68%)	*Starmerella caucasica*	
PLF3203H	*S. caucasica* (96.19%)	*S. caucasica* (89.43%)	*Starmerella caucasica*	

Table 9 (continued)

Strain no.	Identification by		Identification species	References
	LSU D1D2 (identity)	ITS (identity)		
PLF3205H	*S. caucasica* (96.12%)	*S. caucasica* (89.66%)	*Starmerella caucasica*	
PLF3206H	*S. caucasica* (96.12%)	*S. caucasica* (89.87%)	*Starmerella caucasica*	
PLF3301H	*S. apis* (98.20%)	*S. apis* (87.19%)	*Starmerella apis*	
TO2201H	*S. apis* (98.20%)	*S. apis* (88.92%)	*Starmerella apis*	
TO2203H	*S. apis* (98.20%)	*S. apis* (89.28%)	*Starmerella apis*	
TO2802H	*S. apis* (98.20%)	-	*Starmerella apis*	
TO2803H	*S. apis* (98.20%)	-	*Starmerella apis*	
TO2804H	*S. apis* (98.20%)	*S. apis* (89.20%)	*Starmerella apis*	

nd, not determined; -, failed sequencing

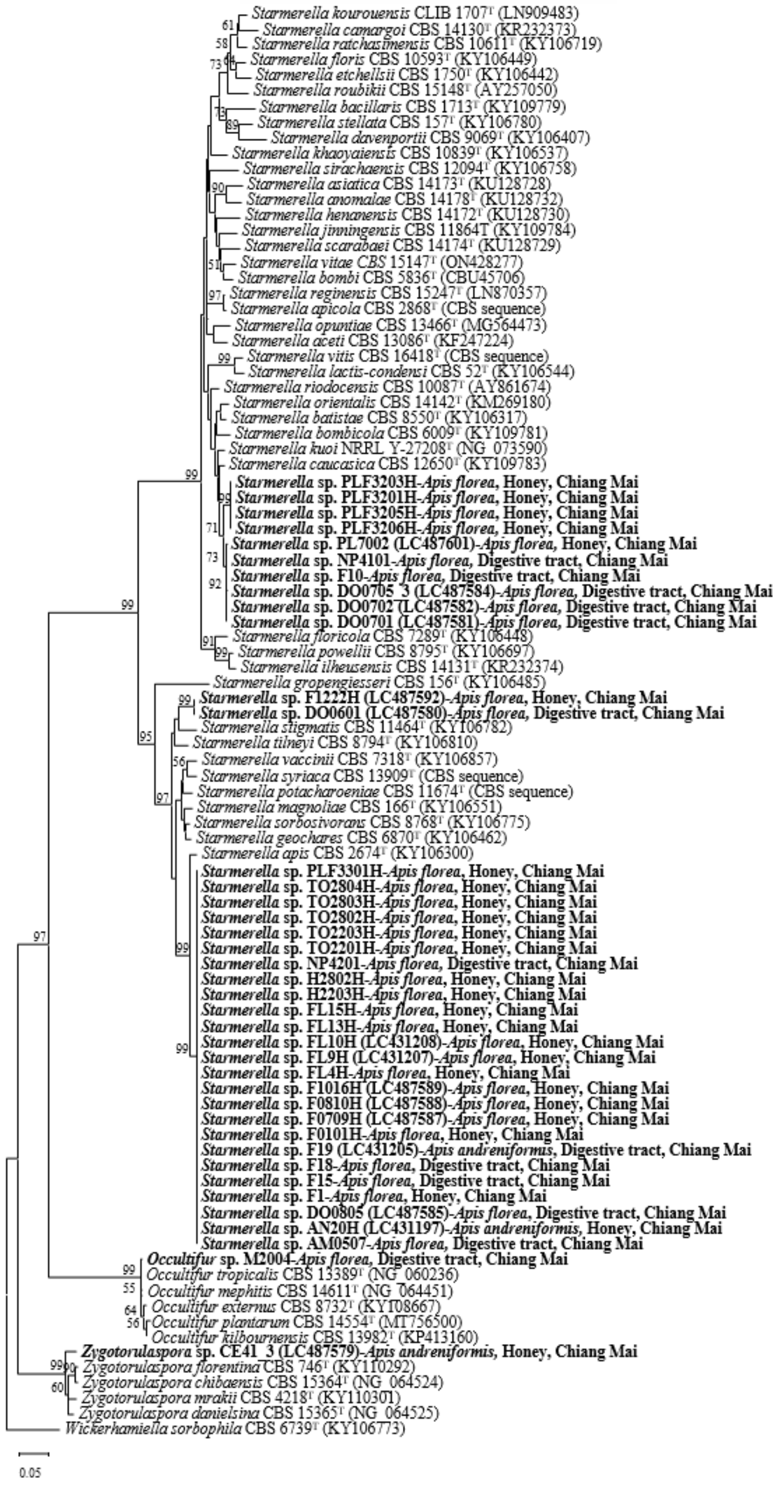

Fig. 1 Neighbor-joining (NJ) tree based on LSU D1/D2 sequences showing phylogenetic positions of yeast strains and their closest relatives. *Wickerhamiella sorbophila* CBS 6739^{T} was used as an outgroup. Number at each node is the percentage bootstrap value obtained from 1,000 replicates (only values >50% are shown). Scale bar shows 0.05 substitutions per nucleotide position.

Fig. 2 Neighbor-joining (NJ) tree based on ITS sequences showing phylogenetic positions of yeast strains and their closest relatives. *Wickerhamiella sorbophila* CBS 6739^{T} was used as an outgroup. Number at each node is the percentage bootstrap value obtained from 1,000 replicates (only values >50% are shown). Scale bar shows 0.05 substitutions per nucleotide position.

4.3 Yeast Diversity

From 93 hives of native Thai bees, 13 (14%) hives were found to contain a candidate assumed new species near *Starmerella apis*. Therefore, this yeast was the species with the highest occurrence, followed by *S. caucasica*, which was detected in 4 (4.3%) hives (Table 10). The highest numbers of two yeast species were found in 3 hives of *Apis florea* (hive no. 5, 7 and 32; Appendix A, Table 16). The 22 remaining hives contained one yeast species. Yeasts were common in sugar-rich habitats associated with bees, including floral nectar and honey provision (Rutkowski et al., 2023). The *Starmerella* clade comprised yeasts isolated mainly from bees and their related environments (Daniel et al., 2014; Rosa et al., 2003). When visiting flowers, bees could come into contact with the yeasts (Alimadadi et al., 2016; Lachance, 2011; Li et al., 2013; Sipiczki, 2015). Several *Starmerella* species have been noted for their specific interactions with insects and occurrence in insect-visiting flowers, suggesting a strong reliance of yeasts on insect hosts and vectors (Lachance et al. 2001). Bee-associated yeasts were generally osmotolerant (Brysch-Herzberg 2004) that made them well adapted to sugar-rich habitats (El Sohaimy et al. 2015). In addition, fructophily also exhibited a preference for fructose over glucose (Leandro et al. 2014: Gonçalves et al. 2020). This trait might contribute to the yeast success in honey-associated habitats, which often contained more fructose than glucose (Cheng et al. 2019; De-Melo et al. 2017; El Sohaimy et al. 2015).

Table 10 Taxonomic summary of honeybee yeast strains from 93 hives and their frequencies of occurrence

Yeast species	Bee species					
	A. andreniformis	*A. cerana*	*A. dorsata*	*A. florea*	No. of yeasts	No. of hives (% FO)
A. thailandense	1	0	0	0	1	1 (1.1%)
F. mali	1	0	0	1	2	2 (2.2%)
K. ohmeri	0	1	0	0	1	1 (1.1%)
O. mephitis	0	0	0	1	1	1 (1.1%)
P. kudriavzevii	0	1	0	0	1	1 (1.1%)
S. apicola	0	0	0	2	2	1[b] (1.1%)
S. apis	2	0	0	24	26	13[ac] (14%)
S. caucasica	0	0	0	9	9	4[abc] (4.3%)
S. meliponinorum	0	0	1	0	1	1 (1.1%)
S. stigmatis	0	0	0	2	2	2 (2.2%)
Z. mrakii	0	1	0	0	1	1 (1.1%)
Total no.	4	3	1	39	47	25

FO, Frequency of occurrence (%) = number of hives, where a particular yeast species was observed, as a proportion of the total number of hives; [a-c], two yeast species per hives

4.4 Yeast Antagonistic Activity

Thirty-nine candidates assumed new yeast species were screened for antagonistic activity against four Gram-positive bacteria (*Bacillus cereus* TISTR 687, *B. subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885 and *S. epidermidis* TISTR 518) and ten Gram-negative bacteria (*Acinetobacter calcoaceticus* TISTR 360, *Escherichia coli* TISTR 887, *Klebsiella oxytoca* TISTR 556, *Proteus mirabilis* TISTR 100, *P. morganii* TISTR 098, *Pseudomonas aeruginosa* TISTR 1287, *P. fluorescens* TISTR 358, *Salmonella enterica* subsp. *enterica* ATCC 10708, *S. enterica* subsp. *enterica* serovar Typhimurium TISTR 292 and *Serratia marcescens* TISTR 1354). Table 4.6 showed a significant potential of yeasts against *A. calcoaceticus* TISTR 360 and the inhibition zone diameters ranged from 10.8±0.4 to 14.6±0.5 mm. One strain of new species near *Starmerella apis* (FL15H) and two strains of new species near *S. caucasica* (PLF3203H and PLF3205H) exhibited a slightly different capacity to inhibit the bacterial growth. Similar to the previous reports by Ma et al. (2022) and Chen et al. (2020), showing that *S. bombicola* had antagonistic activity against Gram-positive bacteria and Gram-negative bacteria. Yeast antagonistic mechanisms could be divided into four major pathways, including competition for nutrients and space, direct parasitism effect, production of antimicrobial compounds, and induction of host resistance (Ma et al., 2023). Some yeast species in the *Starmerella* clade, *S. apicola*, *S. bombicola*, *S. kuoi*, *S. riodocensis* and *S. stellate*, showed significant sophorolipid production (Kurtzman et al., 2010). In addition, the previous reports suggested that sophorolipids exhibited antimicrobial activity through several mechanisms, including altering and destabilizing cellular membrane permeability (Baek et al., 2003; Gaur et al., 2019; Kim et al., 2002).

Table 11 Antagonistic activity of candidates assumed new yeast species against *Acinetobacter calcoaceticus* TISTR 360 using agar well diffusion on YMA at 37°C for 24 h

Strain no.	New species near	Inhibition zone (mm)
F15	*Starmerella apis*	$12.0±0.7^{b}$
F18	*Starmerella apis*	$11.8±0.4^{ab}$
F19	*Starmerella apis*	$11.0±0.7^{ab}$
FL13H	*Starmerella apis*	$10.8±0.4^{a}$
FL15H	*Starmerella apis*	$13.2±1.8^{c}$
PLF3203H	*Starmerella caucasica*	$13.8±0.4^{cd}$
PLF3205H	*Starmerella caucasica*	$14.6±0.5^{d}$
PLF3206H	*Starmerella caucasica*	$11.4±0.5^{ab}$

Values are mean ± standard deviation from five replications; values followed by the same alphabetical letter are not statistically different by Duncan's test ($p < 0.05$)

CHAPTER 5
CONCLUSION

1. Forty-seven yeast strains considered in this study were isolated from 4 hives of *Apis andreniformis*, 3 hives of *Apis cerana*, 1 hive of *Apis dorsata* and 17 hives of *Apis florea*. Approximately 34% (n = 16) and 66% (n = 31) of yeast strains were obtained from the digestive tract and honey samples, respectively.
2. Most yeast cells were spheroidal to ellipsoidal, 1.5-1.5×12.5-17.5 µm. Budding is monopolar, bipolar or multilateral. Yeast colonies were white to cream, convex, smooth, with an entire margin, and they were osmotolerant.
3. According to the LSU D1/D2 sequence analysis, the yeasts from native Thai bees were divided into 2 groups. The first group contained the known species in the genera *Aureobasidium*, *Filobasidium*, *Kodamaea*, *Pichia* and *Starmerella*. Whereas, the second group included the candidates assumed new species in the genera *Occultifur*, *Starmerella* and *Zygotorulaspora*. The species with the highest occurrence was a candidate assumed new species near *Starmerella apis* that exhibited a strong association with *Apis florea*.
4. Thirty-nine candidates assumed new yeast species were screened for antagonistic activity against four Gram-positive bacteria and ten Gram-negative bacteria using agar well diffusion method. Eight strains, identified as *S. apis* and *S. caucasica*, exhibited a capacity to inhibit *Acinetobacter calcoaceticus* TISTR 360 and the inhibition zone diameters ranged from 10.8±0.4 to 14.6±0.5 mm.

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APPENDIX

APPENDIX A

Table 12 Cell morphology of investigated yeasts

Strain no.	Cell morphology		References
	Previous results	Recent results	
AM0507	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-5.0 µm, bipolar	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-7.5 µm, multilateral	Silakam (2018)
AN20H	Spheroidal to ellipsoidal, 1.3-2.5 × 2.5-3.8 µm, monopolar	Spheroidal to ellipsoidal, 3.75-3.75 ×3.75-5.0 µm, multilateral	Photinakae (2015)
CE41_3	Spherical, 2.5-5.0 × 2.5-5.0 µm, multilateral	Spheroidal to ellipsoidal, 5.0-6.25 × 5.0-10.0 µm, bipolar	Photinakae (2015)
DO0601	Spheroidal, 3.5-4.5 × 3.5-4.5 µm, multilateral	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral	Dumsuwan (2016)
DO0701	Ellipsoidal, 2.5-3.0 × 4.0-4.5 µm, bipolar	Ellipsoidal, 2.5-3.75 × 5.0-10.0 µm, bipolar	Dumsuwan (2016)
DO0702	Ellipsoidal, 2.75-3.25 × 3.75-4.25 µm, multilateral	Ellipsoidal, 1.5-1.5 × 5.0-10.0 µm, bipolar	Dumsuwan (2016)
DO0705_3	Ellipsoidal, 2.5-3.5 × 4.0-4.75 µm, bipolar	Ellipsoidal, 2.5-2.5 × 3.75-7.5 µm, monopolar	Dumsuwan (2016)
DO0805	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, bipolar, pseudomycelium	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral	Dumsuwan (2016)
F0101H	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-7.5 µm, multilateral	Sangprasert (2016)
F0709H	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 2.5-2.5 × 3.75-7.5 µm, multilateral	Sangprasert (2016)
F0810H	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 2.5-5.0 × 7.5-7.5 µm, multilateral	Sangprasert (2016)
F1	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral	Spheroidal to ellipsoidal, 2.5-3.75 × 3.75-5.0 µm, multilateral	Thongnum (2015)
F10	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Ellipsoidal, 2.5-2.5 × 5.0-7.5 µm, monopolar	Thongnum (2015)

Table 12 (continued)

Strain no.	Cell morphology		
	Previous results	Recent results	References
F1016H	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 3.75-3.75 × 3.75-5.0 µm, multilateral	Sangprasert (2016)
F1222H	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 3.75-3.75 × 3.75-5.0 µm, multilateral	Sangprasert (2016)
F15	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral	Thongnum (2015)
F18	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-5.0 µm, multilateral	Thongnum (2015)
F19	Spheroidal, 2.5-5.0 × 2.5-5.0 µm, multilateral	Spheroidal, 2.5-7.5 × 2.5-7.5 µm, multilateral	Thongnum (2015)
FL4H	Spheroidal to ellipsoidal, 1.3-2.5 × 2.5-5.0 µm, monopolar	Spheroidal to ellipsoidal, 2.5-5.0 × 2.5-7.5 µm, multilateral	Photinakae (2015)
FL9H	Spheroidal to ellipsoidal, 1.3-2.5 × 2.5-3.8 µm, multilateral	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-5.0 µm, multilateral	Photinakae (2015)
FL10H	Spheroidal to ellipsoidal, 2.5-3.8 × 3.8-5.0 µm, multilateral	Spheroidal to ellipsoidal, 2.5-5.0 × 2.5-12.5 µm, multilateral	Photinakae (2015)
FL13H	Spheroidal to ellipsoidal, 2.5-3.8 × 3.8-5.0 µm, multilateral	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-6.25 µm, multilateral	Photinakae (2015)
FL15H	Spheroidal, 2.5-3.8 × 2.5-3.8 µm, multilatera	Spheroidal to ellipsoidal, 2.5-6.25 × 2.5-6.25 µm, multilateral	Photinakae (2015)
H2203H	Spheroidal to ellipsoidal, monopolar	Spheroidal, 3.75-5.0 × 3.75-5.0 µm, multilateral	Laksitanon (2018)
H2802H	Spheroidal to ellipsoidal, monopolar	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral	Laksitanon (2018)
M2004	Spheroidal to ellipsoidal, monopolar	Ellipsoidal, 5.0-6.25 × 7.5-12.5 µm, bipolar	Laksitanon (2018)

Table 12 (continued)

Strain no.	Cell morphology		References
	Previous results	Recent results	
NP4101	Spheroidal to ellipsoidal, 2.5-3.75 × 3.75-5.0 µm, monopolar	Ellipsoidal, 2.5-2.5 × 2.5-7.5 µm, monopolar	Thipsawek (2021)
NP4201	Spheroidal, 2.5-3.75 × 2.5-5.0 µm, monopolar	Spheroidal, 3.75-6.25 × 3.75-6.25 µm, multilateral	Thipsawek (2021)
PL0702	Ellipsoidal, 2.25-2.25 × 5.0-10 µm, monopolar	Ellipsoidal, 1.25-3.75 × 5.0-10 µm, monopolar	Charoenphol (2018)
PLA0701H	Spheroidal, 5.0-7.5 × 5.0-7.5 µm, monopolar	Spheroidal to ellipsoidal, 5.0-7.5 × 13.75 µm, monopolar	Sumkaew (2021)
PLA0801	Ellipsoidal, 5.0-7.5 × 7.5-10.0 µm, monopolar	Ellipsoidal, 2.5-5.0 × 5.0-25 µm, bipolar	Sumkaew (2021)
PLC3201	Spheroidal to ellipsoidal, 2.5-5.0 × 5.0-7.5 µm, monopolar, pseudohyphae[i]	Spheroidal to ellipsoidal, 2.5-3.75 × 7.5-15 µm, monopolar, pseudohyphae	Sumkaew (2021)
PLC3401	Ellipsoidal, 2.5-5.0 × 5.0-10.0 µm, monopolar	Ellipsoidal, 2.5-3.75 × 5.0-15.0 µm, monopolar	Sumkaew (2021)
PLD0901H	Spheroidal, 2.0-7.5 × 5.0-7.5 µm, monopolar	Spheroidal to ellipsoidal, 5.0-5.0 × 5.0-7.5 µm, multilateral	Buddama (2021)
PLF3201H	Spheroidal, 2.5-3.75 × 5.0 µm, monopolar	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-7.5 µm, monopolar	Buddama (2021)
PLF3202H	Spheroidal, 2.5-3.75 × 2.5-3.75 µm, monopolar	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0-7.5 µm, monopolar	Buddama (2021)
PLF3203H	Ellipsoidal, 2.5-3.75 × 5.0 µm, monopolar	Ellipsoidal, 2.5 -2.5 × 5.0-7.5 µm, monopolar	Buddama (2021)
PLF3204H	Spheroidal, 2.5-3.75 × 2.5-3.75 µm, monopolar	Spheroidal to ellipsoidal, 1.25-2.5 × 3.75-6.25 µm, monopolar	Buddama (2021)
PLF3205H	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0 µm, monopolar	Ellipsoidal, 2.5-2.5 × 5.0-7.5 µm, monopolar	Buddama (2021)

Table 12 (continued)

Strain no.	Cell morphology		References
	Previous results	Recent results	
PLF3206H	Spheroidal to ellipsoidal, 2.5-3.75 × 5.0 µm, monopolar	Ellipsoidal, 2.5-2.5 × 5.0-6.25 µm, monopolar	Buddama (2021)
PLF3301H	Spheroidal, 2.5-3.75 × 2.5-3.75 µm, monopolar	Spheroidal to ellipsoidal, 2.5-3.75 × 6.25-7.5 µm, multilateral	Buddama (2021)
TO2201H	Spheroidal, 2.5 × 6.2 µm, bipolar	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral	Kulee (2018)
TO2203H	Spheroidal, 2.5 × 5.0 µm, bipolar	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral	Kulee (2018)
TO2301H	Spheroidal, 3.7 × 3.7 µm, multilateral	Spheroidal to ellipsoidal, 5.0-6.25 × 5.0-12.5 µm, monopolar	Kulee (2018)
TO2802H	Spheroidal, 2.5 × 3.7 µm, bipolar	Spheroidal to ellipsoidal, 3.75-3.75 × 5.0-7.5 µm, multilateral	Kulee (2018)
TO2803H	Spheroidal, 3.7 × 6.2 µm, bipolar	Spheroidal to ellipsoidal, 2.5-3.75 × 2.5-6.25 µm, multilateral	Kulee (2018)
TO2804H	Spheroidal, 3.7× 6.2 µm, multilateral[g]	Spheroidal to ellipsoidal, 3.75-5.0 × 5.0-6.25 µm, multilateral	Kulee (2018)

After 7 days incubation at 25ºC on YMB

Table 13 Colony morphology of investigated yeasts

Strain no.	Colony morphology		
	Previous results	Recent results	References
AM0507	White, glistening, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Silakam (2018)
AN20H	Cream, glistening, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Photinakae (2015)
CE41_3	White to tannish-white, shiny, convex, smooth, entire margin	White to tannish-white, shiny, convex, smooth, entire margin	Photinakae (2015)
DO0601	Cream, convex, smooth, entire margins	White, slightly glistening, convex, smooth, entire margin	Dumsuwan (2016)
DO0701	Cream, convex, smooth, entire margins	Cream, slightly glistening, convex, smooth, entire margin	Dumsuwan (2016)
DO0702	White, convex, smooth, entire margins	White, slightly glistening, convex, smooth, entire margin	Dumsuwan (2016)
DO0705_3	White, convex, smooth, entire margins	White, glistening, convex, smooth, entire margin	Dumsuwan (2016)
DO0805	White, convex, smooth, entire margins	Cream, mat, convex, smooth, entire margin	Dumsuwan (2016)
F0101H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Sangprasert (2016)
F0709H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Sangprasert (2016)
F0810H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Sangprasert (2016)
F1	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Thongnum (2015)
F10	White, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Thongnum (2015)
F1016H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Sangprasert (2016)
F1222H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Sangprasert (2016)
F15	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Thongnum (2015)
F18	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Thongnum (2015)
F19	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Thongnum (2015)

Table 13 (continued)

Strain no.	Colony morphology: Previous results	Colony morphology: Recent results	References
FL4H	Cream, convex or umbonate, glistening, entire margin	White, slightly glistening, convex, smooth, entire margin	Photinakae (2015)
FL9H	Cream, convex or umbonate, glistening, entire margin	White, mat, convex, smooth, entire margin	Photinakae (2015)
FL10H	Cream, convex or umbonate, glistening, entire margin	White, slightly glistening, convex, smooth, entire margin	Photinakae (2015)
FL13H	Cream, convex, glistening, entire margin	White, mat, convex, smooth, entire margin	Photinakae (2015)
FL15H	Cream, convex, glistening, entire margin	White, slightly glistening, convex, smooth, entire margin	Photinakae (2015)
H2203H	Orange, convex, glistening, entire margin	White, slightly glistening, convex, smooth, entire margin	Laksitanon (2018)
H2802H	White, convex, glistening, entire margin	White, slightly glistening, convex, smooth, entire margin	Laksitanon (2018)
M2004	Orange, butyrous, glistening, convex, entire margin	Orange, butyrous, glistening, convex, smooth, entire margin	Laksitanon (2018)
NP4101	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Thipsawek (2021)
NP4201	White, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Thipsawek (2021)
PL0702	White, convex, smooth, entire margin	White, glistening, convex, smooth, entire margin	Charoenphol (2018)
PLA0701H	Gray-cream, convex, smooth, entire margin	Gray-cream, mucoid, shiny, convex, smooth, entire margin	Sumkaew (2021)
PLA0801	Cream, flat, smooth, entire margin	Cream, flat, smooth, irregular margin	Sumkaew (2021)
PLC3201	White, convex, rough, undulate margin	White, wrinkled, rough, irregular margin	Sumkaew (2021)

Table 13 (continued)

Strain no.	Colony morphology		
	Previous results	Recent results	References
PLC3401	White, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Sumkaew (2021)
PLD0901H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Buddama (2021)
PLF3201H	White, glistening, convex, entire margin	White, slightly glistening, convex, smooth, entire margin	Buddama (2021)
PLF3202H	White, glistening, convex, entire margin	White, slightly glistening, convex, smooth, entire margin	Buddama (2021)
PLF3203H	White, glistening, convex, entire margin	White, glistening, convex, smooth, entire margin	Buddama (2021)
PLF3204H	White, glistening, convex, entire margin	White, mat, convex, smooth, entire margin	Buddama (2021)
PLF3205H	White, glistening, convex, entire margin	White, slightly glistening, convex, smooth, entire margin	Buddama (2021)
PLF3206H	White, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Buddama (2021)
PLF3301H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Buddama (2021)
TO2201H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Kulee (2018)
TO2203H	White, convex, smooth, entire margin	White, slightly glistening, convex, smooth, entire margin	Kulee (2018)
TO2301H	Gray-cream, convex, smooth, entire margin	Gray-cream, mucoid, convex, smooth, entire margin	Kulee (2018)
TO2802H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Kulee (2018)
TO2803H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Kulee (2018)
TO2804H	White, convex, smooth, entire margin	White, mat, convex, smooth, entire margin	Kulee (2018)

After 7 days incubation at 25ºC on YMA

Table 14 Growth at high sugar concentration of investigated yeasts

Strain no.	Growth at high sugar concentration										References
	50% glucose					60% glucose					
	W1	W2	W3	W4	Result	W1	W2	W3	W4	Result	
AM0507	++	+++	+++	++++	+(s)	+	+	++	++	+(w)	Silakam (2018)
AN20H	++	+++	++++	++++	+(s)	+	++	+++	+++	+(w)	Photinakae (2015)
CE41_3	++	++	+++	+++	+(w)	+	+	++	++	+(w)	Photinakae (2015)
DO0601	++	+++	+++	+++	+(s)	++	++	++	++	+(w)	Dumsuwan (2016)
DO0701	++	+++	+++	+++	+(s)	++	++	++	++	+(w)	Dumsuwan (2016)
DO0702	++	+++	+++	+++	+(s)	++	++	++	++	+(w)	Dumsuwan (2016)
DO0705_3	++	+++	+++	+++	+(s)	++	++	++	++	+(w)	Dumsuwan (2016)
DO0805	+	++	++	+++	+(s)	+	++	++	++	+(w)	Dumsuwan (2016)
F0101H	+	++	++	++	+(w)	+	++	++	++	+(w)	Sangprasert (2016)
F0709H	++	+++	+++	+++	+(s)	+	++	++	++	+(w)	Sangprasert (2016)
F0810H	++	++	++	++	+(w)	+	++	++	++	+(w)	Sangprasert (2016)
F1	+	++	++	++	+(w)	+	++	++	++	+(w)	Thongnum (2015)
F10	+	++	+++	+++	+(s)	+	++	+++	+++	+(s)	Thongnum (2015)
F1016H	++	+++	+++	+++	+(w)	+	++	++	++	+(w)	Sangprasert (2016)
F1222H	++	+++	+++	+++	+(w)	+	++	++	++	+(w)	Sangprasert (2016)
F15	+	++	++	++	+(w)	+	++	++	++	+(w)	Thongnum (2015)
F18	+	++	++	++	+(w)	+	++	++	++	+(w)	Thongnum (2015)
F19	+	++	++	++	+(w)	+	+	++	++	+(w)	Thongnum (2015)
FL4H	+	+++	++++	++++	+(s)	+	++	+++	+++	+(w)	Photinakae (2015)
FL9H	++	+++	+++	++++	+(s)	+	++	++	+++	+(w)	Photinakae (2015)
FL10H	++	+++	+++	+++	+(w)	+	++	++	+++	+(w)	Photinakae (2015)
FL13H	++	+++	++++	++++	+(s)	+	++	++	++	+(w)	Photinakae (2015)
FL15H	++	++++	++++	++++	+(s)	+	++	++	++	+(w)	Photinakae (2015)
H2203H	+	++	+++	++++	+	++	+++	++++	++++	+(s)	Laksitanon (2018)

Table 14 (continued)

Strain no.	Growth at high sugar concentration										References
	50% glucose					60% glucose					
	W1	W2	W3	W4	Result	W1	W2	W3	W4	Result	
H2802H	+	++	+++	++++	+	++	+++	+++	++++	+(s)	Laksitanon (2018)
M2004	-	-	-	-	-	-	-	-	-	-	Laksitanon (2018)
NP4101	+++	++++	++++	++++	+	+++	++++	++++	++++	+	Thipsawek (2021)
NP4201	+++	+++	++++	++++	+	+++	+++	+++	+++	+	Thipsawek (2021)
PL0702H	++	+++	+++	+++	+(s)	+	++	++	++	+(w)	Charoenphol (2018)
PLA0701H	-	-	-	-	-	-	-	-	-	-	Sumkaew (2021)
PLA0801	+++	++++	++++	++++	+	++	++++	++++	++++	+	Sumkaew (2021)
PLC3201	+++	++++	++++	++++	+	+++	++++	++++	++++	+	Sumkaew (2021)
PLC3401	+++	+++	++++	++++	+	++	+++	+++	++++	+(s)	Sumkaew (2021)
PLD0901H	++	++	++	++	+(w)	++	++	++	++	+(w)	Buddama (2021)
PLF3201H	+++	+++	+++	++++	+	+++	+++	+++	++++	+	Buddama (2021)
PLF3202H	+++	+++	+++	++++	+	+++	+++	+++	+++	+	Buddama (2021)
PLF3203H	+++	+++	++++	++++	+	+++	+++	++++	++++	+	Buddama (2021)
PLF3204H	+++	+++	+++	+++	+	+++	+++	+++	+++	+	Buddama (2021)
PLF3205H	+++	+++	+++	++++	+	+++	+++	+++	+++	+	Buddama (2021)
PLF3206H	+++	+++	+++	++++	+	+++	+++	+++	+++	+	Buddama (2021)
PLF3301H	++	++	+++	+++	+(s)	++	+++	+++	+++	+(s)	Buddama (2021)
TO2201H	++	+++	+++	+++	+(s)	+++	+++	+++	+++	+(s)	Kulee (2018)
TO2203H	+++	+++	+++	+++	+(w)	+++	+++	+++	+++	+(w)	Kulee (2018)
TO2301H	-	-	-	-	-	-	-	-	-	-	Kulee (2018)
TO2802H	++	+++	+++	+++	+(s)	++	+++	+++	+++	+(s)	Kulee (2018)
TO2803H	-	+	+	++	+(w)	+	+	+	++	+(w)	Kulee (2018)
TO2804H	-	-	-	-	-	-	-	-	-	-	Kulee (2018)

After 1 month incubation at 25ºC; W1, 1 week; W2, 2 weeks; W3, 3 weeks; W4, 4 weeks; +(s), slow; +(w), week

Table 15 Honeybee yeast strains and their LSU D1/D2 and ITS sequence similarity to those of their relatives

Strain no.	Gene	Substitution	Yeast species	Identity	Gaps	References
AM0507	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	-				
AN20H	D1/D2	8	*Starmerella apis* CBS 2674^T	428/436 (98.17%)	0	Aonwimon (2017)
	ITS	48	*Starmerella apis* CBS 2674^T	369/417 (88.49%)	17	Aonwimon (2017)
CE41_3	D1/D2	35	*Zygotorulaspora mrakii* CBS 4218^T	559/594 (94.11%)	6	Tangcham (2018)
	ITS	83	*Zygotorulaspora mrakii* CBS 4218^T	403/486 (82.92%)	58	Tangcham (2018)
DO0601	D1/D2	24	*Starmerella stigmatis* CBS 11464^T	425/449 (94.65%)	1	Chalangsut (2017)
	ITS	-				
DO0701	D1/D2	11	*Starmerella caucasica* CBS 12650^T	453/464 (97.63%)	0	Chalangsut (2017)
	ITS	49	*Starmerella caucasica* CBS 12650^T	425/474 (89.66%)	21	Chalangsut (2017)
DO0702	D1/D2	11	*Starmerella caucasica* CBS 12650^T	453/464 (97.63%)	0	Chalangsut (2017)
	ITS	49	*Starmerella caucasica* CBS 12650^T	410/459 (89.32%)	21	Chalangsut (2017)
DO0705_3	D1/D2	11	*Starmerella caucasica* CBS 12650^T	453/464 (97.63%)	0	Chalangsut (2017)
	ITS	49	*Starmerella caucasica* CBS 12650^T	412/461 (89.37%)	21	Tangcham (2018)
DO0805	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Aonwimon (2017)
	ITS	42	*Starmerella apis* CBS 2674^T	338/380 (88.95%)	17	Aonwimon (2017)
F0101H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Aonwimon (2017)
	ITS	42	*Starmerella apis* CBS 2674^T	318/360 (88.33%)	17	Aonwimon (2017)
F0709H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Aonwimon (2017)
	ITS	47	*Starmerella apis* CBS 2674^T	388/435 (89.20%)	17	Aonwimon (2017)
F0810H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Aonwimon (2017)
	ITS	48	*Starmerella apis* CBS 2674^T	387/435 (88.97%)	18	Aonwimon (2017)
F1	D1/D2	8	*Starmerella apis* CBS 2674^T	433/441 (98.19%)	0	Lahwthong (2016)
	ITS	-				

Table 15 (continued)

Strain no.	Gene	Substitution	Yeast species	Identity	Gaps	References
F10	D1/D2	11	*Starmerella caucasica* CBS 12650^T	453/464 (97.63%)	0	Tangcham (2018)
	ITS	53	*Starmerella caucasica* CBS 12650^T	425/478 (88.91%)	25	Tangcham (2018)
F1016H	D1/D2	9	*Starmerella apis* CBS 2674^T	435/444 (97.97%)	0	Aonwimon (2017)
	ITS	42	*Starmerella apis* CBS 2674^T	306/348 (87.93%)	17	Aonwimon (2017)
F1222H	D1/D2	24	*Starmerella stigmatis* CBS 11464^T	425/449 (94.65%)	1	Chalangsut (2017)
	ITS	-				
F15	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Lahwthong (2016)
	ITS	48	*Starmerella apis* CBS 2674^T	366/414 (88.41%)	18	Lahwthong (2016)
F18	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Lahwthong (2016)
	ITS	48	*Starmerella apis* CBS 2674^T	366/414 (88.41%)	18	Lahwthong (2016)
F19	D1/D2	8	*Starmerella apis* CBS 2674^T	435/443 (98.19%)	0	Lahwthong (2016)
	ITS	46	*Starmerella apis* CBS 2674^T	351/397 (88.41%)	17	Lahwthong (2016)
FL4H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	48	*Starmerella apis* CBS 2674^T	368/416 (88.46%)	19	
FL9H	D1/D2	8	*Starmerella apis* CBS 2674^T	428/436 (98.17%)	0	Lahwthong (2016)
	ITS	-				
FL10H	D1/D2	8	*Starmerella apis* CBS 2674^T	428/436 (98.10%)	0	Aonwimon (2017)
	ITS	-				
FL13H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Aonwimon (2017)
	ITS	-				
FL15H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	Lahwthong (2016)
	ITS	48	*Starmerella apis* CBS 2674^T	369/417 (88.49%)	17	Lahwthong (2016)
H2203H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	47	*Starmerella apis* CBS 2674^T	381/428 (89.02%)	17	
H2802H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	16	*Starmerella apis* CBS 2674^T	252/268 (94.03%)	6	

Table 15 (continued)

Strain no.	Gene	Substitution	Yeast species	Identity	Gaps	References
M2004	D1/D2	10	*Occultifur mephitis* CBS 14611^T	568/578 (98.27%)	1	
	ITS	38	*Occultifur mephitis* CBS 14611^T	507/545 (93.03%)	10	
NP4101	D1/D2	12	*Starmerella caucasica* CBS 12650^T	463/475 (97.47%)	1	
	ITS	49	*Starmerella caucasica* CBS 12650^T	411/460 (89.35%)	18	
NP4201	D1/D2	8	*Starmerella apis* CBS 2674^T	445/453 (98.23%)	0	
	ITS	-				
PL0702	D1/D2	12	*Starmerella caucasica* CBS 12650^T	452/464 (97.41%)	0	
	ITS	49	*Starmerella caucasica* CBS 12650^T	425/474 (89.66%)	22	
PLA0701H	D1/D2	4	*Filobasidium mali* CBS 15651^T	622/626 (99.36%)	4	Sumkaew (2021)
	ITS	nd				
PLA0801	D1/D2	0	*Aureobasidium thailandense* CBS 133856^T	527/527 (100%)	0	Sumkaew (2021)
	ITS	nd				
PLC3201	D1/D2	1	*Kodamaea ohmeri* CBS 1950^T	527/528 (99.81%)	0	Sumkaew (2021)
	ITS	nd				
PLC3401	D1/D2	2	*Pichia kudriavzevii* CBS 573^T	586/588 (99.66%)	1	Sumkaew (2021)
	ITS	nd				
PLD0901H	D1/D2	2	*Starmerella meliponinorum* CBS 9117^T	499/501 (99.60%)	0	Buddama (2021)
	ITS	nd				
PLF3201H	D1/D2	19	*Starmerella caucasica* CBS 12650^T	445/464 (95.91%)	0	
	ITS	48	*Starmerella caucasica* CBS 12650^T	417/465 (89.68%)	19	
PLF3202H	D1/D2	0	*Starmerella apicola* CBS 2868^T	480/480 (100%)	0	Buddama (2021)
	ITS	nd				
PLF3203H	D1/D2	18	*Starmerella caucasica* CBS 12650^T	455/473 (96.19%)	0	
	ITS	50	*Starmerella caucasica* CBS 12650^T	423/473 (89.43%)	19	
PLF3204H	D1/D2	0	*Starmerella apicola* CBS 2868^T	502/502 (100%)	0	Buddama (2021)
	ITS	nd				

Table 15 (continued)

Strain no.	Gene	Substitution	Yeast species	Identity	Gaps	References
PLF3205H	D1/D2	18	*Starmerella caucasica* CBS 12650^T	446/464 (96.12%)	0	
	ITS	49	*Starmerella caucasica* CBS 12650^T	425/474 (89.66%)	20	
PLF3206H	D1/D2	18	*Starmerella caucasica* CBS 12650^T	446/464 (96.12%)	0	
	ITS	48	*Starmerella caucasica* CBS 12650^T	426/474 (89.87%)	20	
PLF3301H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	56	*Starmerella apis* CBS 2674^T	381/437 (87.19%)	22	
TO2201H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	47	*Starmerella apis* CBS 2674^T	377/424 (88.92%)	18	
TO2203H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	46	*Starmerella apis* CBS 2674^T	383/429 (89.28%)	17	
TO2301H	D1/D2	0	*Filobasidium mali* CBS 15651^T	619/619 (100%)	0	
	ITS	nd				
PLF3205H	D1/D2	18	*Starmerella caucasica* CBS 12650^T	446/464 (96.12%)	0	
	ITS	49	*Starmerella caucasica* CBS 12650^T	425/474 (89.66%)	20	
PLF3206H	D1/D2	18	*Starmerella caucasica* CBS 12650^T	446/464 (96.12%)	0	
	ITS	48	*Starmerella caucasica* CBS 12650^T	426/474 (89.87%)	20	
PLF3301H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	56	*Starmerella apis* CBS 2674^T	381/437 (87.19%)	22	
TO2201H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	47	*Starmerella apis* CBS 2674^T	377/424 (88.92%)	18	
TO2203H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	46	*Starmerella apis* CBS 2674^T	383/429 (89.28%)	17	
TO2301H	D1/D2	0	*Filobasidium mali* CBS 15651^T	619/619 (100%)	0	
	ITS	nd				
TO2802H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	-				

Table 15 (continued)

Strain no.	Gene	Substitution	Yeast species	Identity	Gaps	References
TO2803H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	-				
TO2804H	D1/D2	8	*Starmerella apis* CBS 2674^T	436/444 (98.20%)	0	
	ITS	46	*Starmerella apis* CBS 2674^T	380/426 (89.20%)	17	

nd, not determined; -, failed sequencing

Table 16 Particular yeast species observed in bee hives and their GenBank accession numbers

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
27 Oct 2012	*Apis ndreniformis*	3	1	0	nd			
			3	1	AN20H	Honey	*Starmerella apis*	LC431197
			4	0	nd			
20 Jan 2013	*A. cerana*	4	7	0	nd			
			8	0	nd			
			9	0	nd			
			10	0	nd			
	A. florea	1	3	2	FL9H	Honey	*Starmerella apis*	LC431207
					FL10H	Honey	*Starmerella apis*	LC431208
19 Oct 2013	*A. andreniformis*	1	2	1	F19	Digestive tract	*Starmerella apis*	LC431205
	A. florea	1	2	0	nd			
20 Jan 2014	*A. cerana*	3	12	0	nd			
			14	0	nd			
			15	0	nd			

Table 16 (continued)

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
25 Jan 2014	*A. andreniformis*	1	5	0	nd			
	A. cerana	6	1	0	nd			
			2	0	nd			
			3	0	nd			
			4	0	nd			
			5	0	nd			
			13	1	CE41_3	Honey	*Zygotorulaspora mrakii*	LC487579
	A. florea	3	1	3	F0101H	Honey	*Starmerella apis*	
					F1	Honey	*Starmerella apis*	
					FL4H	Honey	*Starmerella apis*	
			4	1	F18	Digestive tract	*Starmerella apis*	
			5	4	F10	Digestive tract	*Starmerella caucasica*	
					F15	Digestive tract	*Starmerella apis*	
					FL13H	Honey	*Starmerella apis*	
					FL15H	Honey	*Starmerella apis*	
26 Jan 2014	*A. cerana*	1	6	0	nd			
28 Jan 2014	*A. cerana*	1	11	0	nd			
19 May 2014	*A. dorsata*	2	1	0	nd			
			2	0	nd			
19 Jul 2014	*A. andreniformis*	1	6	0	nd			
	A. cerana	3	16	0	nd			
			17	0	nd			
			18	0	nd			

Table 16 (continued)

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
19 Apr 2015	*A. cerana*	6	19	0	nd			
			20	0	nd			
			21	0	nd			
			22	0	nd			
			23	0	nd			
			24	0	nd			
	A. florea	7	6	1	DO0601	Digestive tract	*Starmerella stigmatis*	LC487580
			7	5	DO0701	Digestive tract	*Starmerella caucasica*	LC487581
					DO0702	Digestive tract	*Starmerella caucasica*	LC487582
					DO0705_3	Digestive tract	*Starmerella caucasica*	LC487584
					F0709H	Honey	*Starmerella apis*	LC487587
					PL0702	Honey	*Starmerella caucasica*	LC487601
			8	2	DO0805	Digestive tract	*Starmerella apis*	LC487585
					F0810H	Honey	*Starmerella apis*	LC487588
			9	0	nd			
			10	1	F1016H	Honey	*Starmerella apis*	LC487589
			11	0	nd			
			12	1	F1222H	Honey	*Starmerella stigmatis*	LC487592

Table 16 (continued)

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
28 Jan 2017	*A. cerana*	7	25	0	nd			
			26	0	nd			
			27	0	nd			
			28	0	nd			
			29	0	nd			
			30	0	nd			
			31	0	nd			
	A. florea	1	27	0	nd			
9 Apr 2017	*A. dorsata*	2	7	0	nd			
			8	0	nd			
	A. florea	12	18	0	nd			
			19	0	nd			
			20	1	M2004	Digestive tract	*Occultifur mephitis*	
			21	0	nd			
			22	4	AM0507	Digestive tract	*Starmerella apis*	
					H2203H	Honey	*Starmerella apis*	
					TO2201H	Honey	*Starmerella apis*	
					TO2203H	Honey	*Starmerella apis*	
			23	1	TO2301H	Honey	*Filobasidium mali*	
			24	0	nd			
			25	0	nd			
			26	0	nd			

Table 16 (continued)

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
9 Apr 2017			28	4	H2802H	Honey	*Starmerella apis*	
					TO2802H	Honey	*Starmerella apis*	
					TO2803H	Honey	*Starmerella apis*	
					TO2804H	Honey	*Starmerella apis*	
			29	0	nd			
			30	0	nd			
4 Apr 2019	*A. andreniformis*	2	7	1	PLA0701H	Honey	*Filobasidium mali*	
			8	1	PLA0801	Digestive tract	*Aureobasidium thailandense*	
	A. cerana	4	32	2	PLC3201	Digestive tract	*Kodamaea ohmeri*	
			33	0	nd			
			34	2	PLC3401	Digestive tract	*Pichia kudriavzevii*	
			35	0	nd			
	A. dorsata	2	9	1	PLD0901H	Honey	*Starmerella meliponinorum*	
			10	0	nd			
	A. florea	3	31	0	nd			
			32	6	PLF3201H	Honey	*Starmerella caucasica*	
					PLF3202H	Honey	*Starmerella apicola*	
					PLF3203H	Honey	*Starmerella caucasica*	
					PLF3204H	Honey	*Starmerella apicola*	
					PLF3205H	Honey	*Starmerella caucasica*	
					PLF3206H	Honey	*Starmerella caucasica*	
			33	1	PLF3301H	Honey	*Starmerella apis*	

Table 16 (continued)

Collecting date	Bee species	Total hives	Hive no.	No. of yeasts	Strain no.	Sample	Yeast species	GenBank accession no.
27 Jul 2019	*A. andreniformis*	3	9	0	nd			
			10	0	nd			
			11	0	nd			
	A. cerana	4	36	0	nd			
			37	0	nd			
			38	0	nd			
			39	0	nd			
	A. florea	9	34	0	nd			
			35	0	nd			
			36	0	nd			
			37	0	nd			
			38	0	nd			
			39	0	nd			
			40	0	nd			
			41	1	NP4101	Digestive tract	*Starmerella apis*	
			42	1	NP4201	Digestive tract	*Starmerella apis*	

nd, not determined

Table 17 Antagonistic activity of candidates assumed new yeast species against *Acinetobacter calcoaceticus* TISTR 360 from five replications

Strain no.	Inhibition zone					
	1	2	3	4	5	Mean
F15	13	12	12	11	12	12.0±0.7
F18	12	11	12	12	12	11.8±0.4
F19	12	10	11	11	11	11.0±0.7
FL13H	11	11	11	11	10	10.8±0.4
FL15H	16	12	12	14	12	13.2±1.8
PLF3203H	13	14	14	14	14	13.8±0.4
PLF3205H	14	15	15	15	14	14.6±0.5
PLF3206H	12	11	12	11	11	11.4±0.5

Agar well diffusion on YMA at 37ºC for 24 h

Table 18 Statistical analysis by Duncan test at significant level of 0.05

Oneway

ANOVA

Inhibition zone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	67.175	7	9.596	14.217	.000
Within Groups	21.600	32	.675		
Total	88.775	39			

Post Hoc Tests
Homogeneous Subsets

Inhibition zone

Duncan[a]

Strain	N	Subset for alpha = 0.05			
		1	2	3	4
FL13H	5	10.80			
F19	5	11.00	11.00		
PLF3206H	5	11.40	11.40		
F18	5	11.80	11.80		
F15	5		12.00		
FL15H	5			13.20	
PLF3203H	5			13.80	13.80
PLF3205H	5				14.60
Sig		.087	.087	.257	.133

Means for groups in homogeneous subsets are displayed; [a], Uses Harmonic Mean

APPENDIX B

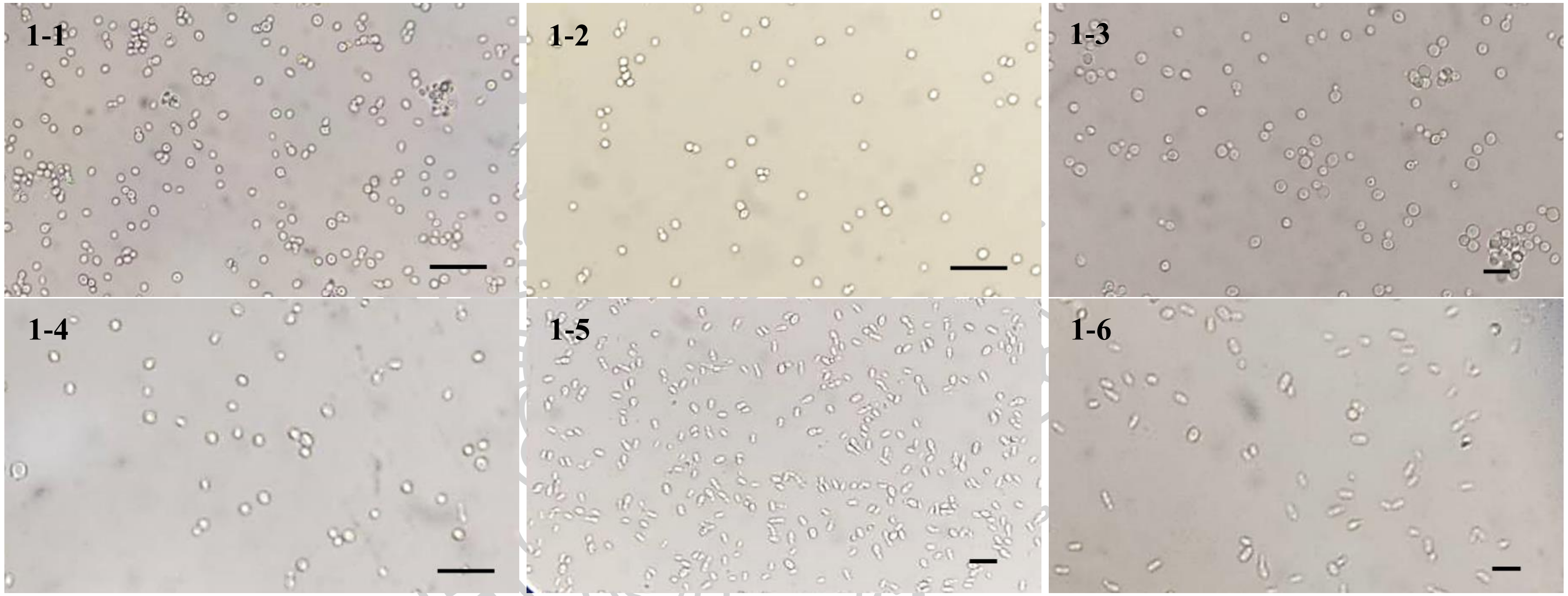

Fig. 3 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-1, AM0507; 1-2, AN20H; 1-3, CE41H; 1-4, DO0601; 1-5, DO0701; 1-6, DO0702; scale bar, 10 μm

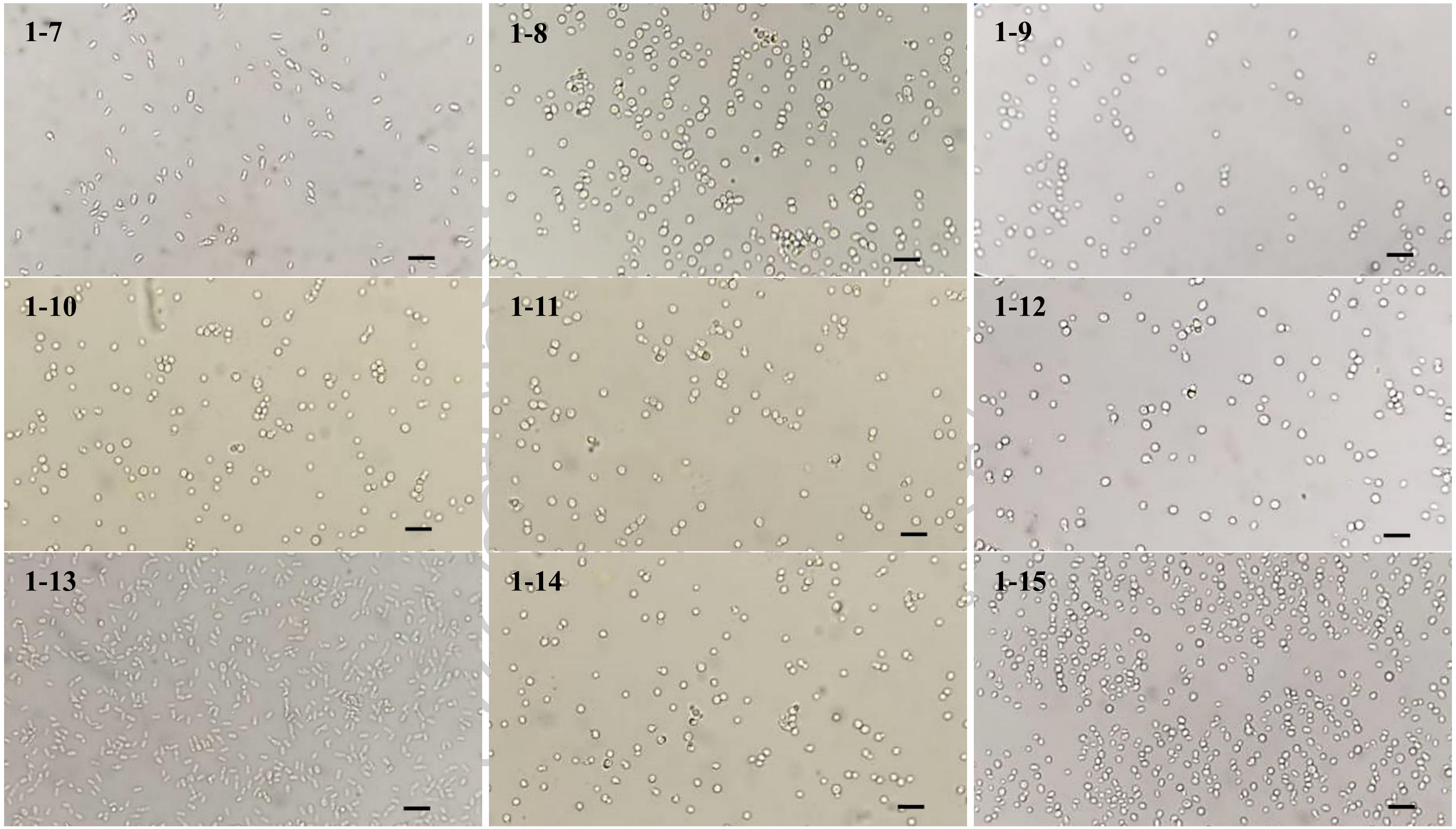

Fig. 4 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-7, DO0705_3; 1-8, DO0805; 1-9, F0101H; 1-10, F0709H; 1-11, F0810H; 1-12, F1; 1-13, F10; 1-14, F1016H; 1-15, F1222H; scale bar, 10 μm

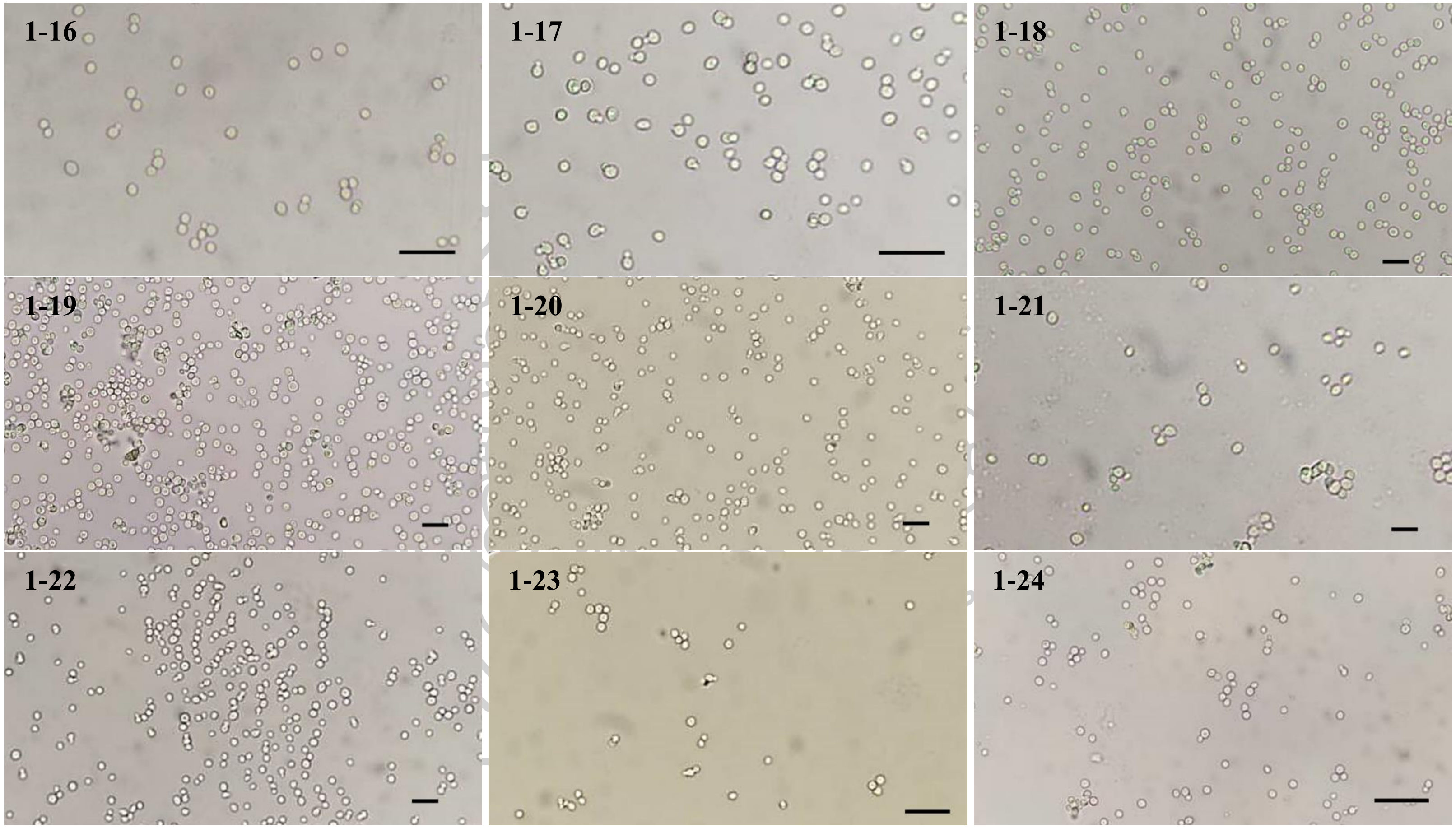

Fig. 5 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-16, F15; 1-17, F18; 1-18, F19; 1-19, FL4H; 1-20, FL9H; 1-21, FL10H; 1-22, FL13H; 1-23, FL15H; 1-24, H2203H; scale bar, 10 µm

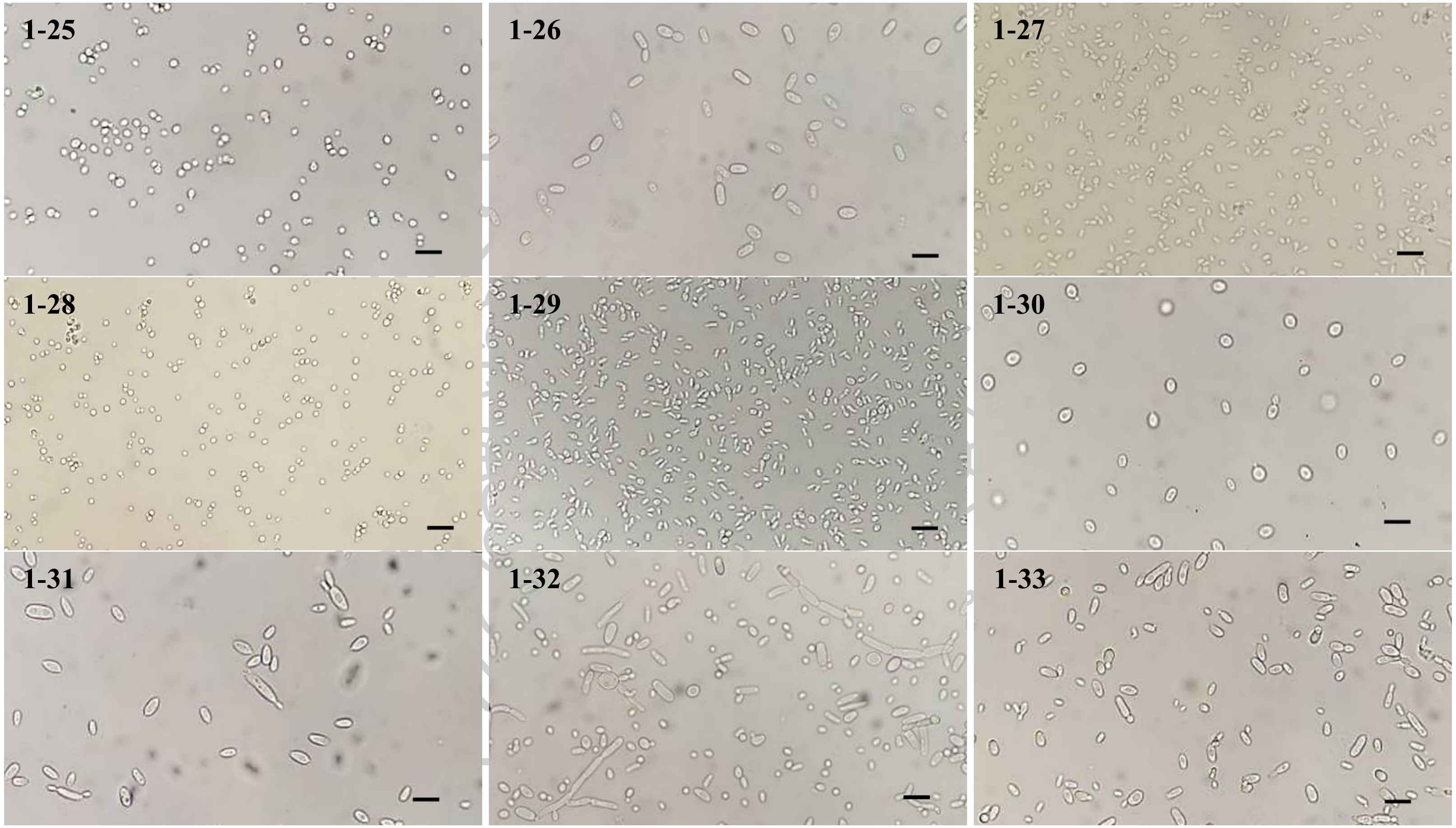

Fig. 6 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-25, H2802H; 1-26, M2004; 1-27, NP4101; 1-28, NP4201; 1-29, PL0702; 1-30, PLA0701H; 1-31, PLA0801; 1-32, PLC3201; 1-33, PLC3401; scale bar, 10 μm

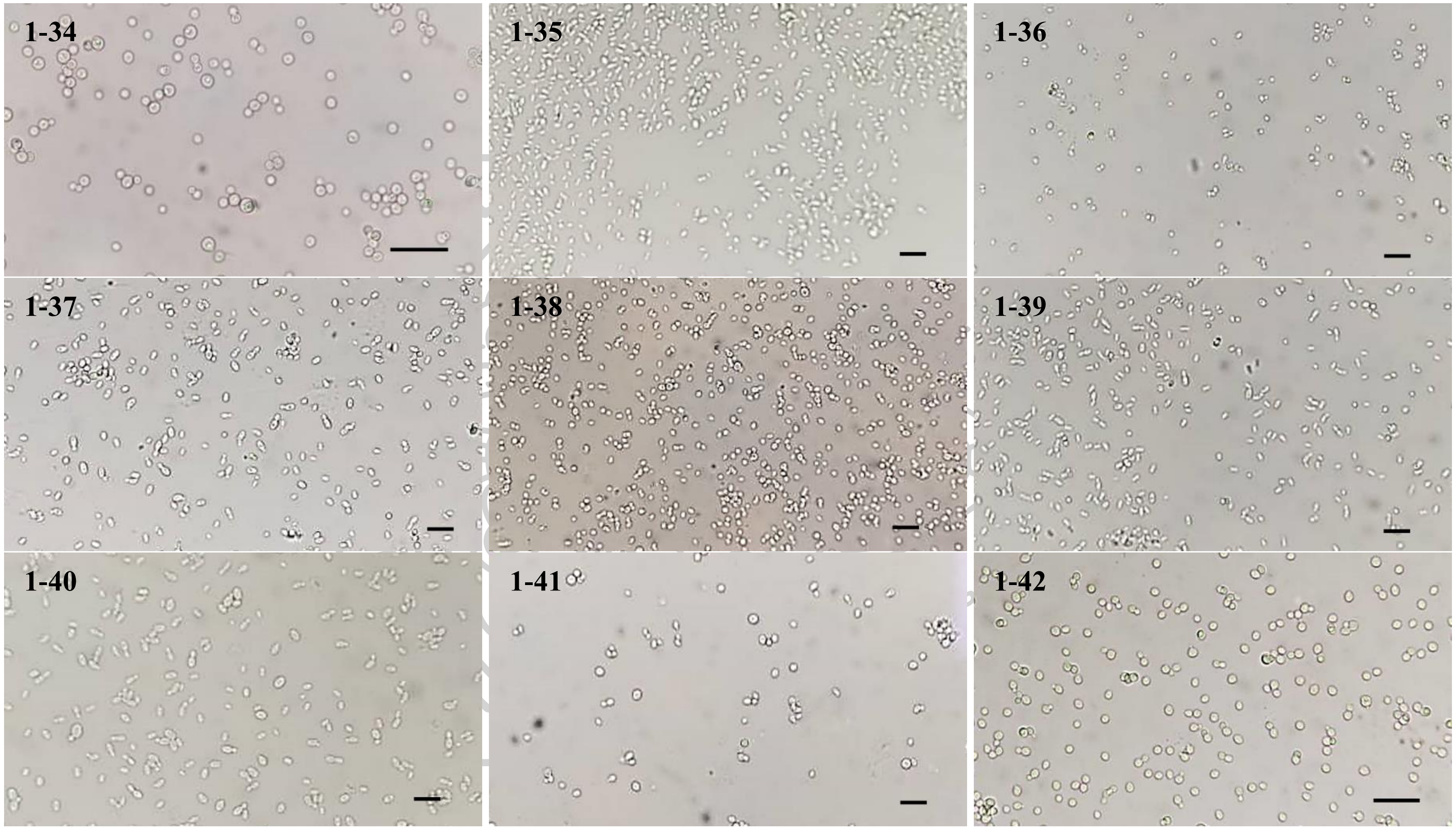

Fig. 7 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-34, PLD0901H; 1-35, PLF3201H; 1-36, PLF3202H; 1-37, PLF3203H; 1-38, PLF3204H; 1-39, PLF3205H; 1-40, PLF3206H; 1-41, PLF3301H; 1-42, TO2201H; scale bar, 10 μm

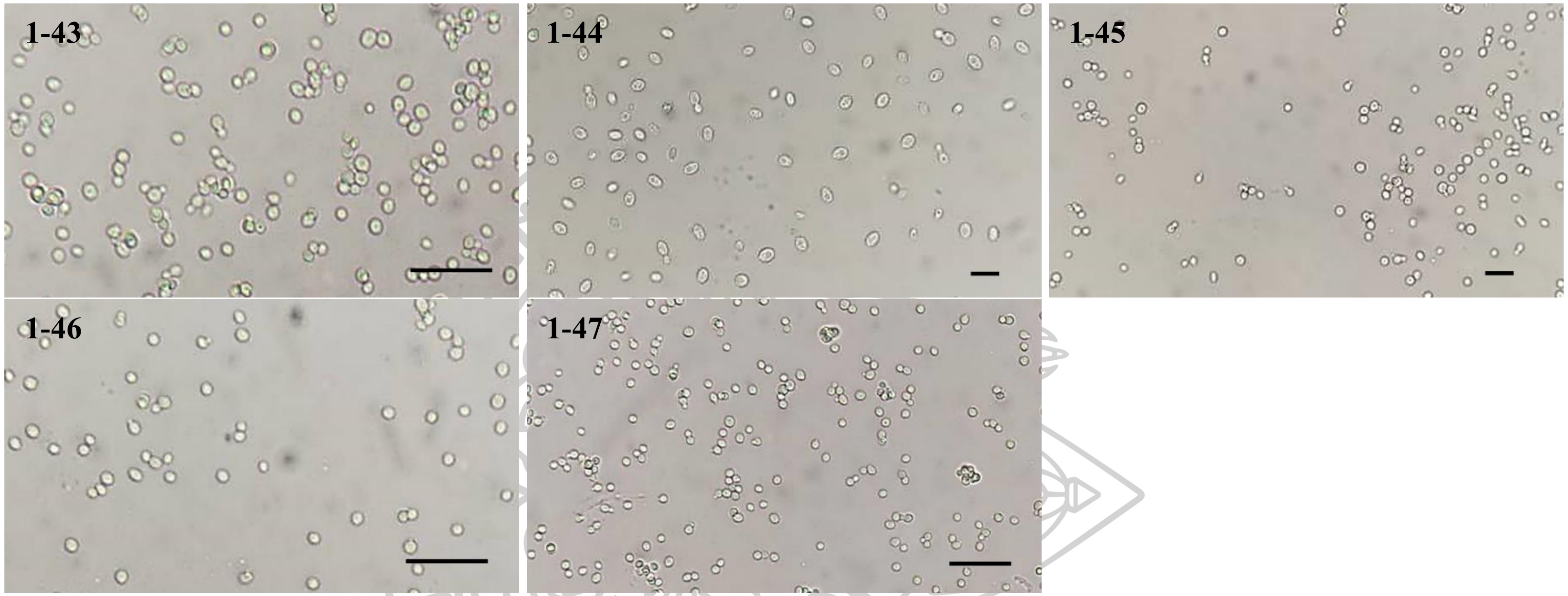

Fig. 8 Cell morphology of investigated yeasts in YMB at 25ºC for 7 days. 1-43, TO2203H; 1-44, TO2301H; 1-45, TO2802H; 1-46, TO2803H; 1-47, TO2804H; scale bar, 10 µm

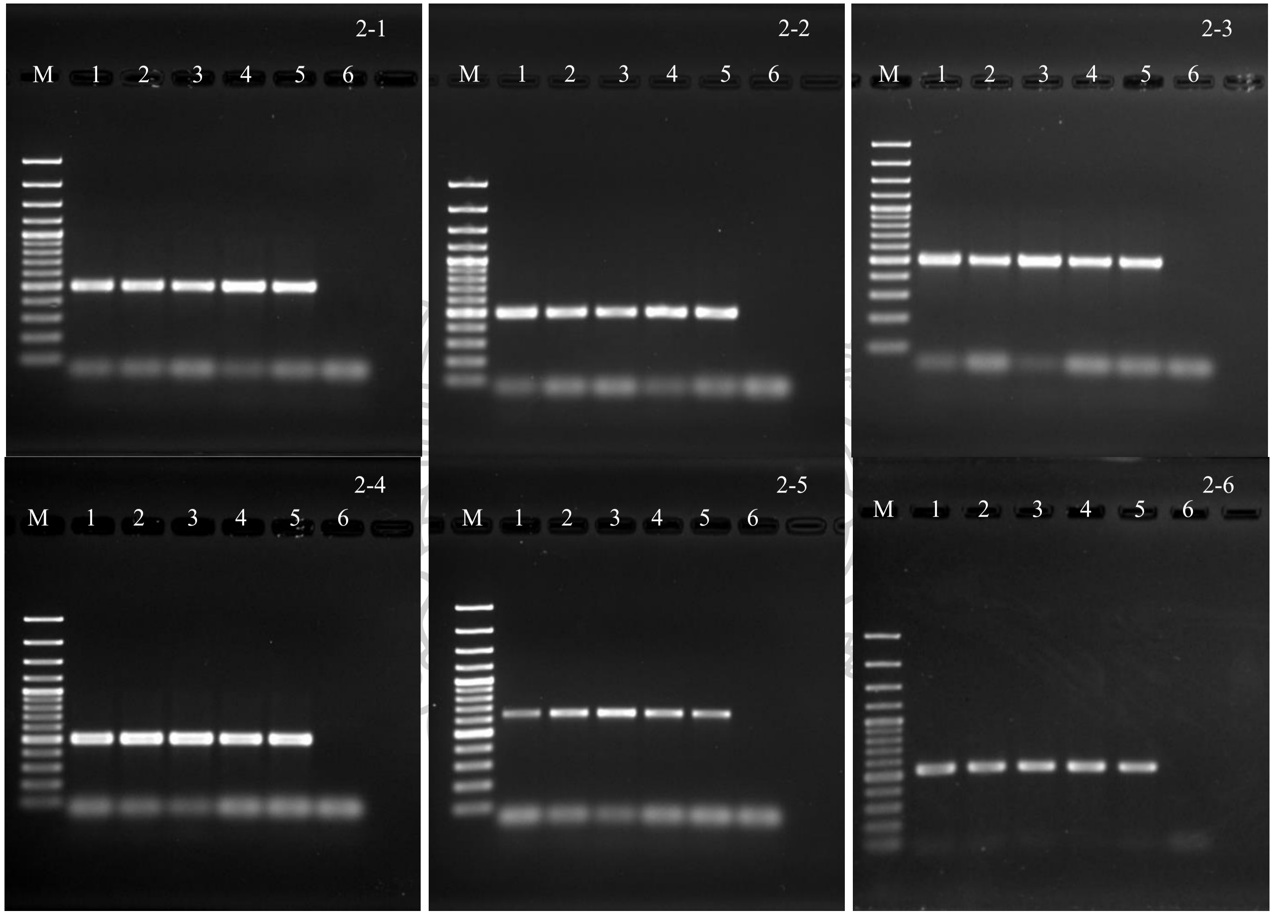

Fig. 9 Agarose gel of PCR products of LSU D1/D2 domains. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 2-1, AM0507; 2-2, FL4H; 2-3, H2203H; 2-4, H2802H; 2-5, M2004; 2-6, NP4101

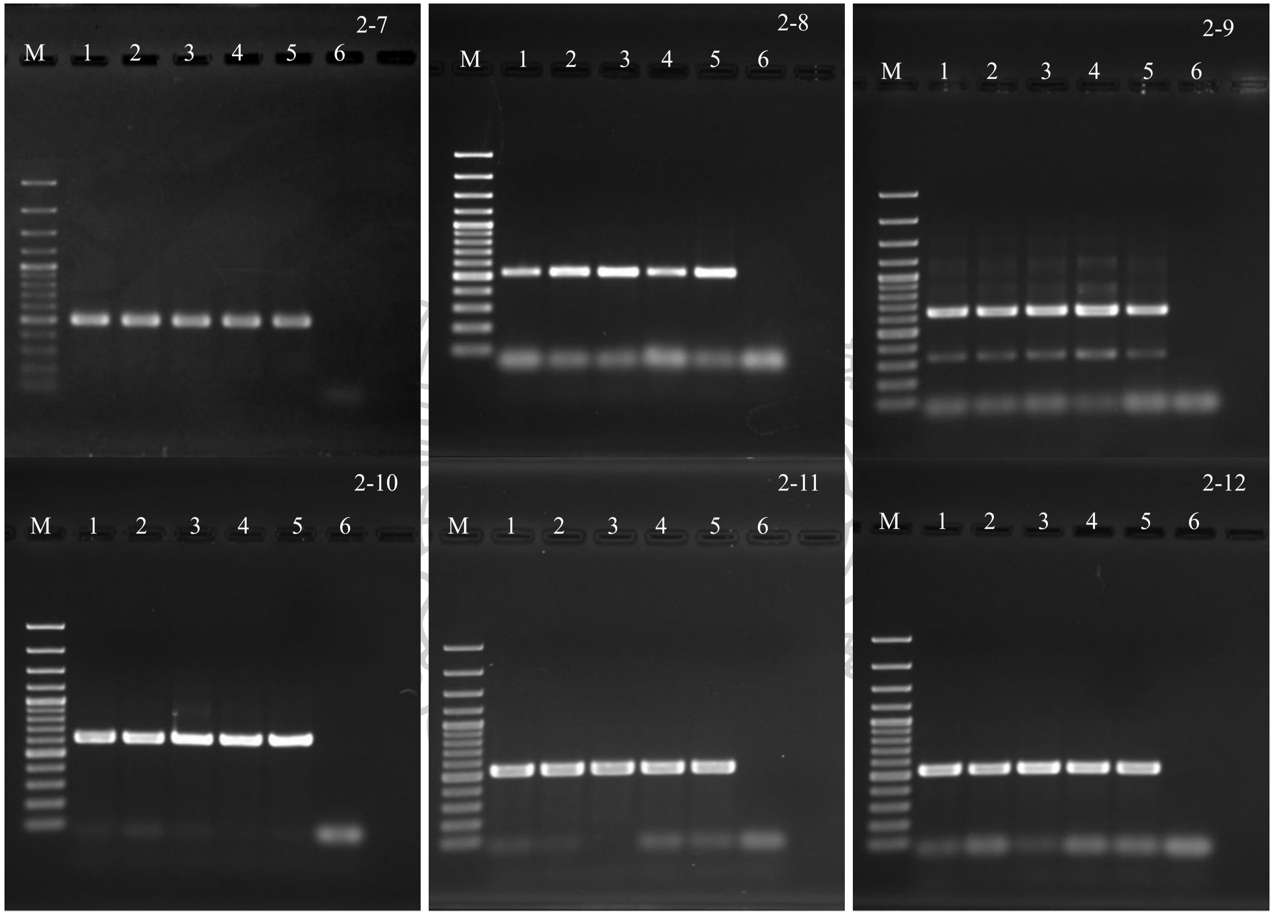

Fig. 10 Agarose gel of PCR products of LSU D1/D2 domains. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 2-7, NP4201; 2-8, PL0702; 2-9, PLC3301H; 2-10, PLC3401; 2-11, PLF3201H; 2-12, PLF3203H

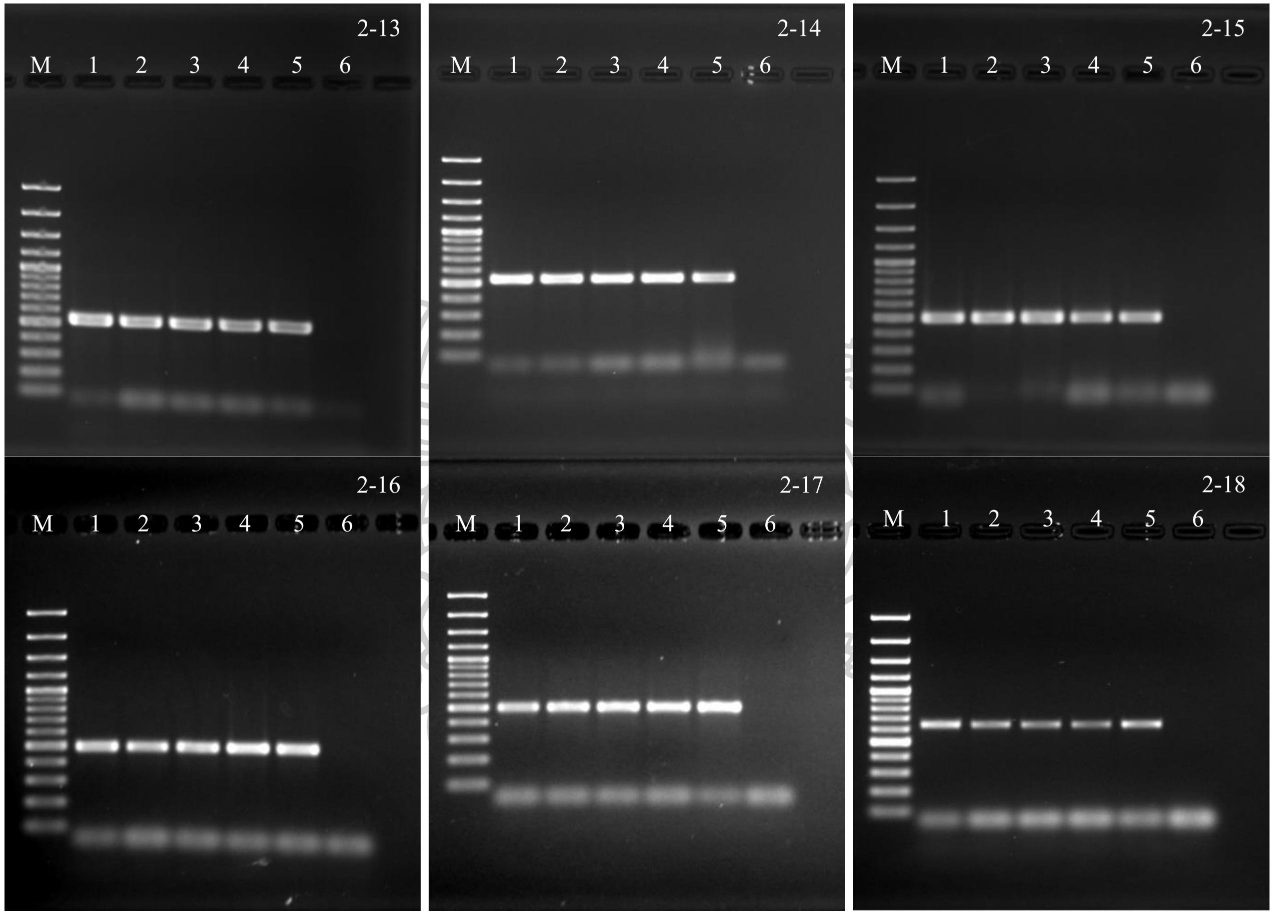

Fig. 11 Agarose gel of PCR products of LSU D1/D2 domains. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 2-13, PLF3205H; 2-14, PLF3206H; 2-15, PLF3301H; 2-16, TO2201H; 2-17, TO2203H; 2-18, TO2301H

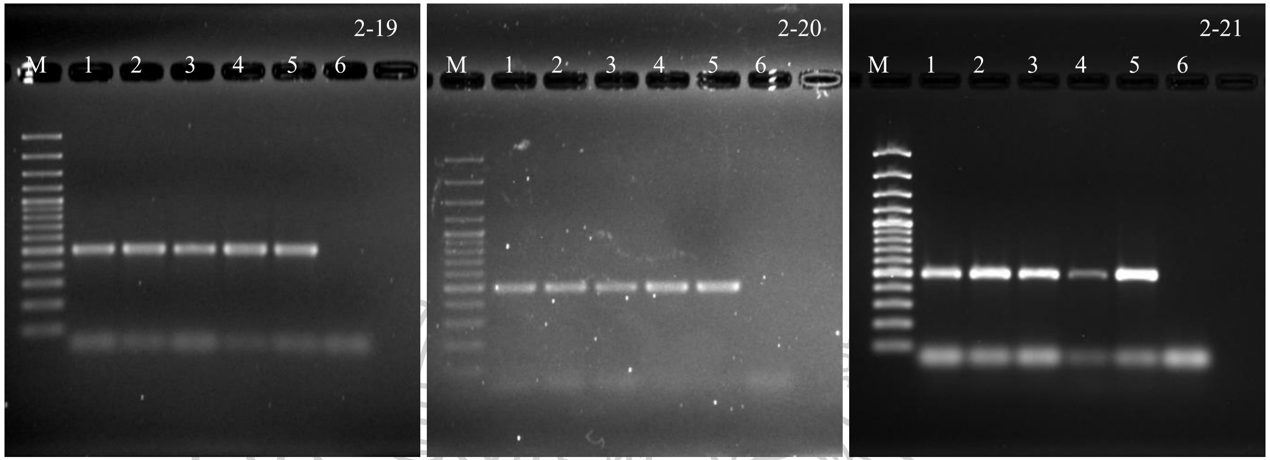

Fig. 12 Agarose gel of PCR products of LSU D1/D2 domains. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 2-19, TO2802H; 2-20, TO2803H; 2-21, TO2804H

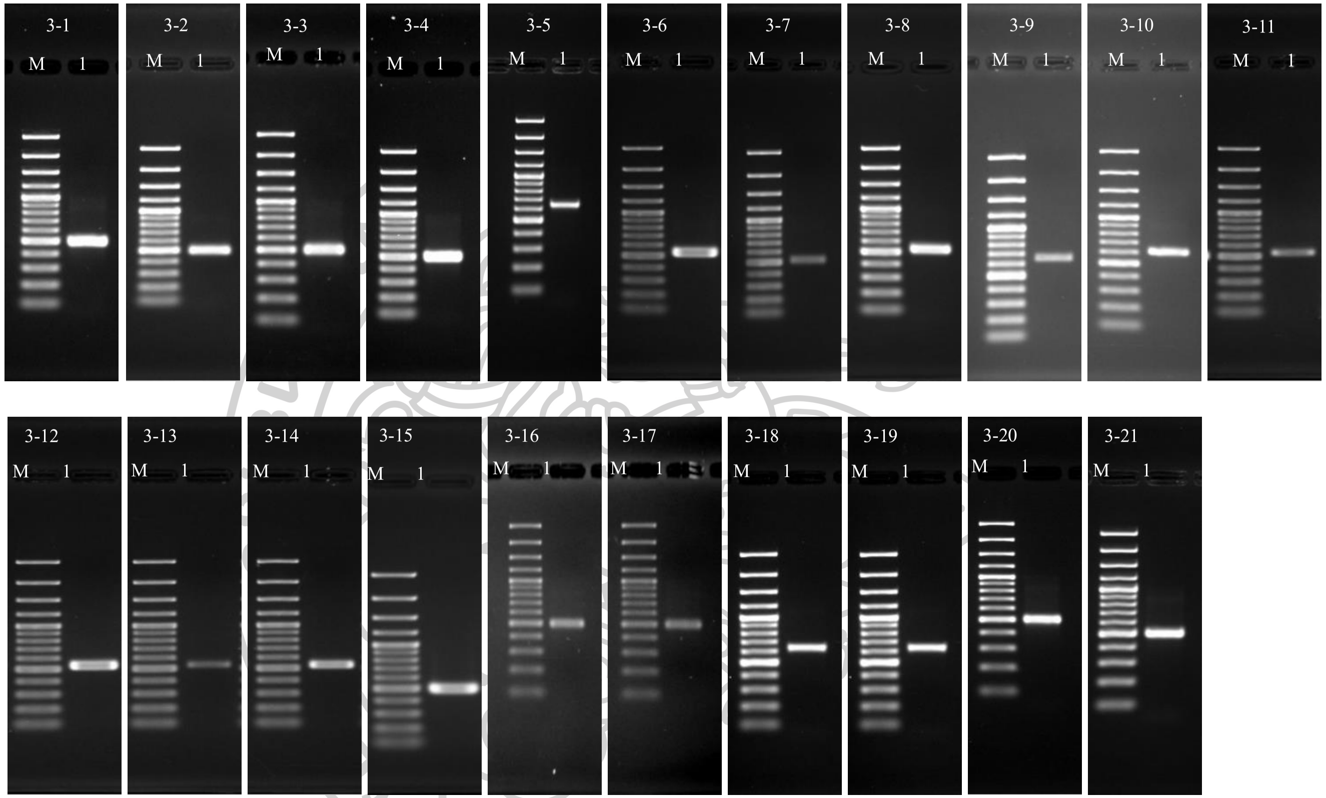

Fig. 13 Agarose gel of purified PCR products of LSU D1/D2 domains. Lane M, 100 bp plus DNA ladder; Lanes 1, purified PCR products; 3-1, AM0507; 3-2, FL4H; 3-3, H2203H; 3-4, H2802H; 3-5, M2004; 3-6, NP4101; 3-7, NP4201; 3-8, PL0702; 3-9, PLC3301H; 3-10, PLC3401; 3-11, PLF3201H; 3-12, PLC3203H; 3-13, PLF3205H; 3-14, PLF3206H; 3-15, PLF3301H; 3-16, TO2201H; 3-17, TO2203H; 3-18, TO2301H; 3-19, TO2802H; 3-20, TO2803H; 3-21, TO2804H

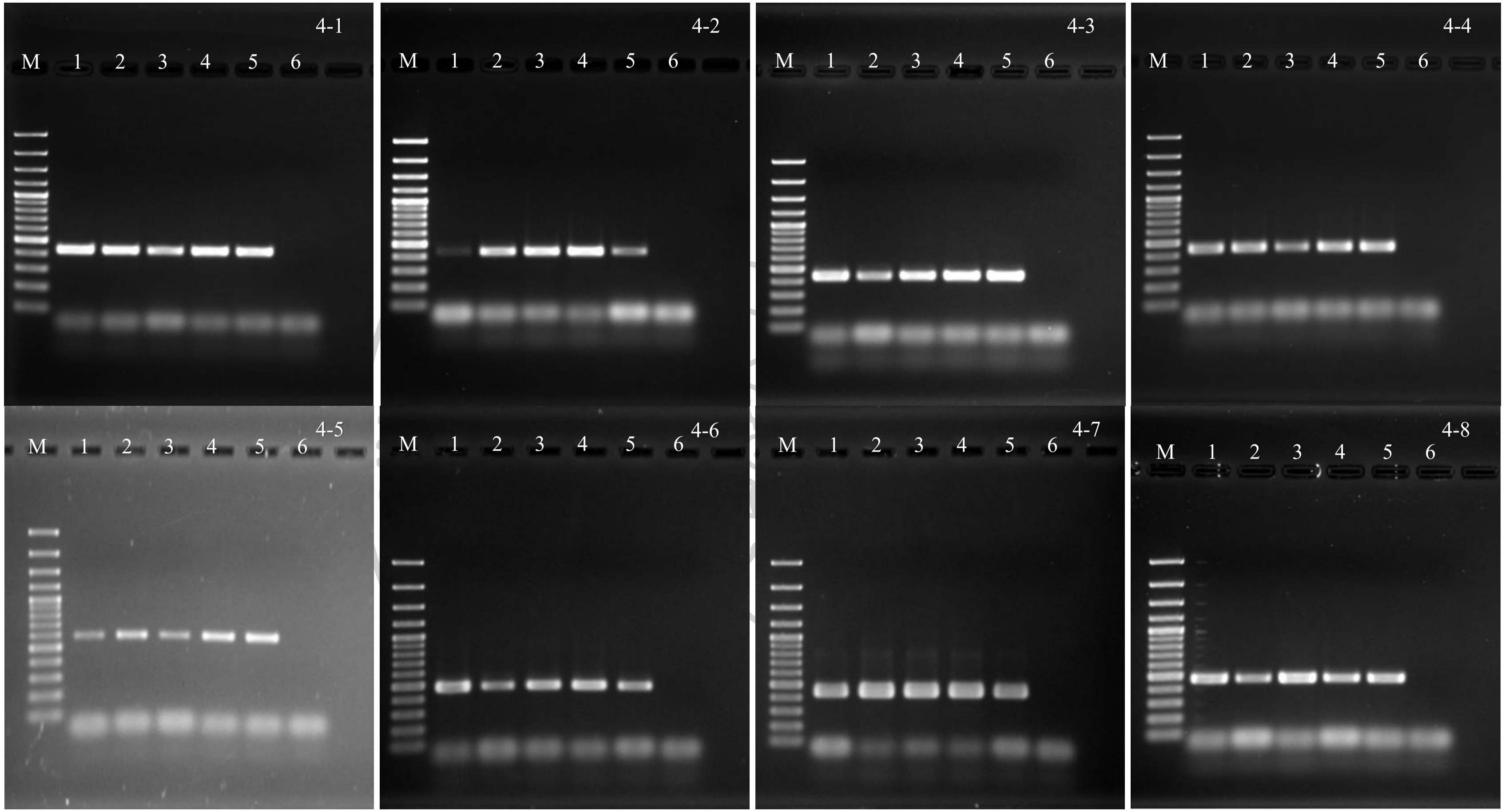

Fig.14 Agarose gel of PCR products of ITS regions. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 4-1, AM0507; 4-2, FL4H; 4-3, H2203H; 4-4, H2802H; 4-5, M2004; 4-6, NP4101; 4-7, NP4201; 4-8, PL0702

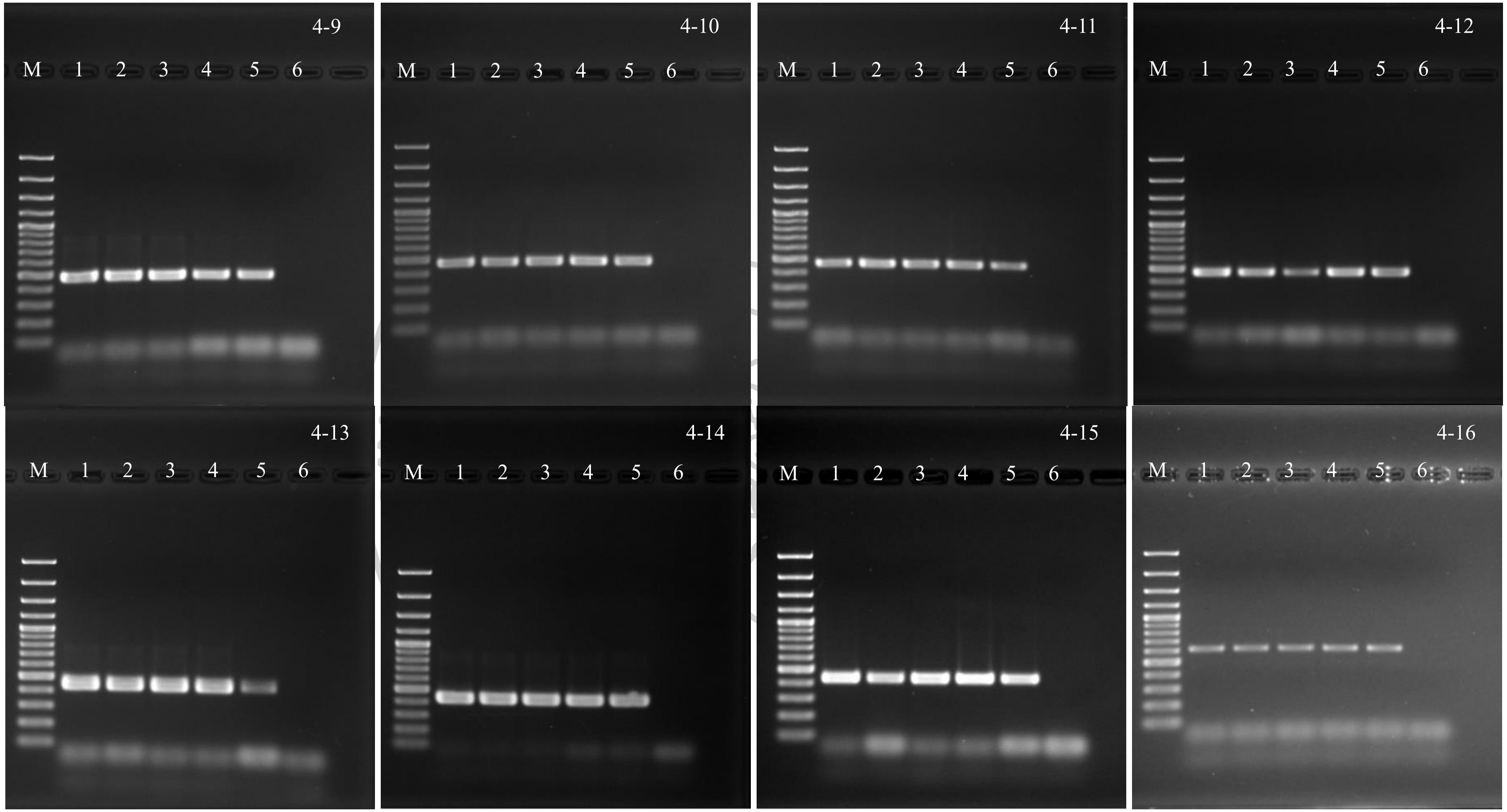

Fig. 15 Agarose gel of PCR products of ITS regions. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 4-9, PLF3201H; 4-10, PLF3203H; 4-11, PLF3205H; 4-12, PLF3206H; 4-13, PLF3301H; 4-14, TO2201H; 4-15, TO2203H; 4-16, TO2301H

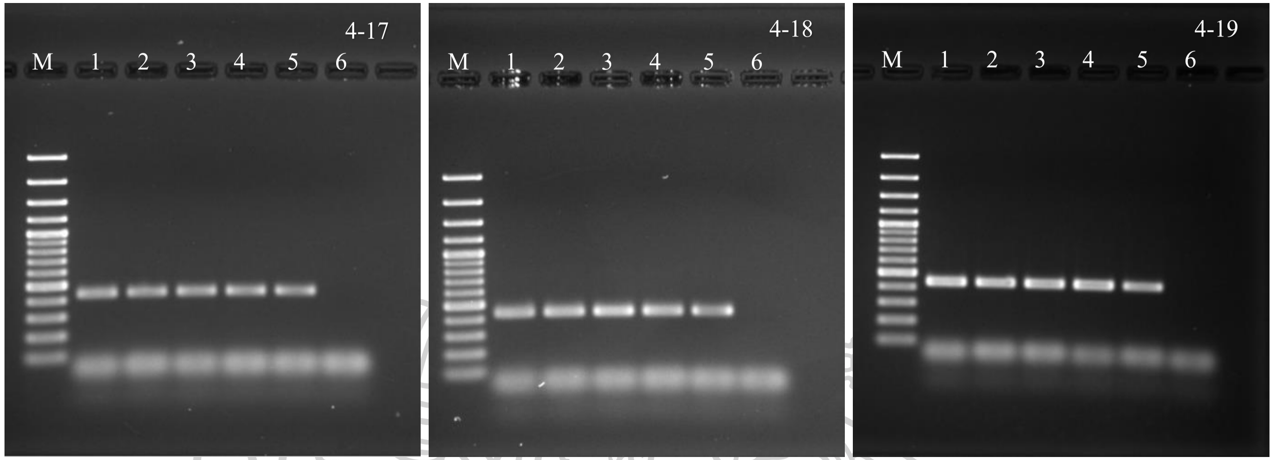

Fig. 16 Agarose gel of PCR products of ITS regions. Lane M, 100 bp plus DNA ladder; Lanes 1-5, PCR products; Lanes 6, negative control; 4-17, TO2802H; 4-18, TO2803H; 4-19, TO2804H

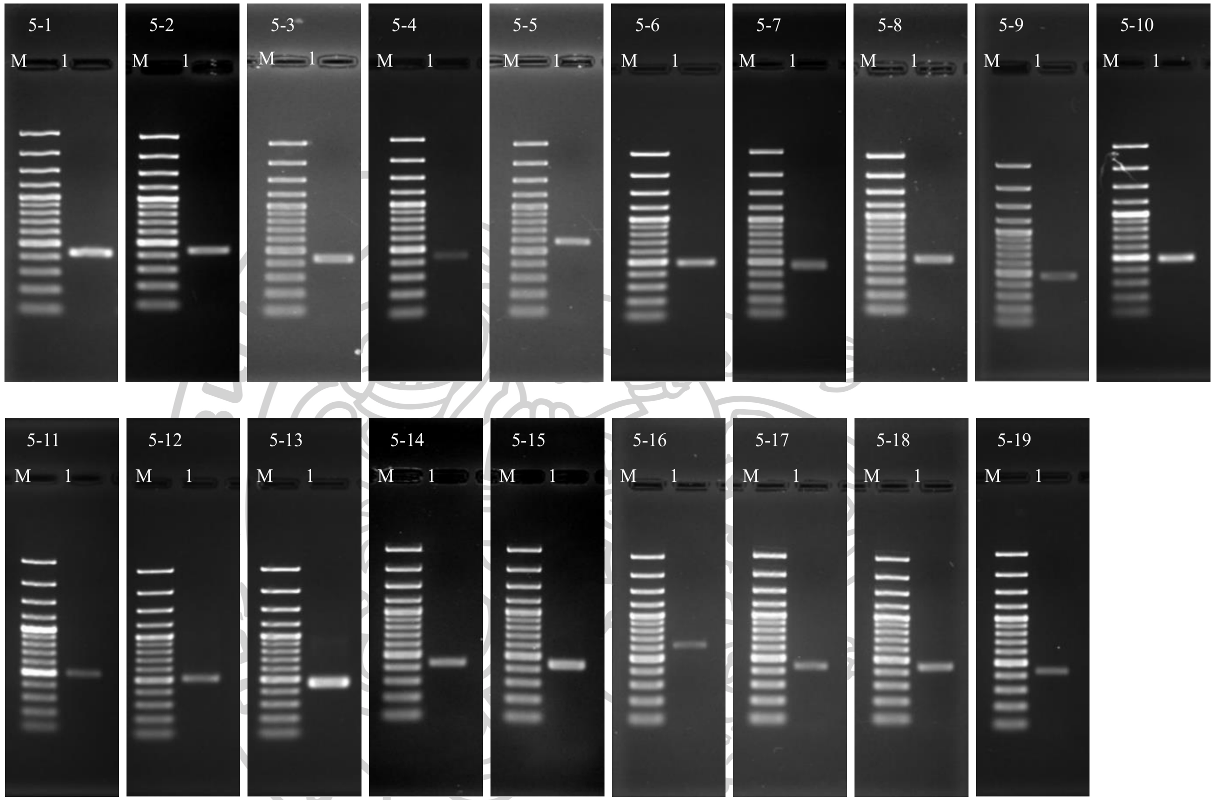

Fig. 17 Agarose gel of purified PCR products of ITS regions. Lane M, 100 bp plus DNA ladder; Lanes 1, purified PCR products; 5-1, AM0507; 5-2, FL4H; 5-3, H2203H; 5-4, H2802H; 5-5, M2004; 5-6, NP4101; 5-7, NP4201; 5-8, PL0702; 5-9, PLF3201H; 5-10, PLF3203H; 5-11, PLF3205H; 5-12, PLF3206H; 5-13, PLF3301H; 5-14, TO2201H; 5-15, TO2203H; 5-16, TO2301H; 5-17, TO2802H; 5-18, TO2803H; 5-19, TO2804H

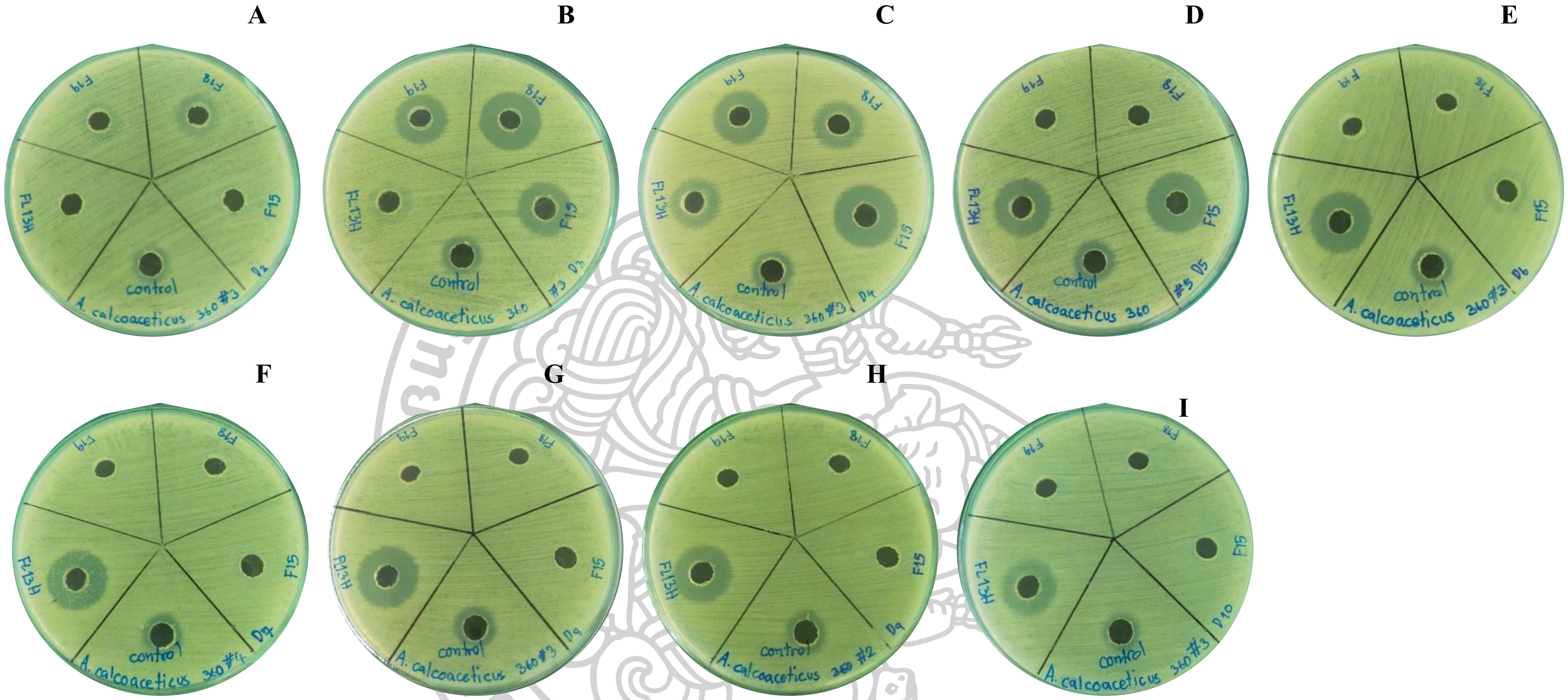

Fig. 18 Antagonistic activity of four yeast strains (F15, F18, F19 and FL13H) against *Acinetobacter calcoaceticus* TISTR 360 on YMA plate at 37ºC for 24 h. A, 2 days; B, 3 days; C, 4 days; D, 5 days; E, 6 days; F, 7 days; G, 8 days; H, 9 days; I, 10 days of yeast cultivation; kanamycin was used as a positive control

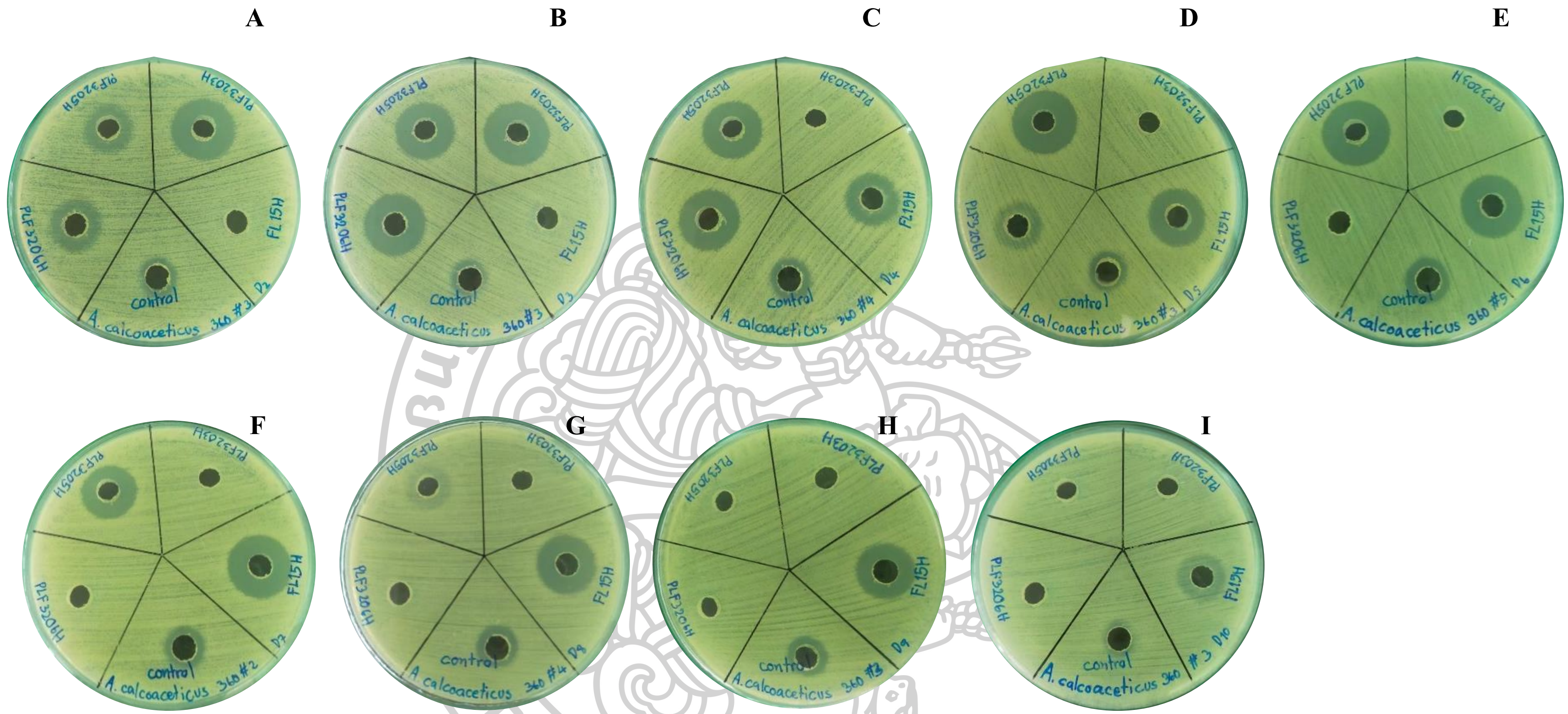

Fig. 19 Antagonistic activity of four yeast strains (FL15H, PLF3203H, PLF3205H and PLF3206H) against *Acinetobacter calcoaceticus* TISTR 360 on YMA plate at 37ºC for 24 h. A, 2 days; B, 3 days; C, 4 days; D, 5 days; E, 6 days; F, 7 days; G, 8 days; H, 9 days; I, 10 days of yeast cultivation; kanamycin was used as a positive control

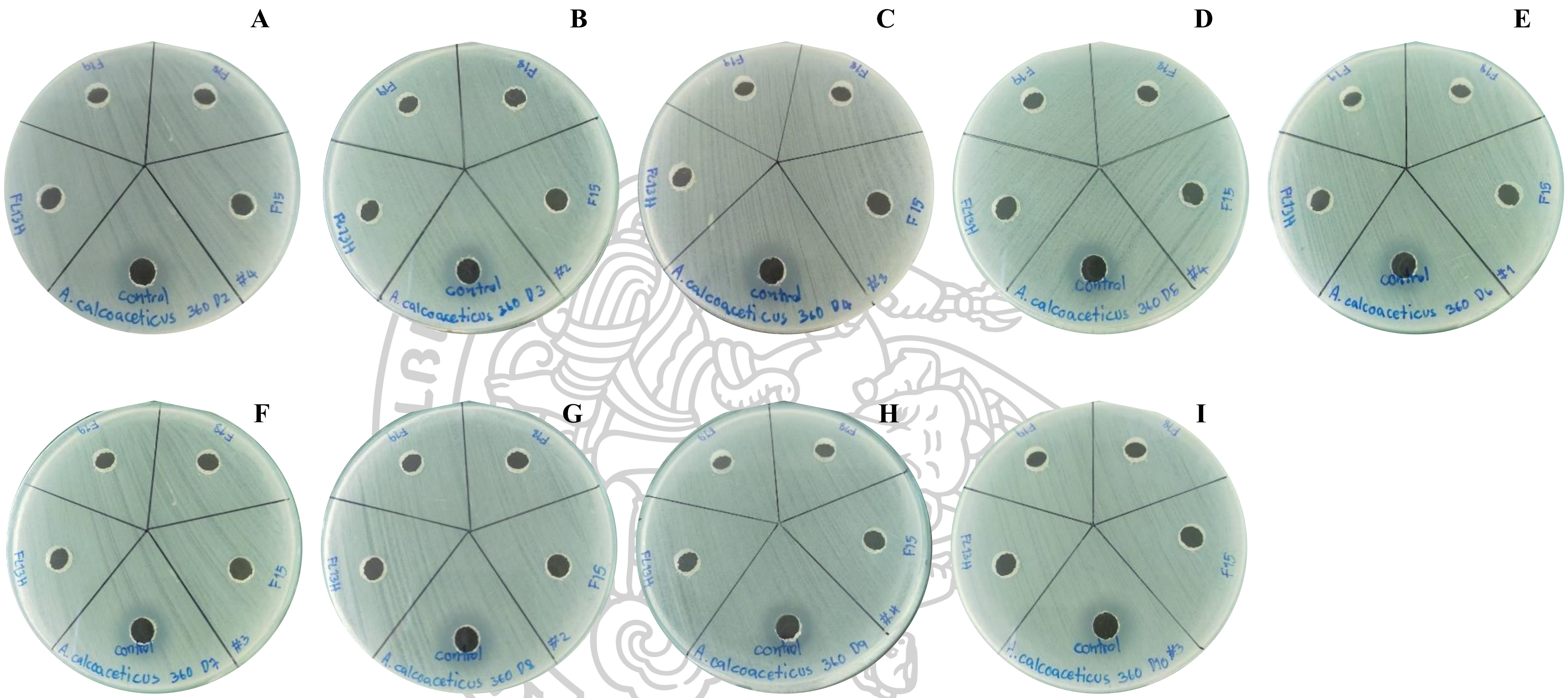

Fig. 20 Antagonistic activity of four yeast strains (F15, F18, F19 and FL13H) against *Acinetobacter calcoaceticus* TISTR 360 on NA plate at 37ºC for 24 h. A, 2 days; B, 3 days; C, 4 days; D, 5 days; E, 6 days; F, 7 days; G, 8 days; H, 9 days; I, 10 days of yeast cultivation; kanamycin was used as a positive control

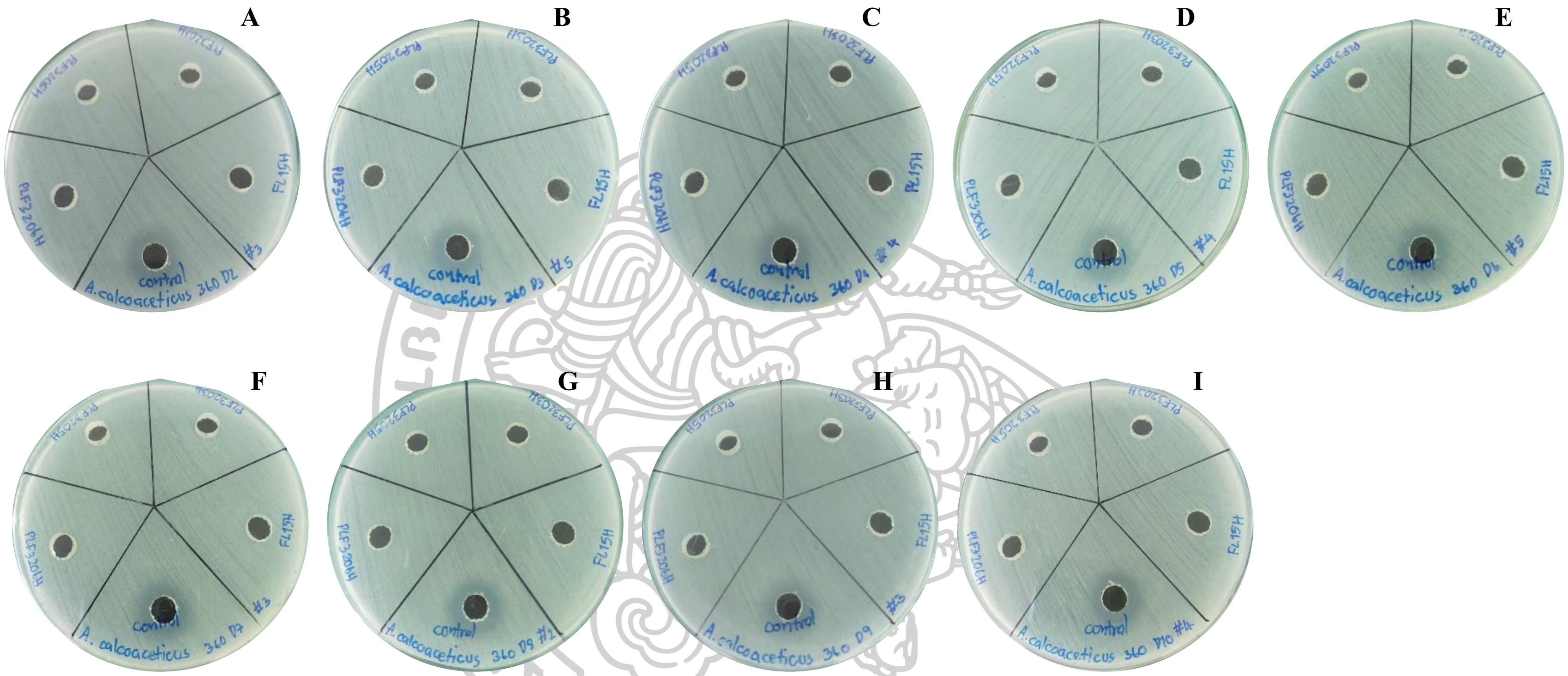

Fig. 21 Antagonistic activity of four yeast strains (FL15H, PLF3203H, PLF3205H and PLF3206H) against *Acinetobacter calcoaceticus* TISTR 360 on NA plate at 37ºC for 24 h. A, 2 days; B, 3 days; C, 4 days; D, 5 days; E, 6 days; F, 7 days; G, 8 days; H, 9 days; I, 10 days of yeast cultivation; kanamycin was used as a positive control

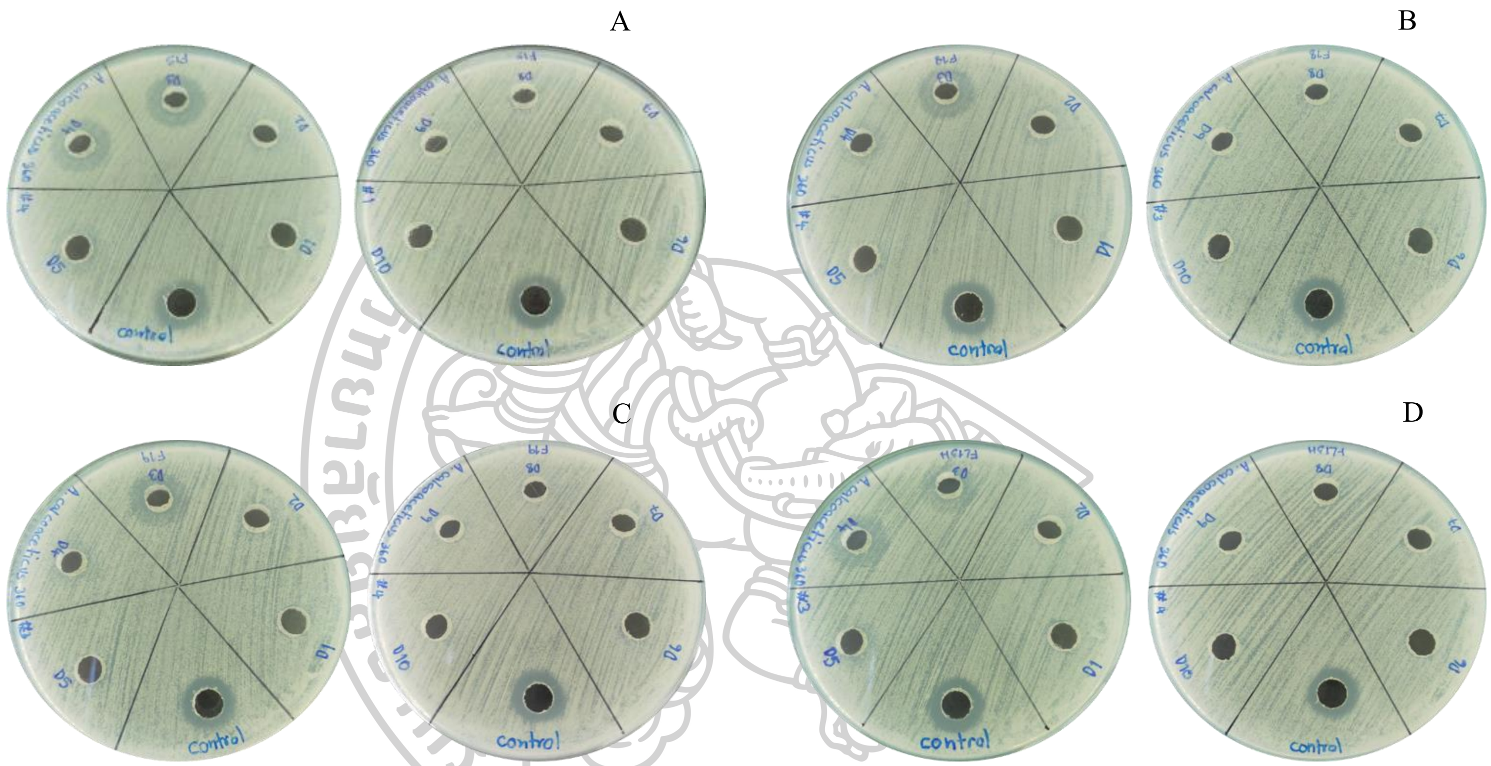

Fig. 22 Antagonistic activity of four yeast strains against *Acinetobacter calcoaceticus* TISTR 360 on YMA plate at 37ºC for 24 h. A, F15; B, F18; C, F19; D, FL13H; daily sampling for up to 10 days; kanamycin was used as a positive control

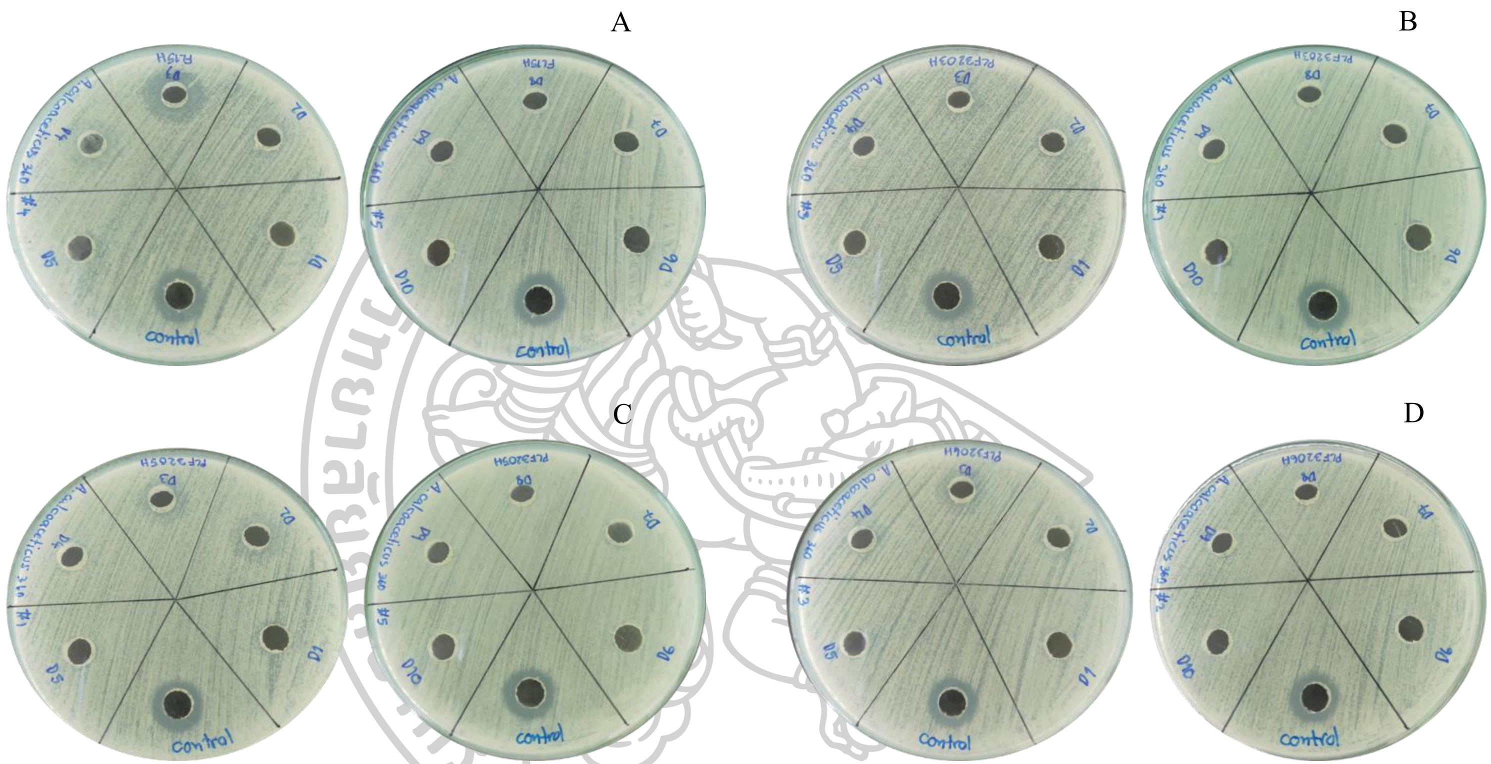

Fig. 23 Antagonistic activity of four yeast strains against *Acinetobacter calcoaceticus* TISTR 360 on YMA plate at 37ºC for 24 h. A, FL15H; B, PLF3203H; C, PLF3205H; D, PLF3206H; daily sampling for up to 10 days; kanamycin was used as a positive control

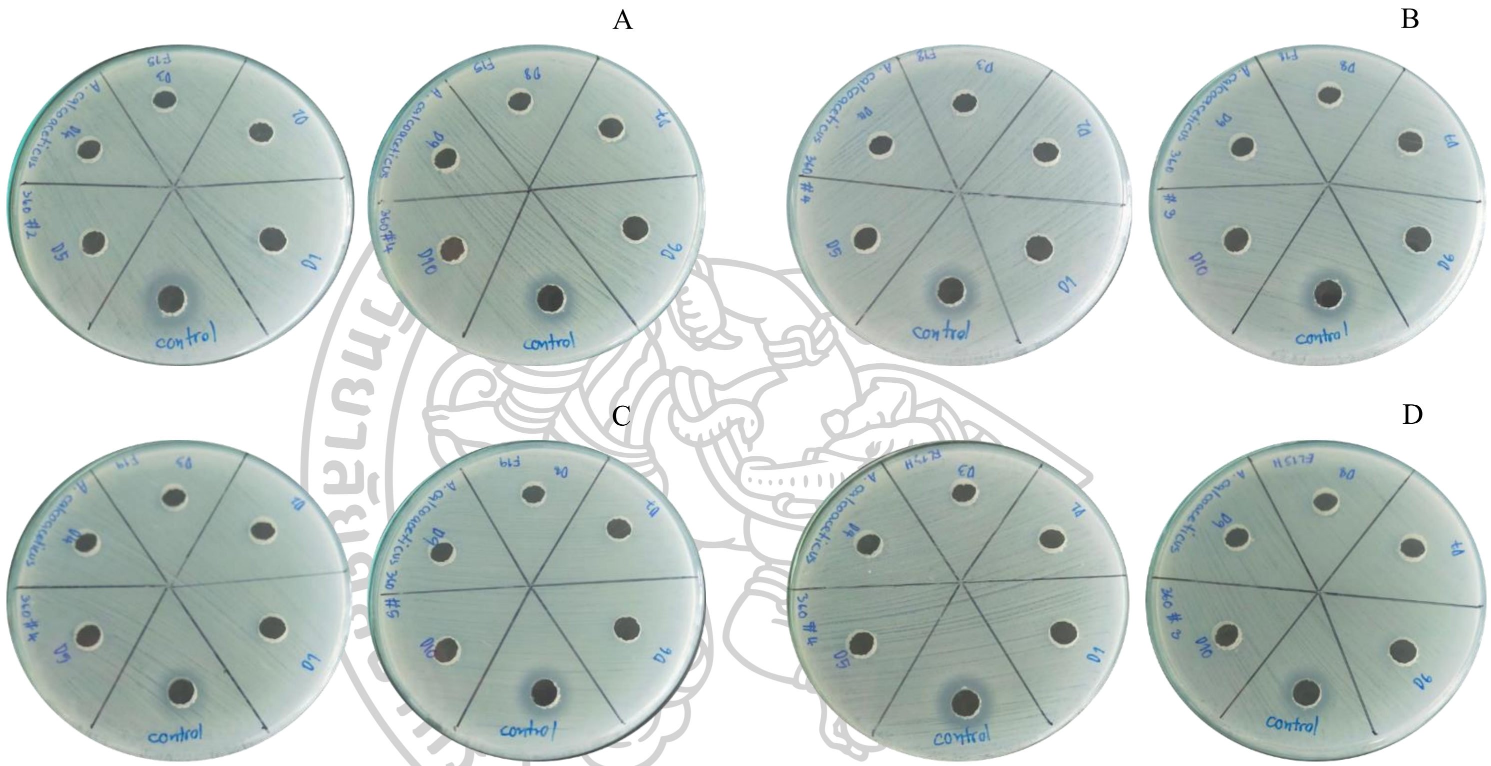

Fig. 24 Antagonistic activity of four yeast strains against *Acinetobacter calcoaceticus* TISTR 360 on NA plate at 37ºC for 24 h. A, F15; B, F18; C, F19; D, FL13H; daily sampling for up to 10 days; kanamycin was used as a positive control

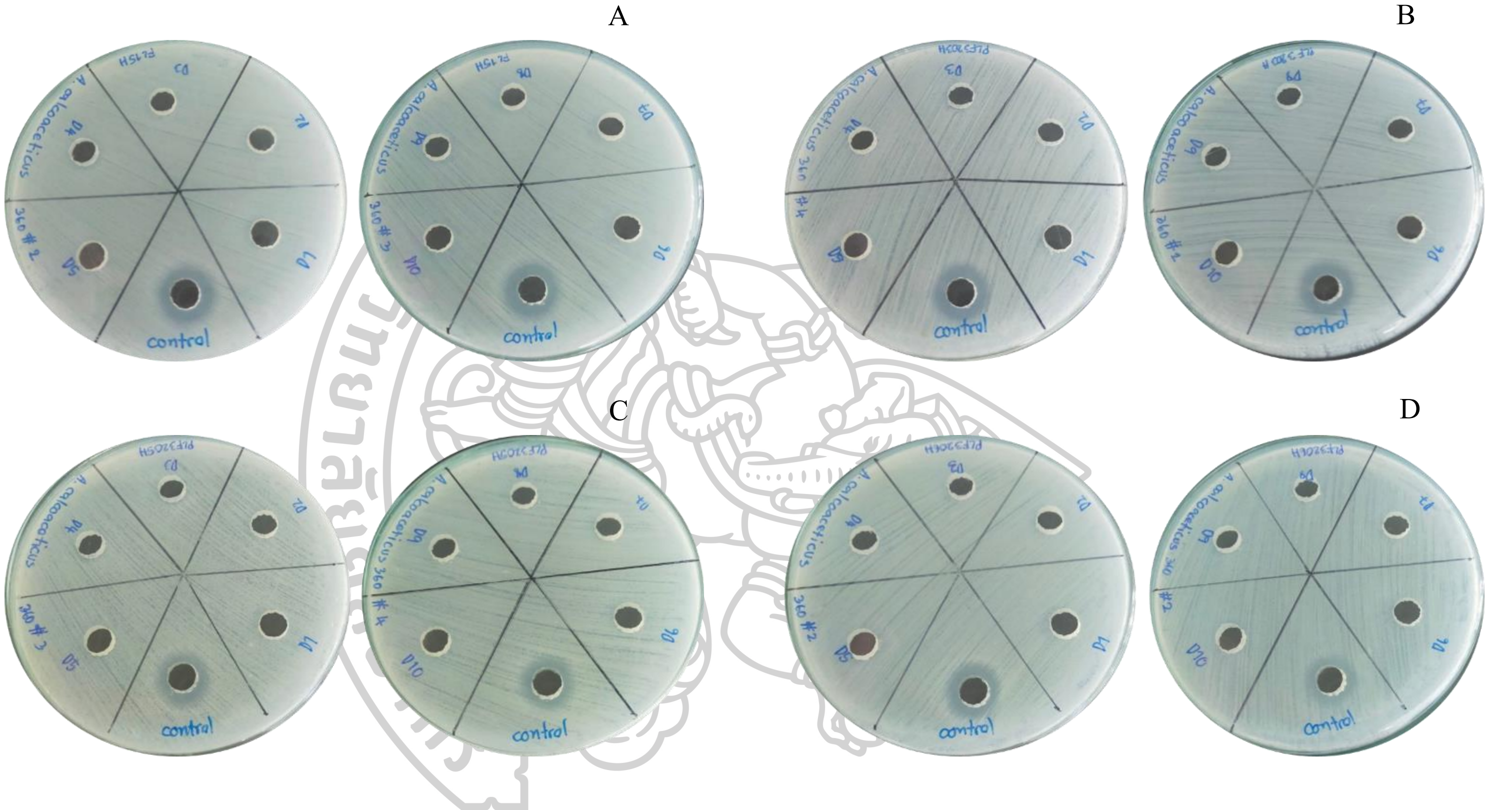

Fig. 25 Antagonistic activity of four yeast strains against *Acinetobacter calcoaceticus* TISTR 360 on NA plate at 37ºC for 24 h. A, FL15H; B, PLF3203H; C, PLF3205H; D, PLF3206H; daily sampling for up to 10 days; kanamycin was used as a positive control

APPENDIX C

Publications

BIO-P10

ANTAGONISTIC ACTIVITY OF *Filobasidium* sp. TO2301* ISOLATED FROM *Apis florea* Fabricius, 1787 HONEY AGAINST *Staphylococcus aureus* Tistr 885

Nawarat Charoenphol[1,2], Saran Promsai[3], Yaowanoot Promnuan[3], Eakaphun Bangyeekhun[1] and Sujinan Meelai[1,*]

The aim of this study was to investigate the antagonistic potential of yeasts isolated from combs of the red dwarf honeybee (*Apis florea* Fabricius, 1787). In total, 10 isolates were screened for antagonistic activity against four Gram-positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus* and *S. epidermidis*) and five Gram-negative bacteria (*Escherichia coli*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Salmonella enterica*, *S. typhimurium*). Following screening using an agar well diffusion method, only one isolate showed a potential to inhibit the growth of *S. aureus* TISTR 885 (11.33 ± 1.53 mm). The analysis of the *D1/D2* domain of large subunit *rRNA* gene (LSU rDNA D1/D2) sequence similarity and the phylogenetic tree based on the neighbor-joining (NJ) algorithm showed that the isolate TO2301* was closely related to *Filobasidium mali* CBS 15651^T (EU002805, 100%). This is the first report on antagonistic activity against pathogenic bacteria from yeast associated with native Thai bee.

Keywords: Antagonistic Yeasts, Native Thai Bee, Pathogenic Bacteria, Red Dwarf Honeybee

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การประชุมวิชาการระดับชาติ ครั้งที่ 19 มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน วันที่ 8-9 ธันวาคม 2565

Antagonistic activity of *Starmerella* species isolated from dwarf honey bees against *Acinetobacter calcoaceticus* TISTR 360

Nawarat Charoenphol[1], Saran Promsai[2], Yaowanoot Promnuan[2], Eakaphun Bangyeekhun[1], and Sujinan Meelai[1]

Abstract

The aim of this study was to investigate the antagonistic potential of yeasts isolated from dwarf honey bees (*Apis andreniformis* and *Apis florea*). In total, 8 yeast isolates were screened for antagonistic activity against four Gram-positive bacteria (*Bacillus cereus* TISTR 687, *Bacillus subtilis* TISTR 008, *Staphylococcus aureus* TISTR 885 and *Staphylococcus epidermidis* TISTR 518) and six Gram-negative bacteria (*Acinetobacter calcoaceticus* TISTR 360, *Escherichia coli* TISTR 887, *Klebsiella oxytoca* TISTR 556, *Proteus mirabilis* TISTR 100, *Salmonella enterica* subsp. *enterica* ATCC 10708 and *Salmonella enterica* subsp. *enterica* ser. Typhimurium TISTR 292). Following screening using an agar well diffusion method, seven isolates have shown a potential to inhibit the growth of *A. calcoaceticus* TISTR 360, with the inhibitory zones ranging from 8.8±0.4 to 13.8±0.4 mm. The analysis of the D1/D2 domain of large subunit rRNA gene (LSU rDNA D1/D2) sequence similarity and the phylogenetic tree based on the neighbor-joining (NJ) algorithm showed that the isolates F15, F18, F19, FL13H and FL15H were closely related to *Starmerella apis* CBS 2674T (KY106300, 98.20%), while the isolates PLF3203H, PLF3205H and PLF3206H were closest relatives in *Starmerella kuoi* NRRL Y-27208T (NG 073590, 96.07%). This is the first report on antagonistic activity against *A. calcoaceticus* TISTR 360 from yeast associated with native Thai bees.

Keyword : antagonistic yeasts, native Thai bees, bacteria, dwarf honey bees

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INSTITUTIONS ATTENDED	Bachelor of Science Microbiology
PUBLICATION	Charoenphol, N, Promsai, S, Promnuan, Y, Bangyeekhun, E, Meelai, S. Antagonistic activity of Starmerella species isolated from dwarf honey bees against Acinetobacter calcoaceticus TISTR 360. The 19 th KU KPS National Conference. Nakhon Pathom: Kasetsart University, Kamphaeng Saen Campus, 8-9 December, 2022. Charoenphol, N, Promsai, S, Promnuan, Y, Bangyeekhun, E, Meelai, S. Antagonistic Activity of Filobasidium sp. TO2301* Isolated from Apis florea Fabricius, 1787 Honey Against Staphylococcus aureus TISTR 885. The 13 th National Science Research Conference. Phatthalung: Thaksin University, 12-13 May, 2022.