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Extracellular Enzyme Activity of Yeast Isolates from Gringsingan Flowers (*Hyptis Suaveolens* (L.) Poit) in Kupang as Extracellular Enzyme Producers

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Abstract

Yeast can be found in flowers because flowers contain nectar which is a source of nutrition for yeast. Yeast can produce extracellular enzymes. This study aims to evaluate the extracellular enzyme activity of yeast isolates from Gringsingan flowers (Hyptis suaveolens) in Kupang. Yeast isolation was carried out using the spread plate technique. The ability of yeast to produce extracellular enzymes was evaluated through starch and casein tests. Three types of yeast were obtained: *Kodamaea ohmeri*, *Starmerella floricola*, and *Candida orthopsilosis*. Isolate KB103 can produce amylase, with an amylase index of 2. Isolate Pme can produce protease enzymes, with a protease index of 3.25. Isolates KB103 and Pme were identified as *Candida orthopsilosis*.

Keywords: *Isolate, Yeast, Flowers, Hyptis Suaveolens, Extracellular Enzyme.*

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I. INTRODUCTION

Yeasts are commonly found in external environments, including leaf surfaces, soil, freshwater, air, and insects, but are less prevalent in flowers (Pozo *et al.,* 2012). Flowers host smaller yeast communities compared to insect glossae or plant phylloplanes, with pollinators playing a key role in introducing yeast to floral surfaces. Nectar, rich in sugar and secondary metabolites, supports osmosis-tolerant yeasts such as Debaryomyces, Metschnikowia, Starmerella, and Zygosaccharomyces spp. (Pozo *et al.,* 2012).

Common floral yeast genera include Metschnikowia, Kodamaea, Wickerhamiella, and Starmerella (Lachance *et al.,* 2001). Yeast thrives on floral nectar as an energy source, with insects like bees, beetles, and drosophilids acting as vectors for their distribution. Notable yeast species, such as Saccharomycopsis fibuligera and Pichia burtonii, produce amylase enzymes with indices of 3.8 and 3.18, respectively (Nurhartadi & Rahayu, 2011). Industrially, yeast extracts are widely used as food flavor enhancers, animal feed additives, and components in microbial fermentation media. Yeast fermentation also supports the production of valuable products like biofuels from plant-based resources (Maica, 2020; Tao *et al.,* 2023).

Plants provide microhabitats with different topographic features, nutrients, water availability and microclimatic conditions for the microbiota present on their surfaces (Pozo *et al.,* 2012). Yeast microhabitats on plants include decaying tissues, flowers, nectar or fruits. Yeast colonization on floral surfaces is influenced by pollinating insects, which carry yeasts in their mouthparts and on their glossae. When insects collect nectar, yeasts from the insect mouthparts and glossae are distributed to the flower. The high sugar content of floral nectar acts as a nutrient source to supports microbial growth and creates a selective habitat for osmosis-tolerant yeasts. Nectar contains secondary metabolites that act as nectar-robbing deterrents, thus favoring legitimate visitors. Floral defense compounds such as nectar-robbing deterrents can limit the number of yeast species that form a community on the flower. Osmosis-tolerant Ascomycetous yeast communities found on insect mouthparts include Debaryomyces, Metschnikowia, Starmerella or Zygosaccharomyces spp. (Pozo *et al.,* 2012).

The weed Hyptis suaveolens L. Poit., commonly found in East Nusa Tenggara, thrives in various environments, including roadsides and dry lands. In Kupang City, it is known as a nuisance plant that grows wild along roadsides or on abandoned fields. Hyptis suaveolens (commonly referred to as Gringsingan) belongs to the Lamiaceae family and is an aromatic, upright annual herb that can reach up to 1.5 meters in height. It thrives in lowland areas up to 1,000 meters above sea level and is distributed widely across tropical and subtropical regions. Originally native to Tropical America, this species has become widespread globally due to its rapid propagation ability. H. suaveolens commonly grows in disturbed habitats such as roadsides, grasslands, open forests, riverbanks, floodplains, coastal areas, and waste areas. It is unpalatable to livestock and has a tendency to dominate native grasslands, especially in overgrazed areas, reducing their quality (Ruma *et al.,* 2024).

Gringsingan flowers (Hyptis suaveolens (L.) Poit.) are pollinated with the help of honey bees, as noted by Aluri et al. (2005). The nectar of these flowers contains a variety of components, including glucose, fructose, sucrose, amino acids, and fat, as outlined by Komosinska-Vassev et al. (2015). Additionally, pollen from these flowers comprises glucose, fructose, protein, and fat (Komosinska-Vassev *et al.,* 2015). The presence of glucose in both nectar and pollen provides energy and carbon source for yeast, contributing to the microbial ecosystem found within the flowers.

Yeast is a unicellular eukaryotic organism that can ferment glucose and produce extracellular enzymes. One notable species, Saccharomycopsis fibuligera, is capable of producing the amylase enzyme with an amylase index of 3.8 (Nurhartadi and Rahayu, 2011). Another species, Pichia burtonii, also produces amylase but with a slightly lower index of 3.18 (Nurhartadi and Rahayu, 2011). Yeast extracts are extensively utilized across various industries, serving as additives in animal feed, agents for enhancing food flavor, cosmetic ingredients, etc.

Enzymes have been integral to biological systems and industries for centuries, particularly in producing alcohol and beverages. They are widely used in food, animal feed, beverages, detergents, pharmaceuticals, textiles, paper, and leather processing (Singh *et al.,* 2022; Mondal *et al.,* 2022). Microbial enzymes are favored commercially due to the ease of cultivation and genetic modification compared to plant or animal enzymes. With growing pressure for sustainable development, enzymes are replacing chemicals in various industries due to their specificity, eco-friendliness, and biodegradability. In 2019, microbial enzymes accounted for over 85% of the global enzyme market, which was valued at $9.9 billion in 2020 and is expected to grow at a 7.1% CAGR through 2027 (Singh *et al.,* 2022; Mondal *et al.,* 2022).

Microbial enzymes such as amylases, cellulases, proteases, and lipases are commonly used for various industrial purposes. Amylase is a vital enzyme with extensive applications in laboratories and industries (Singh *et al.,* 2022; Farooq *et al.,* 2021; Mondal *et al.,* 2022). Primarily, α-amylase is produced by microbes such as bacteria, fungi, and yeast. It holds a significant share of the enzyme market and is widely utilized across industries, including starch processing, textiles, detergents, beverages, biofuels, and food. Amylase's versatility lies in its ability to hydrolyze starch into low molecular weight sugars, making it essential for producing sugar syrups, cyclodextrins, and biofuels (Singh *et al.,* 2022; Farooq *et al.,* 2021; Mondal *et al.,* 2022).

Proteases are key industrial biocatalysts, accounting for about 60% of the enzyme market and 40% of global enzyme sales. These enzymes, known for breaking peptide bonds, are widely used in food, detergents, leather, textiles, cosmetics, and pharmaceuticals. Microbial proteases play crucial roles in both protein synthesis and degradation, with

traditional use in food fermentation now expanded to other industries, thanks to advances in genetics and protein engineering. Bacterial and fungal proteases dominate the commercial enzyme sector, offering sustainable, eco-friendly, and economically viable solutions. Researchers continue to innovate, aiming to enhance their industrial applications (Gimenes *et al.,* 2021; Naveed *et al.,* 2021; Liu & Kokare, 2023; Solanki *et al.,* 2021).

This study aims to evaluate the extracellular enzyme activity of yeast isolates from Gringsingan flowers (Hyptis suaveolens) in Kupang, which serve as microhabitats enriched with nectar and pollen, providing energy and carbon sources for yeast growth. By exploring these isolates, the research seeks to uncover novel enzymatic profiles with potential industrial applications, particularly in producing amylases and proteases used in food, pharmaceuticals, and biofuels. Given the dominance of microbial enzymes in the global market and their role in sustainable processes, this study could identify new yeast-derived enzymes suitable for eco-friendly and economically viable biotechnological innovations.

II. MATERIALS AND METHODS

Research activities were conducted from April 2023 to August 2023 at the Bioscience Laboratory, UPT Laboratorium Terpadu, Universitas Nusa Cendana, Kupang. Samples were collected from three locations : Lasiana (Kelapa Lima District, Kupang City), Liliba (Oebobo District, Kupang City) and Noelbaki (Kupang Tengah District, Kupang Regency).

The tools used in this research are glassware, scales, hot plate, magnetic stirrer, anaerobic jar, incubator, micropipette (Gilson, US), vortex mixer (Laquatwin HORIBA, Japan), autoclave (OSK 6500, Ogawa Seiki , Japan), Bio Safety cabinet, light microscope. Materials used in this research are gringsingan flowers, MRS Agar (Merck), MRS Broth (Merck), Anaerogas packs (Merck, Darmstadt, Germany), sterile filter membrane size 0.45 µm, CaCO3 (Merck), NaCl (Merck), Agar Bacto (Oxoid), Aquabidest, disposable petri dishes, Gram stain kit, Hydrogen peroxide, starch, tributyrin.

The research was divided into 4 activities: sampling of Gringsingan flowers, isolation of yeast from Gringsingan flowers, determination of yeast isolates, qualitative extracellular enzyme tests. Gringsingan flower samples were collected from areas in Lasiana, Liliba, and Noelbaki. Isolation of yeast was carried out using Mann Rogosa Sharpe (MRS) agar media supplemented by 1% $CaCO_3$. Extracellular enzyme of yeast isolates activity was tested using the hydrolytic of starch and casein tests.

- *Sampling of Gringsingan Flowers:-*

Sampling of Gringsingan flowers was carried out from three locations which had been randomly determined. The gringsingan flowers that have already bloomed were collected into sterile container, then put in a cool box, and transported to the laboratory. In the laboratory, the sample from three locations were mixed together.

- *Isolation of Yeast from Gringsingan Flowers:-*

The sample from three locations were composited and 25 g of the sample were mixed with 225 mL of sterile physiological NaCl (Rahayu and Nurwitri, 2012). The sample was made into serial dilutions (10^{-1} to 10^{-4}), then 100 µL of the sample from each serial dilution (10^{-1} to 10^{-4}) were cultured on MRS agar media supplemented with 1% $CaCO_3$ (Karyawati *et al.,* 2019). The cultures were incubated in an anaerobic jar with a gas pack at 37°C for 48 hours.

- *Determination of Yeast Isolates:-*

The colony that grew in surface of MRS agar were subculture on MRS agar. The cultures were stained with Gram staining followed by catalase test (Varghese and Joy, 2014). The catalase tests were carried out by dropping distilled water on a glass object and mixing it with 1% the isolate, then dripping it with 2 drops of 3% H_2O_2. Isolate that made clear zone in the around colony, gram positive, and catalase negative was determined as lactic acid bacteria. Yeast isolates can form clear zones around colonies on the surface of MRS agar supplemented by 1% $CaCO_3$. After Gram staining, when the yeast is observed under a microscope, the yeast shows purple cells which shows that it is similar to gram positive bacteria but from the shape of the cells it is clearly yeast, the cells with budding yeast form. Yeast can have the enzyme catalase.

- *Qualitative Extracellular Enzymes Tests:-*

The extracellular enzyme activity tests were carried out using starch hydrolysis, lipid hydrolysis, and casein hydrolysis tests (Sunatmo, 2009). Yeast isolates were inoculated on MRS agar medium which had been supplemented with 1% starch/triglyceride, tributyrin/ casein using the dot method, then incubated at 37°C for 48 hours. In the lipid hydrolysis and casein hydrolysis tests, extracellular enzyme activity can be observed with the formation of a clear zonearound the yeast colony. In the starch hydrolysis test, the incubation results are flooded with Gram's iodine solution for 30 seconds, then the excess Gram's iodine solution is discarded, and then the clear zone around the bacterial colony is observed.

III. RESULTS AND DISCUSSION

Sampling of Gringsingan flowers were carried out at three locations. The first sampling location was in the garden near the Undana Bioscience Laboratory, Lasiana Village, Kelapa Lima District, Kupang City. The second sample was taken in a garden near a resident's house, Liliba Village, Oebobo District, Kupang City. The third sample was taken from a garden near Panmuti Beach, Noelbaki Village, Central Kupang District, Kupang Regency.

The isolation results showed that only a small number of colonies that had been isolated from Gringsingan flowers. Only samples from Lasiana that can be counted, at the 10^{-1} dilution there were 32 colonies, but at the 10^{-2} dilution there were too scanty. In the samples from Liliba and Noelbaki, only a few colonies grew in the 10^{-1} and 10^{-2} dilutions. At dilutions 10^{-3} and 10^{-4}, there was no colony growth for all samples. The number of colonies were 320 cfu/ml, the number of colonies that contained in the

Gringsingan flowers were scanty. This condition may be caused by the size of Gringsingan flowers are very small, the average size of the Gringsingan flowers is 2-3 mm wide and 5-7 mm long. This fact also showed that only a small number of microorganisms can be cultured in the laboratory, and most microorganisms cannot be cultured. The metagenomic technique can be used as a method to determine the microorganism composition of the samples.

Table 1 Clear Zone Formation, Gram Staining And Catalase Test Results Of 6 Isolates

Isolate	Clear zone	Gram	Cell shape	Catalase	Information
KB103	-	+	Oval	+	Yeast
K23	-	+	oval	-	Yeast
K27	-	+	oval	+	Yeast
KU1	-	+	oval	+	Yeast
KU3	+	+	oval	+	Yeast
Pme	+	+	oval	-	Yeast

Table 2 Extracellular Enzyme Activity Test Results: Starch Hydrolysis

Isolate	Colony diameter (mm)	Clear zone diameter (mm)	Amylase Index
KB103	10	20	2
K23	9	0	0
K27	7	0	0
KU1	9	0	0
KU3	8	0	0
Pme	8	0	0

KB103 isolate had extracellular enzyme activity. The activity of this enzyme was demonstrated by the formation of a clear zone around the isolated colony after being flooded with Gram's iodine solution. Starch will be purple after being flooded with Gram's iodine, the clear zone around the isolate indicates the absence of starch because it has been hydrolyzed by the bacterial isolate. The enzyme that hydrolyzes starch is the amylase enzyme. KB103 isolate had the ability to produce amylase enzymes with an amylase index of 2 (Table 2).

Table 3 Extracellular Enzyme Activity Test Results: Casein Hydrolysis

Isolate	Colony diameter (mm)	Clear zone diameter (mm)	Protease Index
KB103	19	0	0
K23	9	0	0
K27	8	0	0
KU1	9	0	0
KU3	9	0	0
Pme	8	26	3.25

Pme isolates had extracellular enzyme activity (Table 3). This extracellular enzyme activity was indicated by the formation of a clear zone around the colony. The medium that contains casein will become white cloudy as a result of the casein have been suspended in the agar medium. The clear zone around the colony shows the activity of the protease enzyme from the bacterial isolate which works by hydrolyzing casein. Pme isolate was capable of producing protease enzymes.

Based on extracellular enzyme tests, yeast isolates from Gringsingan flowers were able to produce the enzymes (amylase and protease). The amylase enzyme was produced by KB103 isolate, while Pme only produced protease enzyme. In this study, the greatest enzyme activity had been showed by Pme isolates that produced protease enzyme with a protease index 3.25.

The identification of amylase-producing yeasts is significant due to the widespread industrial use of this enzyme, particularly in starch processing, biofuel production, and food industries. Amylase's ability to hydrolyze starch into low-molecular-weight sugars makes it essential for producing sugar syrups, cyclodextrins, and biofuels, and its inclusion in the microbial enzyme market has made it a key player in sustainable industrial processes (Singh et al., 2022 ; Farooq et al., 2021). Similarly, protease-producing yeasts offer a valuable contribution to the enzyme market, which accounts for a substantial share in various industries such as food, detergents, textiles, and pharmaceuticals. Proteases, essential for breaking peptide bonds, are integral to processes ranging from food fermentation to the textile and cosmetic industries, where they provide sustainable, eco-friendly solutions (Gimenes et al., 2021; Liu & Kokare, 2023). The discovery of these yeast isolates highlights their potential as bioresource tools for producing enzymes that can be used to replace chemicals in industrial processes, offering cost-effective, environmentally friendly, and biotechnologically viable alternatives. This study contributes to the growing interest in microbial enzymes, especially those derived from novel sources, to support the sustainable development of various industrial sectors.

Three genera of yeast that found in *Hyptis suaveolens* flowers were *Candida orthopsilosis*, *Kodamae ohmeri*, and *Starmerella floricola* (Table 5). *Candida orthopsilosis* was known as fungal pathogen that cause of candidemia.

Kodamae ohmeri and *Starmerella floricola* were known as the yeast in flowers.

Among the yeast isolates from Gringsingan flowers, the Pme isolate has the highest protease index (3.25). The result of molecular identification of the Pme isolate has a similarity of 99 percent to Candida orthopsilosis. The phylogenetic tree of the Pme isolate is shown in Figure 1. Based on the phylogenetic tree, the Pme isolate is in the same line as Candida orthopsilosis.

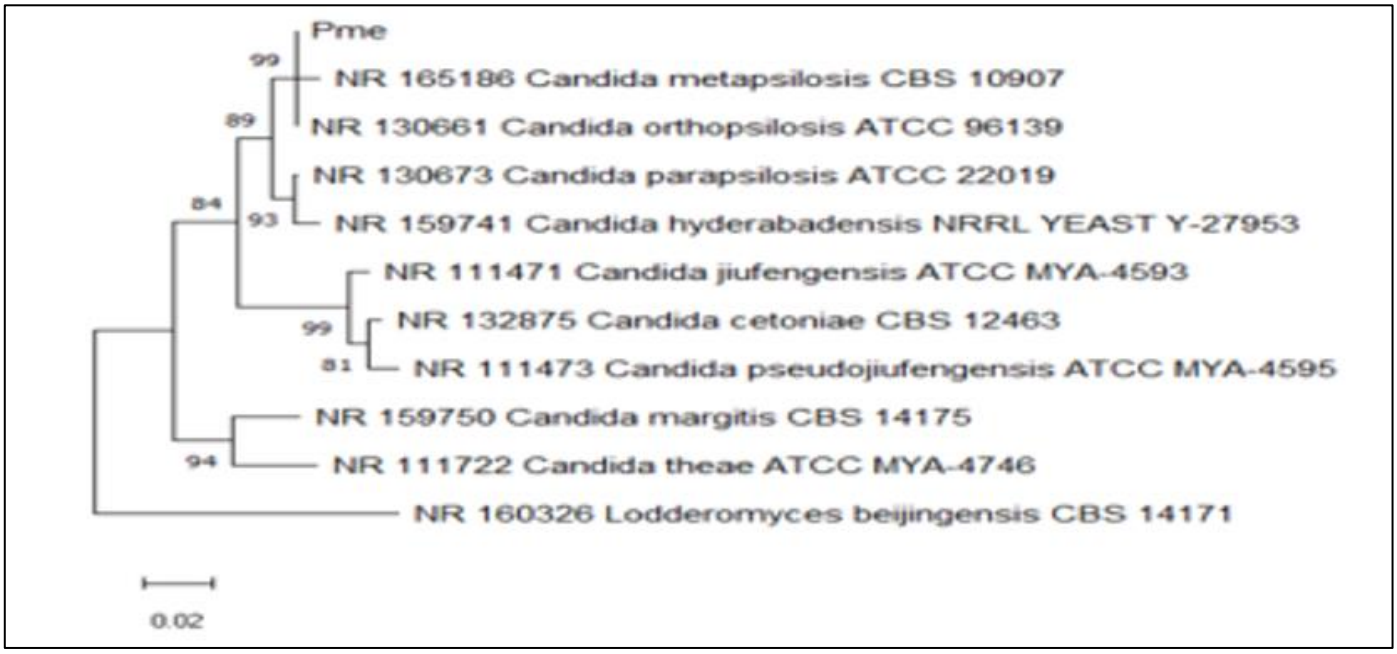

Fig 1 Phylogenetic Tree of Pme Culture Based on ITS Region. Tree Reconstruction Using Neighbor Joining Method, Kimura 2 Parameter Model and Gamma Distribution with 1000x Iteration.

Candida orthopsilosis is one of the fungi that can cause candidemia. Candidemia can be caused by Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, and Candida orthopsilosis. The candidemia has assosiated with high morbidity and mortality (Acosta-Mosquera et al., 2024).

Invasive fungal infections caused by *Candida* species are widely associated with high rates of severe illness and may be responsible for as many as 30% of all deaths from fungal diseases (Branco *et al.,* 2023).

Candida species can produce and secrete several hydrolytic enzymes, including secreted aspartyl proteases (SAPs), lipases (LIPs) and phospholipases (Branco *et al.,* 2023). The activity of the enzymes is closely related to Candida's pathogenisity such as adhesion, cell damage and invasion to host tissues. SAPs can degrade structural and immunological defense proteins of host which fasilitate to invasion and colonization in the host tissues. LIPs catalyze hydrolysis and synthesis of triacylglycerols. Phospholipases hydrolyze phospholipids and fatty acids which facilitate to adhesion.

The *Candida parapsilosis* group consists of 3 species, namely *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida methapsilosis*. Among the 3 members of the *Candida parapsilosis* group, *Candida parapsilosis* is the most common cause of candidaemia. *Candida parapsilosis* had been resistant to voriconazole and fluconazole but *Candida orthopsilosis* and *Candida methapsilosis* were susceptible to voriconazole and fluconazole (Bonfietti *et al*., 2012). Based on the secretion of hydrolytic enzymes, *C. metapsilosis* can secrete urease and esterase, *C. parapsilosis* can secrete protease and urease, while *C. orthopsilosis* can secrete urease. *C. parapsilosis* is the most virulent because *C. parapsilosis* can form pseudohyphae after incubation for 24 hours or 48 hours. *C. metapsilosis* is the least pathogenic (Gago *et al.,* 2014).

Candida orthopsilosis can be used as a biocontrol agent to prevent colonization of *Aspergillus* fungi on post-harvest citrus fruits (Sukmawati *et al*., 2021). Citrus fruits can be damaged or rotten after harvesting. This is caused by the colonization of fungi such as *Aspergillus niger* and *Aspergillus flavus*. Citrus was soaked in *C. orthopsilosis* suspension following a 2 mm wounding. After 7 days of incubation, *C. orthopsilosis* was capable to inhibit the growth of *Aspergillus niger* and *Aspergillus flavus*, and the Citrus still in fresh.

Yeast can inhibit fungal growth through nutrient competition, produce antifungal compounds, and produce lityc enzymes such as glucanase, chitinase and protease. The growth of fungal colonies can be disrupted by a lack of nutrients and space to grow. Lytic enzymes can cause the degradation of protein components from the fungal cell wall and then cell wall growth can be inhibited (Sukmawati *et al.,* 2021).

Based on the cellular structure, the cell wall of *C. albicans*, *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* have a similar wall composition but the arrangement of this component is different among the species (Manuela *et al.,* 2024). The composition of the cell wall consists of phosphomannan, cell wall protein, β-1,3-glucan, N-glicans and O-glicans, chitin and plasma

membrane. When compare to *C. albicans*, the species from *C. parapsilosis* complex (*C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*) showed lower mannan content, simillar protein levels, and increase in chitin and β-1,3-glucan exposure on the cell wall. The distribution of structural polysaccharides in the cell wall of *C. orthopsilosis* exposes all its β-1,3-glucan and chitin at the cell wall surface, making the inner wall polysaccharides is more accessible.

Kodamaea ohmeri belongs to yeasts that formerly known as *Pichia ohmeri* (Mao *et al.,* 2024). *K. ohmeri* was first identified from pleural effusion in 1984. *K. ohmeri* is an emerging fungal pathogen that often infects patients with weakened immunity. The patient presented with fever, shortness breath and letargic within two weeks.

Kodamaea ohmeri has been isolated from enviromental source such as sand, seawater, pools, and fruits (Singh *et al.,* 2024). *K. ohmeri* grows as smooth, dry, pale-white yeast colonies on Sabourad's dextrose agar. Infections due to *K. ohmeri* occur in immuno-compromised patients such as cancer and diabetic patients.

Kodamaea ohmeri were isolated and identified from grapes skin (An R *et al.,* 2024). *K. ohmeri* is a rare yeast. *Kodamaea ohmeri* also known as *Pichia ohmeri*. *K. ohmeri* is pathogenic to human especially for immunocompromised patients.

One of the main yeast that found in colonial cheese is *Kodamaea ohmeri* (Myazaki *et al*., 2023). The main microbiota present in colonial cheese are lactic acid bacteria and yeast. *Lactococcus lactis*, *Enterococcus sp.*, and *Bifidobacterium psychraerophilum* are lactic acid bacteria in colonial cheese that naturally found in milk. *Diutina catelunata*, *Clavispora lusitaniae*, *Kodamaea ohmeri*, *Kluyveromyces marxianus*, and *Candida ethanolica* are the yeast in colonial cheese that naturally found in raws material and the production environment. There may exist sinergical relationship between yeast and lactic acid bacteria. Lactic acid bacteria produce lactate that decrease pH in cheese, yeast elevate the pH of the environment (resulting from lactic acid bacteria metabolism) by utilizing lactate and generating alkaline substances by through proteolysis. The yeasts provide vitamins and amino acids to bacteria.

The immune system must exhibit good specifity and function optimally to act against pathogens without eliminating the benefit microorganism (Myazaki *et al*., 2023). Individuals with good nutritional status have a-well functioning immune system. Some pathogenic bacteria presented in our work may not be a problem for individuals with healthy immune system.

Kodamaea ohmeri ENCB-23, *K. ohmeri* ENCB-8R, and *K. ohmeri* ENCB-VIK were shown to be hydrocarbonoclastic yeast species (Ortiz-Alvarez *et al*., 2019). *K. ohmeri* can metabolize n-alkanes, branched alkanes, cycloalkanes, and n-octanol in the presence of heavy metals and under acidic conditions. The alkanes were main constituent in petroleum and representatif the hydrocarbon contaminant of air, soil and water. Therefore, *K. ohmeri* can be used as reducing alkanes in the environment that were contaminated by petroleum.

IV. CONCLUSION

There were three species of yeast isolates from Gringsingan flowers, namely *Candida orthopsilosis, Kodamaea ohmeri* and *Starmerella floricola*. KB103 isolate produced amylase enzymes and Pme isolate produced protease enzyme. KB103 and PMe isolates were determined as *Candida orthopsilosis*. *Candida orthopsilosis* from Gringsingan flowers had potency to produce extracellular enzymes, especially for production of protease enzyme.

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