INSCIENTECH : JOURNAL OF INDUSTRIAL, SCIENCE AND TECHNOLOGY
ISSN : 2598-3113

YEAST ON EPIPHYTE OF TANGERINES CITRUS FRUIT (*Citrus nobilis* L.) AND THEIR POTENTIAL ANTAGONIST TO *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

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Abstract

One of the most important diseases of citrus plant is anthracnose which caused by *Colletotrichum gloeosporioides* (Penz.) fungi. Yeast is one of microbes which has a good potential to control the disease. This research focused on exploring yeasts on tangerines and examining their efficacy to control *C. gloeosporioides* using *in vitro* and *in vivo* treatments. Observation was started by isolated *C. gloeosporioides* and yeast from tangerines citrus fruit, then followed with *in vitro* and *in vitro* treatment. Both were counted the percentage of antagonist and pathogen incubation stage and also disease incidence, respectively. Result showed that yeast has been isolated from tangerines fruit were *Candida* sp. (isolate 1), *Candida* sp. (isolate 2) and *Pichia* sp. All yeast showed inhibit *C. gloeosporioides* growth and suppressed its development.

Key words: *Colletotrichum gloeosporioides*, *Candida* sp., *Pichia* sp., biocontrol agent

INTRODUCTION

Nowdays, a modern management of agroecosystem play an important role in the our agriculture. Those modern management needed to overcome several pest and disease problem. One of the most important diseases attacking citrus plant is anthracnose, caused by the *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. This anthracnose disease-causing fungus is latent and systemic (Walker, 1957). It's potential to takes a biological control action in the same ecological niche as the *C.gloeosporioides* to manage its growth and existence.

Yeast is one microbe which promise in modern concept because has potential to control anthracnose disease caused by *C. gloeosporioides*. Some yeast species play an important role in degrading fungal cells and spores (Suzzi *et al*., 1995; Kusumaningati, *et. al*., 2013).

This research focused on isolating yeasts from tangerines and examining their efficacy to control *C. Gloeosporioides.*

MATERIALS AND METHODS

We were explored epiphyte yeast from tangerine fruit and test their efficacy *in vitro* and *in vivo* vs *C. gloeosporioides*.

Microbes Isolation

Isolation of *C. gloeosporioides* (fungi: pathogen) was carried out by planting it single spores from tangerine fruit which showed anthracnose symptom on PDA media. While yeast was isolated by wash skin of tangerine fruit using 10 mL sterile water and then take 1 mL of it and mix it with 9 mL of sterile water. Then, the 10 mL suspension was diluted gradually to 10^{-5} dilution. Dilution suspensions of 10^{-3}, 10^{-4} and 10^{-5} were planted in YMA (*Yeast Malt Agar*) media as much as 50 µL (Assis and Mariano, 1999) and then were incubated at 40^{o}C for 90 minutes (Kardos *et al*., 2011).

After treated, then it purified using *strike plate method* and identified manually under reference *"The Yeast 5th Edition".* Manual identification by observing the macroscopic appearance : 1) shape, 2) color, 3) texture, 4) edges, and 5) elevations of the colony. Cell were observed under stereo microscope based on: 1) cell shape 2) cell size, 3) sexual and asexual reproduction type, 4) pattern of reproduction, and 5) presence of pseudohifa.

In vitro* Antagonistic Test of Yeast vs *C. gloeosporioides

Relative Resistance Level (RRL)

In vitro antagonistic test was performed by planting yeast isolate and pathogenic *C. gloeosporioides* on the same PDA media (Sugipriatini, 2009). Observations were carried out for 6 days after monitored inhibit zone width. The percentage of RRL was then calculated by the following formula (Hadiwiyono, 1999):

$$\mathbf{RRL} = \frac{\mathbf{dc} - \mathbf{dt}}{\mathbf{dc}} \times \mathbf{100}\%$$

Information:
RRL = relative resistance level of pathogen growth
dc = total of pathogen colony radius (r1+r2) without yeast treatment (control)
dt = total of pathogen colony radius (r1+r2) with yeast treatment

In vivo* Antagonistic Test of Yeast vs *C. gloeosporioides

In vivo antagonistic test was performed by inoculating pathogen isolate and yeast on tangerine fruit. Observations were made on disease incubation period and the disease incidence. The incubation period was observed every single day till appear first symptom. The percentage of disease incidence was calculated at 6 days after inoculation (DAI) using the following formula: (Korsten and Demoz 2006):

$$\mathrm{DI} = \frac{\mathrm{n}}{\mathrm{N}} \times 100\%$$

Information:
DI = percentage of disease incidence
n = number of points that cause symptoms
N = total number of inoculation points

Data Analysis

The data of efficacy test of yeast vs *C. gloeosporioides* were analyzed using Analysis of Variance (ANOVA) and continued with the Least Significant Difference test (LSD) at 5% level if there was difference.

RESULT

***C. gloeosporioides* and Yeast Growth**

The colony of *C. Gloeosporioides* showed grayish-white color, and thick - dense texture. Microscopically, *C. gloeosporioides* showed a septum and branched hygiene hyphae. Conidia grow on the top of the conidiofor, hyaline, unscapped and cylindrical with blunt ends at the both side. Yeast from tangerine were *Candida* sp. (Isolate 1), *Candida* sp. (Isolate 2), and *Pichia* sp. Macroscopic and microscopic characteristics can be seen in Table 1.

Tabel 1. Macroscopic and Microscopic Characteristics of Yeast

Name of Species	Macroscopic				
	Colony's color	**Colony's texture**	**Colony's surface**	**Colony's elevation**	**Colony's edge**
Candida sp. (isolate 1)	Yellowish white	Granules	Smooth	Rather convex	Jagged flat
Candida sp. (isolate 2)	Pure white	Granules	Dull smooth	Convex	Flat
Pichia sp.	Yellowish white	Granules	Smooth	Convex	Flat
Name of Species	**Microscopic**				
	Cell shape	**Cell size (µm)**	**Reproduction**	**Bud pattern**	**Pseudohypha**
Candida sp. (isolate 1)	Round	1-2,5	Bud	Multilateral	-
Candida sp. (isolate 2)	Round	1-2,49	Bud	Multilateral	-
Pichia sp.	Ovoid round	2-4,04	Bud	Multilateral	-

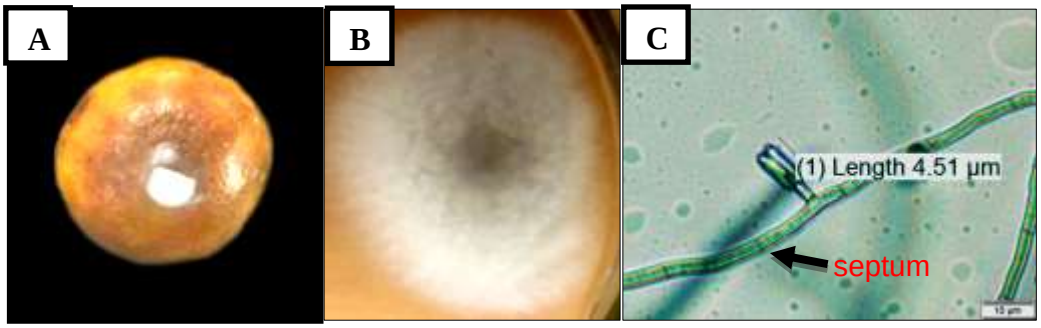

Figure 1. *C. gloeosporioides* on Agar media. A: Symptoms of the disease on citrus fruits. B: Macroscopic appearance of fungal colonies. C: Microscopic hyphae and conidia.

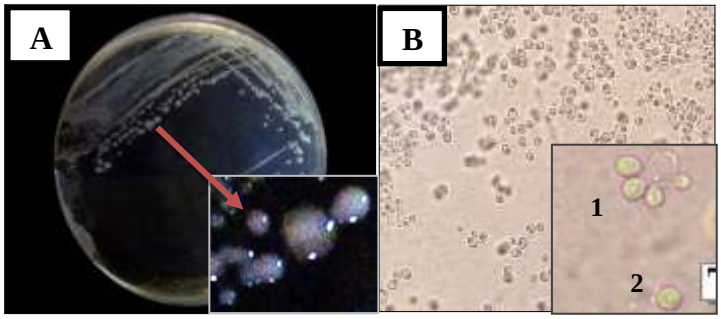

Figure 2. *Candida* sp. (Isolate 1), A. Macroscopic age 5 DAI. B. Microscopic; (1) Multilateral bud pattern (2) Single cell.

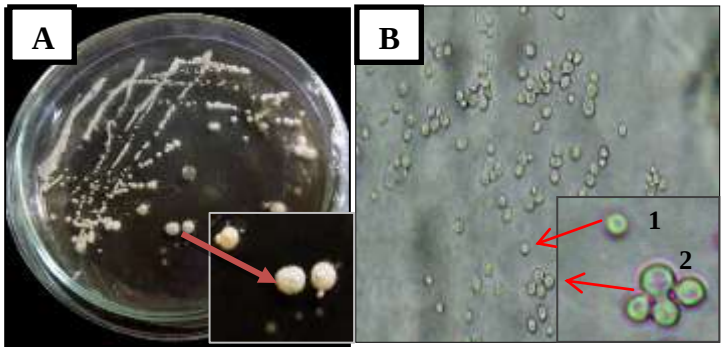

Figure 3. *Candida* sp. A. Macroscopic age 5 DAI. B. Microscopic; (1) Single cell (2) Multilateral bud pattern

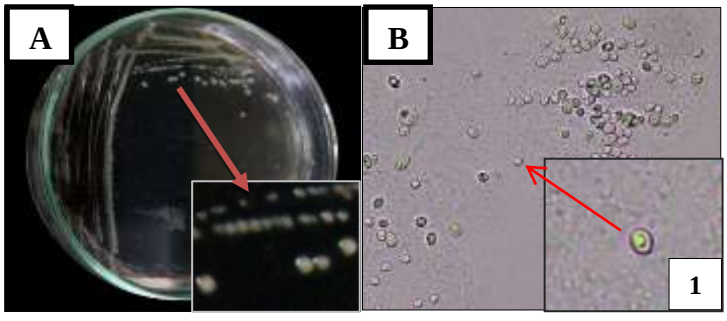

Figure 4. *Pichia* sp. A. Macroscopic age 5 DAI. B. Microscopic; (1) Single cell.

In vitro* Antagonist Test Result of Yeast vs *C. gloeosporioides

Test of antagonist with calculation of relative resistance level (RRL)

The results of *in vitro* antagonistic test by calculating the percentage of RRL can be seen in Table 2. Treatment using yeast indicates a barrier occurring since 4-6 DAI. The results of the analysis using Least Significant Difference test at the 5% error level (5% LSD) presented in Table 2. shows the significant different effect between *Candida* sp. (Isolate 1) with *Candida* sp. (Isolate 2), and *Pichia* sp. with *Candida* sp. (Isolate 2). But there was no real difference between *Candida* sp. (Isolate 1) with *Pichia* sp. treatment to inhibiting the growth of *C. gloeosporioides* at 6 DAI.

Tabel 2. Percentage of Relative Inhibition of Yeast vs *C. gloeosporioides* Growth for 6 DAI

Treatments	Percentage of inhibition (%)					
	1 DAI	2 DAI	3 DAI	4 DAI	5 DAI	6 DAI
Candida sp. (isolat 1)	18,86	20,14	14,86	10,28 b	24,57 b	27,14 b
Candida sp. (isolat 2)	7,86	14,43	10,43	4,71 a	7,86 a	14,14 a
Pichia sp.	25,14	21	12,57	12,71 b	20 b	23,14 b

Information: The numbers followed by the same letter show the results are not significantly different (5% LSD)

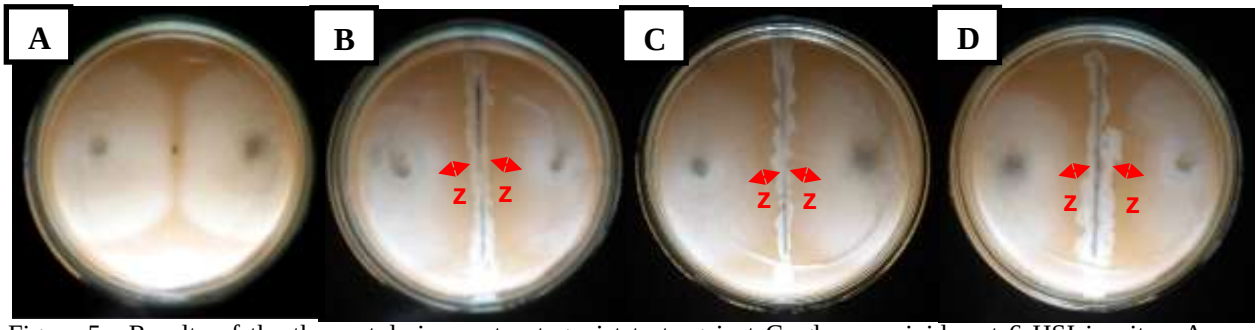

Figure 5. Results of the thermotoleric yeast antagonist test against C. gloeosporioides at 6 HSI in vitro. A: control treatment, B: *Candida* sp. (Isolate 1) treatment, C: *Candida* sp. (Isolate 2) treatment, D: treatment of *Pichia* sp., z: Clear zone.

In vivo* Antagonist Test Result of Yeast vs *C. gloeosporioides

Treatment using *Candida* sp. (Isolate 1) showed disease incidence rate of 81.14%, treatment using yeast was able to suppress disease incidence rate of 18.86%. While, treatment using *Candida* sp. (Isolate 2) showed disease incidence rate of 90.57%, indicating a 9.43% incidence of disease incidence rate. Treatment using *Pichia* sp. Resulting in a disease incidence rate of 85.86%, indicating a 14.14% incidence of disease incidence rate. There was an emphasis on the incidence rate of the disease by the three yeast treatments, but the suppression value was very small (not significant) so it was not seen quantitatively.

The result of incubation period showed significant difference between yeast treatment and control treatment (without yeast), but there was no significant difference between the three yeast treatments (Table 3).

Tabel 3. The effect of yeast on the incidence of disease and the incubation period of the pathogen

Treatments	Incidence of disease (%)	Incubation Period (Days)
Control	100	2 a
Candida sp. (isolate 1)	81.14	3.43 b
Candida sp. (isolate 2)	90.57	3 b
Pichia sp.	85.86	3.28 b

Information: The numbers followed by the same letter show the results are not significantly different (5% LSD)

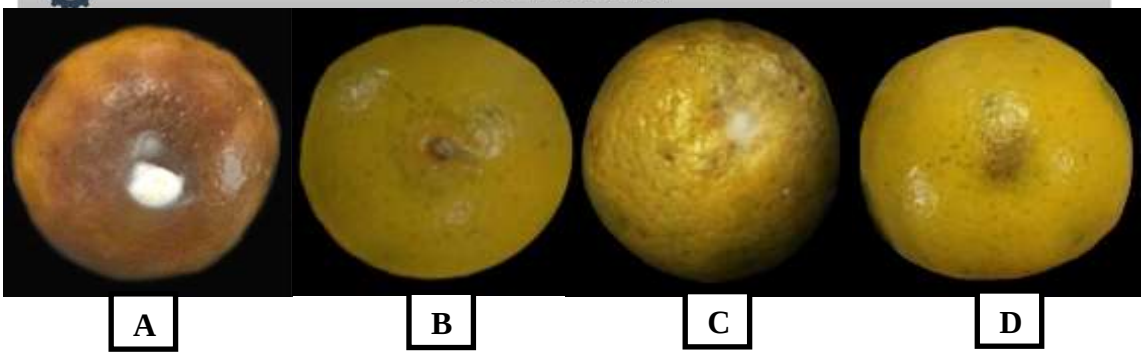

Figure 6. The results of the *in vivo* antagonist test between thermotolerant yeast against *C. gloeosporioides* at 6 DAI. A: control treatment, B: *Candida* sp. (Isolate 1) treatment, C: Candida sp. (Isolate 2) treatment, D: *Pichia* sp. treatment

DISCUSSION

Research showed that at temperature range 25°C - 34°C *Colletotrichum* sp. was developed optimally (Boyette *et al*., 2012).But, *Colletotrichum* spores can also survive at temperatures above 35°C (Fernando *et al*., 1999). It takes a yeast with the ability to live at higher temperatures to increase the odds of the antagonistic power of yeast against the pathogen, so that the researchers only choose yeast that can survive at 40°C.

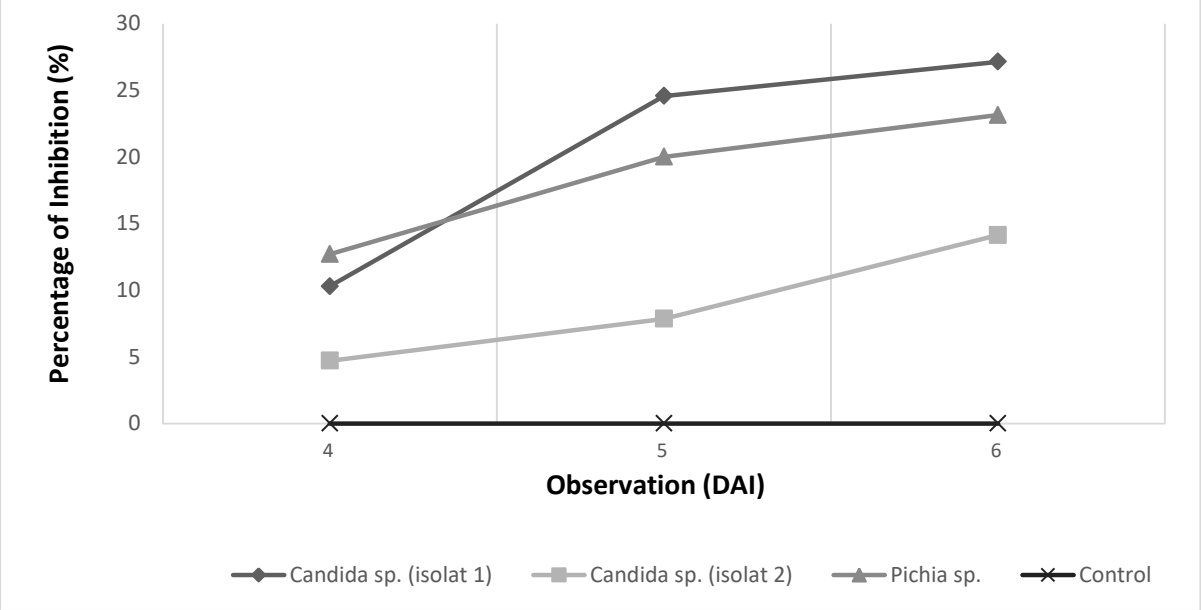

Figure 7. The average percentage of yeast inhibition against *C. gloeosporioides* from 4 DAI to 6 DAI

The results showed that there are three yeasts tested were potentially inhibit the growth of pathogens *C. gloeosporioides*. Percentage value of three yeasts greater than control. Control treatment has the lowest percentage resistance, at 0%, indicating that there were no obstacles to this treatment. The highest resistance percentage at 6 DAI was shown by the yeast treatment of *Candida* sp. (Isolate 1), meanwhile treatment of yeast *Candida* sp. (Isolate 2) shows the percentage of least resistance lower than those of the other two yeasts treatment (Figure 7).

Antagonistic mechanism was indicated as an antibiotic mechanism. It was showed by presence of clear zones between yeast and *C. gloeosporioides* which indicate an obstacle to the growth of pathogen. Those antibiosis mechanism because yeast secretes secondary metabolite compounds or other toxic compounds inhibiting pathogen growth. One of them is through the enzymatic mechanism. The enzyme which produced by yeast is chitinase, this enzyme can degrade chitin which make up the fungus cell wall. Haggag and Mohamed (2007) reported that the yeast antibiosis mechanism involves the use of secondary metabolites such as enzymes pelisis, volatile compounds, siderophores or other toxic substances that can cause fungistatik, lysis of the cell wall (Dewi, *et. al.*, 2013; Aliah, *et. al.*, 2015), or necrotic, so that fungal growth is inhibited.

Incidence rate analysis showed that there was no significant difference between the treatments of yeast in control *C. Gloeosporioides* growth (table 3). Meanwhile, if analyzed qualitatively based on observations, the three yeast treatments have the potential to suppress the incidence rate of the disease in the fruit. This was suspected because the pathogens and yeasts come from the same habitat. According Golubev (2006); Shofiani, *et. al*., (2015), the ability of yeast or fungi antagonism will be more increased to microorganisms from different habitats. Microorganisms from different habitats are considered as new competitors who must be defeated in order to dominate the space and nutrients available.

The average incubation period of *C. gloeosporioides* without yeast treatment is 2 days after the inoculation. The incubation rate of pathogenic *C. gloeosporioides* with yeast treatment ranged 3 days after inoculation. This result showed that the treatment of the three yeasts were able to slow the incubation period of the pathogen. Mean of incubation period of pathogen with treatment of *Candida* sp. (Isolate 1) was at 3.43 days after inoculation. Mean of pathogen incubation period with treatment of *Candida* sp. (Isolate 2) was at 3 days after inoculation. Mean of pathogen incubation period with treatment of *Pichia* sp. was at 3.28 days after inoculation. It was seen that there was no significant difference among three treatments.

Qualitative observation showed that the spraying method (using yeast suspension) on citrus fruit surface provides other benefits. Citrus fruits sprayed with yeast suspension become more durable because of its inhibition ability to pathogen infection. The method of spraying on the surface of citrus fruits is known as bioedible coating. Greener and Fennema (1989) explained that the coating of the fruit can be done in several ways, namely dipping (dip application), spray application, foam application, and drip application.

CONCLUSION

Based on this research, it can be concluded that there were thermotolerant yeast on tangerine fruit which can survive at 40^{o}C. The yeasts obtained were *Candida* sp. (Isolate 1), *Candida* sp. (Isolate 2) and *Pichia* sp. The three yeasts can inhibit the growth of *C. gloeosporioides* pathogens *in vitro*. The three yeasts were also able to suppress the development of *C. gloeosporioides in vivo*.

SUGGESTION

From the research that has been conducted, it is necessary to conduct further research on the identification of yeast molecularly to the species level to strengthen the results of identification. It is necessary to test the antagonistic mechanism of yeast using slide culture method under a microscope to ascertain the antagonistic mechanism that occurs. Testing of metabolic compounds contained by yeasts in suppressing the growth of pathogens is also required.

REFERENCES

Aliah, N.U. L. Sulistyowati, A. Muhibbudin. 2015. Hubungan ketebalan lapisan epidermis daun terhadap serangan jamur (*Mycosphaerella musicola*) penyebab penyakit bercak daun sigatoka pada sepuluh kultivar pisang. Jurnal Hama dan Penyakit Tumbuhan 3 (1), pp. 35-43.

Assis, S. M. P., and R. L. R. Mariano. 1999. Antagonism of Yeasts to *Xanthomonas campetris* pv. Campestris on Cabbage phylloplane in Field. Rev. Microbiol. 30: 191-195.

Boyette, C. D., R. E. Hoagland, M. A. Weafer, and K. Stetina. 2012. Biological Control Pontential of *Colletotrichum gloeosporioides* for Coffee Senna (Cassia occidentalis). American Journal of Plant Sciences 3: 430-436.

Dewi, I.M. A. Cholil, A. Muhibuddin. 2013. Hubungan Karakteristik Jaringan Daun dengan Tingkat Serangan Penyakit Blas Daun (Pyricularia Oryzae Cav.) pada Beberapa Genotipe Padi (Oryza Sativa L.). Jurnal Hama dan Penyakit Tumbuhan 1 (2), pp. 10-18.

Fernando, T. H. P. S., C. K. Jayasinghe, and R. L. C. Wujesunera. 1999. Factors Affecting Spore Production, Germination and Viability of *Colletotrichum* Isolates from Havea brasiliensis. Mycological Research. 104(6) : 681-685.

Golubev, W. I. 2006. Antagonistic Anteractions among Yeast. Springer, Germany: 197-219.

Guarro, J., J. Gene, and A. M. Stchigel. 1998. Development in Fungal Taxonomy. Clinical Microbiology Reviews. 12 (3): 454-500.

Hadiwiyono. 1999. Jamur Akar Gada (*Plasmodiphora brassicae* Wor.) pada Cruciferae: Uji Toleransi Inang dan Pengendaliannya secara Hayati dengan *Trichoderma*. Universitas Jenderal Soedirman.

Haggag, W. M., and H. A. A. Mohamed. 2007. Biotecnological Aspects of Microorganisms Used in Plant Biological Control. American-Eurasian Journal of Sustainable Agriculture. 1 (1): 7-12.

Kardos, L., A. Juhasz, G. Y. Palko, J. Olah, J. Barkacs, and G. Y. Zaray. 2011. Comparing Mesophilic and Thermophilic Anaerobic Fermented Sewage Sludge Based on Chemical and Biochemical Tests. Appl Ecol Environ Res 9: 293-302.

Korsten, L., and T. B. Demoz. 2006. Bacillus subtilis Attachment, Colonization, and Survival on Avocado Flowers and Its Mode of Action on Stem end Rot Pathogens. Biological Control. 37: 68-74.

Kurtzman, C. P., and J. W. Fell. 1998. The Yeast: A Taxonomic Study, 3rd ed. Elsevier, Amsterdam: P. 1035.

Kusumaningati, M.A, S. Nurhatika, A. Muhibuddin. 2013. Pengaruh Konsentrasi Inokulum Bakteri Zymomonas mobilis dan Lama Fermentasi Pada Produksi Etanol dari Sampah Sayur dan Buah Pasar Wonokromo Surabaya. Jurnal Sains dan Seni ITS 2 (2), pp 218-223.

Shofiana, R.H. L. Sulistyowati, A. Muhibuddin. 2015. Eksplorasi jamur endofit dan khamir pada tanaman cengkeh (Syzygium aromaticum) serta uji potensi antagonismenya terhadap jamur akar putih (Rigidoporus microporus). Jurnal Hama dan Penyakit Tumbuhan 3 (1), pp. 75-83.

Sugipriatini, D. 2009. Potensi Penggunaan Khamir dan Kitosan untuk Pengendalian Busuk Buah *Lasiodiplodia theobromae* (Pat.) Griffon dan Maubl. (syn. Botryodiplodia theobromae Pat.) pada Buah Mangga selama Penyimpanan (Tesis). Bogor: Sekolah Pascasarjana Institut Pertanian Bogor.

Suzzi G., P. Romano, I. Ponti, and C. Montuschi. 1995. Natural Wine Yeast as Biocontrol Agent. J. Appl. Bacteriology 78: 304-308.

Walker, J. C. 1957. Plant Pathology. : Mc Graw-Hill Book Company, inc.