



Phosphate Solubilizing Endophytic Yeasts of Soursop and its Suppressive Effect on Aflatoxigenic *Aspergillus flavus*

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ABSTRACT

Background: Many microorganisms isolated from plant surfaces and inside tissues are ecofriendly and beneficial for biofertilizer and biopesticides. Recent studies showed that colonization of plants by endophytic yeast have been studied due to their capacity to solubilize phosphate and produce organic acids that may be used to control toxigenic fungi. The aim this research was to investigate endophytic yeasts of soursop fruit to solubilize phosphate and their organic acid against toxigenic *A. flavus*.

Methods: The fresh healthy ripe fruit were harvested and followed by surface sterilization. The inner tissue was homogenized and a serial dilution up to 10^{-11} CFU/ml. All dilution were pour plated with malt extract agar medium (MEA). Three replications were made for each dilution. All plates were incubated for 7 days at 29°C. Each yeast isolate was initially enumerated, isolated and examined for morphological characteristics. The potential of endophytic yeast to dissolve phosphate was observed in Pikovskaya's medium. Coconut agar medium contain 30% organic acid produced by each isolate was used to determine mycelial growth and aflatoxin production of *Aspergillus flavus*.

Result: Showed four endophytic yeasts were isolated and *Rhodotorula* sp. was the most dominant species (27×10^{11} CFU/ml), whereas, *Candida* sp. was the highest P solubilization index (7.71), organic acid producer (0,00354%) and inhibit mycelial growth and aflatoxin production of *A. flavus*.

Key words: Aflatoxigenic, *Aspergillus flavus*, Endophytic yeasts, Phosphate solubilization, Soursop.

INTRODUCTION

Endophytic yeasts refer to unicellular fungi that reside within healthy plant tissue such as leaves, stem, roots and fruit without causing harm to the host plant (Doty, 2013 and Rai *et al.*, 2021). As an endosymbiont, yeasts excrete secondary metabolites that ameliorate microbial invasion and the host plant provide nutrients and stable environmental condition for the growth of yeasts (Ling *et al.*, 2020; Raimi and Adeleke 2021). Fruits constitutes suitable habitats for endophyte yeasts particularly due to their low pH and availability of nutrients, however, these traits are varied depend on type and maturity of the fruit. Yeasts are widely distributed in natural environment. Soil yeasts such as *Meyerozyma* and *Rhodotorula* have been studied for phosphate solubilization (Nakayan *et al.*, 2013). Phosphate solubilizing yeasts are important in dissolving fertilizer phosphorus for plant growth ((Dhillon and Kaur, 2021). Phosphorus available in soil is often insufficiency due to its strong binding in insoluble forms by complex chemical and biochemical processes. Yeasts genera isolated from rhizosphere soil and fruits such as *Rhodotorula* sp., *Kloeckera* sp. *Saccharomyces* sp., *Hansenula* sp. and *Debaryomyces hansenii* were studied and potential as phosphate solubilizer (Narsian *et al.*, 2010).

Aspergillus flavus is one of postharvest fungi that infect most agricultural crops and feed products (Nurtjahja *et al.*, 2022). Many efforts to suppress mycelial growth and aflatoxin production of *A. flavus* by yeasts. Previous study by Afsah-Hejri (2013) and Moradi *et al.*, 2020) revealed that endophytic yeasts such as *Pseudozyma fusiformata*,

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Cryptococcus albidus, *Rhodotorula fragaria*, *R. hinula*, *Cryptococcus hungaricus* and *Saccharomyces cerevisiae* isolated from pistachio fruits, leaves and soil have biocontrol activities against the growth and aflatoxin production of a toxigenic *Aspergillus flavus*. Organic compounds of endophytic yeasts isolated from crops particularly *Candida nivariensis* was the most effective inhibit mycelial growth and aflatoxin production of *A. flavus* (Jaibangyang *et al.*, 2020). Yeast strains isolated from soil belonging to *Saccharomyces cerevisiae*, *Suhyomyces xylopsoci*, *Pichia kudriavzevii* and *Candida tropicalis* reduce mycelial growth and aflatoxin production of *A. flavus* (Natarajan *et al.*, 2022). Therefore, the present study was

aimed to isolate, identify and evaluate of indigenous endophytic yeasts on healthy and ripe soursop fruit that potential to solubilize phosphate and as biocontrol to reduce mycelial growth of aflatoxigenic *A. flavus*.

MATERIALS AND METHODS

Enumeration and isolation of endophytic yeasts

The research was conducted from April 2024 to February 2025. The fresh healthy ripe fruits of soursop were collected directly from their tree during dry season May 2024 at local subsistence farmer in Medan, North Sumatera-Indonesia. The fruit then was washed with sterile distilled water and then subjected to consecutive 1 minute washes with 1% sodium hypochlorite, 70% ethanol and sterile distilled water. After the exocarp was removed using a steril scalpel, the inner tissue was aseptically deliquesced with homogenizer and 25 g the suspension was put on to 1000 ml flask then steril distilled water was add until the volume up to 250 ml. The suspension was shaken on a Kotterman 4020 (D3165 Hanigsen, W. Germany) at 250 rpm for 10 minutes. By using a serological pipette, one ml of the aliquots was transfered onto a petri dish (diam 9 cm) and pour plated with malt extract agar (MEA) medium. A serial dilution was made up to 10^{-11} CFU/ml. Each dilution was replicate three times. All plates then were incubated at 29°C for 7 days. Separate yeast colonies were enumerated as colony forming unit (CFU/ml). Individual yeast colony were sub-cultured for 3 days on MEA and identified morphologically (Pitt and Hocking, 1983).

Phosphate solubilization

The potential of endophytic yeasts to solubilize inorganic phosphate was determine in vitro by using Pikovskaya's medium with composition 10 g glucose, 5 g Ca_3HPO_4 , 0.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g KCl, 0.1 g MgSO_4 , 0.1 g H_2O , 0.5 g yeast extract, 25 mg MnSO_4 , 25 mg FeSO_4 25 dan 20 g bacto agar in 1 litre distilled water. The fresh culture of yeast isolates were inoculated on petri dish (9 cm in diameter) containing the solid medium. All plates were incubated at 29°C for 3 weeks. The phosphate solubilization efficiency unit of each strain was calculated as the diameter of the entire visible halo zone divided by the diameter of the zone with yeast colonies.

Aspergillus flavus isolates

A pure culture of aflatoxigenic *Aspergillus flavus* strain (aflatoxin producer) used for experiment was culture collection of Microbiology Laboratory, Universitas Sumatera Utara, isolated from dried stored spices. Their toxigenic was analyzed at SEAMEO BIOTROP, Bogor West Java by cultural method using 10% coconut agar medium (CAM) (36 g/l bacto agar and 100 ml/l coconut cream extracted from freshly shredded coconut endosperm (Davis *et al.*, 1987). The pH medium was adjusted to pH 7.0 using 2 N NaOH and sterilized at 120°C for 20 minutes. A small fragment mycelia of the sub-culture *A. flavus* was

inoculated at the center of a petri dish (9 cm in diameter) containing CAM and incubated at 29°C for 5 days. Aflatoxigenic *A. flavus* strains was signed by the appearance of yellow pigment at reverse side of the petri dish (Lin and Dianese, 1976).

Determination of total organic acids

The yeast isolates tested for their organic acid production were conducted by culturing in flask 500 ml containing 150 mL potato broth medium. Each isolate was inoculated on the medium then was placed in shaker (*Orbital shaker*, Gallenkamp®, England) 120 rpm for 10 days at 29°C. The pH value of each suspension was determined using pH meter, then it was filtered using Whatman filter paper # 42. Boehm titration using 0.1 N NaOH was conducted. Total organic acids produced were determined using formula:

Total organic acid (%) =

$$\frac{(V \text{ NaOH} \times N \text{ NaOH}) \times \text{ew}}{\text{mass sample (mg)}} \text{df} \times 100\%$$

V = Volume of NaOH (ml).

N = NaOH normality (0.098).

ew = Equivalent weight (60).

df = Dilution factor (100/20).

Organic acids againts mycelial growth

The ability of organic acids produced by yeast to supress mycelial growth of *A. flavus* was determined by inoculation of small fragment of the fungal mycelia at the center of a petri dish (9 cm in diameter) containing 10% CAM with 30% the organic acid. Coconut agar medium with no organic acid was performed as control. All plates were subsequently incubated at 29°C for 5 days. Each treatment was conducted in triplicate. Mycelial growth was monitored by means of colony diameter and the presence of yellow pigment on colony at the reverse side of the petri dish to determine qualitative ability of aflatoxin production.

Statistical analysis

All the observed data were analyzed by analysis of variance (ANOVA) for statistically significant differences, followed by Duncan's multiple range test at the 5% probability level. Statistical analysis SPSS software, version 22 (IBM Inc. New York, USA) was used and the results are expressed.

RESULTS AND DISCUSSION

Colony morphology and population

The internal tissues fresh ripe of soursop are mainly represented by basidiomycetes yeasts (*Rhodotorula mucilaginosa*, *Rhodotorula* sp.) and ascomycetes yeasts *i.e.* *Kloeckera* sp. (anamorph of *Hanseniaspora* sp.) and *Candida* sp. (Table 1). Among of the yeasts, *Rhodotorula* sp. was the most predominant genera isolated with count of 27×10^{11} CFU/ml (33.8%) followed by *Kloeckera* sp. 20×10^{11} CFU/ml (25.0%), *Candida* sp. 18×10^{11} CFU/ml (22.5%) and *R. mucilaginosa* 15×10^{11} CFU/ml (18.7%).

The number of basidiomycetes yeast is more than that of ascomycetes. High number of endophytic yeasts might caused by the ripeness of the fruit, low pH and the availability of starch, water, sugar and acidity being favorable to ploriferation of yeasts, the other reason fruit sample was obtained from the subsistence farmers under organic cultivation. Several studies reported that the abundance of endophytic yeasts was increased quantitatively through fruit maturation (Glushakova and Kachalkin, 2017). Previous studies revealed that the presence of endophytic yeasts was severely affected by agrochemical effects (Cammatti-Sartori *et al.*, 2005) and Wachowska *et al.*, 2018). *Rhodotorula* is a common endophytic yeast on fruit, others findings observed that *R. mucilaginosa* also colonize soil (Yadav *et al.*, 2014), fruits such as *Annona crassiflora* (Vale *et al.*, 2021), plum, apple, pea, kiwi and melon (Kachalkin *et al.*, 2022).

Phosphate solubilization

In this study all endophytic yeasts isolates tested showed solubilizing activity of phosphate signed by distinct halos (clear zone) surrounding their colonies (Fig 1).

Among of the yeasts *Candida* sp. showed the highest phosphate solubilization efficiency unit (7.71) and significantly different from *Kloeckera* sp. (6.36) *Rhodotorula* sp, (4.53), *R. mucilaginosa* (3.39) (Table 2).

The endophytic yeasts of the soursop fruit might originated from soil environment. Soil yeasts can enter plant tissues through mechanical micro-injuries of root epidermal cells. Previous study revealed that *Candida rugosa*, *Rhodotorula* sp. *R. mucilaginosa* and *Kloeckera* sp. isolated from rhizospheric soil and and healthy plant effective to solubilize phosphate (Narsian *et al.*, 2010).

In vitro effect organic acids of endophytic yeasts on mycelial growth of *A. flavus*

The presence of yellow pigment with no organic acid as control treatment was the most visible (Fig 2a), however, distinct yellow pigment still present on CAM medium on genera *Rhodotorula* (Fig 2b and 2c). The yellow pigment is absent on *A. flavus* treated by *Kloeckera* (Fig 2d), indicate that the organic acid suppress aflatoxin biosynthesis.

Table 3 shows the antifungal activities of organic acids produced by 4 yeast isolates tested on mycelial growth of aflatoxigenic *A. flavus*. Colony diameter at control treatment was 62 mm (100%), however, colony diameter contain organic acids by *Candida* sp. was the most inhibited (0.0%) followed by *R. mucilaginosa* 16 mm (25.8%) and *Kloeckera* sp. 21 mm (33.8%) and *Rhodotorula* sp. 25 mm (40.3%).

Generally, organic acids produced by all yeasts during sugar fermentation significantly inhibited mycelial growth

Table 1: Colony morphology and population of endophytic yeasts in soursop ripe fruit.

Yeast isolates	Shape	Elevation	Margin	Color	Colony diameter (mm)	Cell shape	Population (CFU/ml)
<i>Rhodotorula mucilaginosa</i>	circular	convex	entire	pink	2.52	ellipsoid	15×10 ¹¹
<i>Rhodotorula</i> sp.	circular	convex	entire	pink	2.37	ellipsoid	27×10 ¹¹
<i>Kloeckera</i> sp.	circular	convex	entire	White yellow	2.10	ellipsoid	20×10 ¹¹
<i>Candida</i> sp.	round	raised	entire	white	2.38	ovoid	18×10 ¹¹

Table 2: Phosphate solubilization by endophytic yeasts of soursop fruit on Pikovskaya's medium for 3 weeks at 29°C.

Yeast isolates	Colony diameter (cm)	Halo zone diameter (cm)	Phosphate solubilization efficiency unit
<i>Rhodotorula mucilaginosa</i>	1.13	2.71	3.39 ^c
<i>Rhodotorula</i> sp.	0.76	2.68	4.53 ^{bc}
<i>Kloeckera</i> sp.	0.45	2.41	6.36 ^{ab}
<i>Candida</i> sp.	0.38	2.55	7.71 ^a

Numbers followed by the same letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test (DMRT).

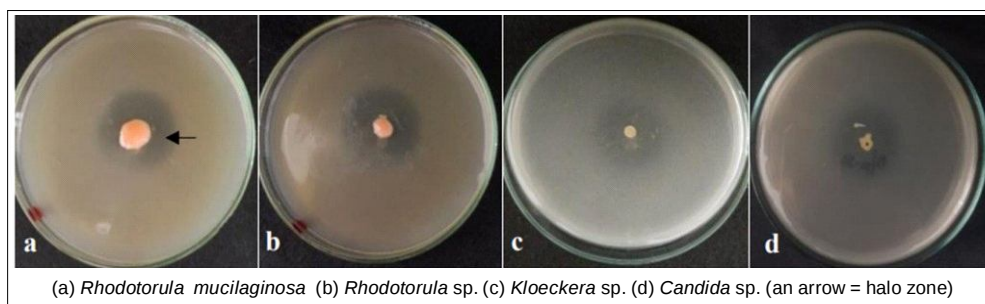


Fig 1: Colony and phosphate solubilization characteristics of endophytic yeasts of soursop fruit on Pikovskaya's medium for 3 weeks at 29°C.

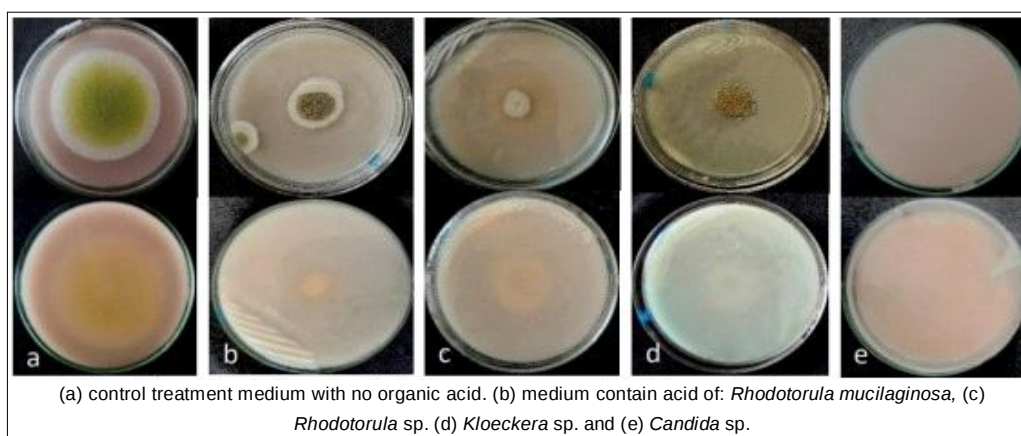


Fig 2: Colony of aflatoxigenic *A. flavus* and a reverse side of the petri dish (showing yellow pigment) on coconut agar medium containing organic acid of endophytic yeasts for 5 days (29°C).

Table 3: The percentage of total organic acid produced by endophytic yeast of soursop fruit on potato broth in inhibiting toxigenic *Aspergillus flavus* in coconut agar medium (CAM) for 5 days at 29°C.

Endophytic yeasts	Total organic acid (%)	pH	Colony diameter (mm)	Mycelial growth of <i>A. flavus</i> (%)	Yellow pigment
Control (no yeasts)	0	6.8	62	100 ^a	+
<i>Rhodotorula mucilaginosa</i>	0.00235	4.0	16	25.8 ^c	+
<i>Rhodotorula</i> sp.	0.00353	4.3	25	40.3 ^b	+
<i>Kloeckera</i> sp.	0.00266	4.2	21	33.8 ^b	-
<i>Candida</i> sp.	0.00354	4.2	0	0.0 ^d	-

Numbers followed by the same letters are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test (DMRT); +, yellow pigment present; -, no yellow pigment.

of *A. flavus* and the growth was the most inhibited by *Candida* sp. In contrast to control treatments, fungal inhibition by yeasts was closely related to pH. pH medium around 4.3 to 4.0 completely inhibit of the fungal growth. A previous study found that organic acids particularly acetic, oxalic, malic and citric acids significantly inhibit the growth of phytopathogenic fungi (Kang *et al.*, 2003). Whereas, Hassan *et al.* (2012) reported that formic, acetic and propionic acid had the antifungal agent against *A. flavus*. Antagonism effects of *Rhodotorula* and *Kloeckera* against filamentous fungi was similar to previously reported by Tryfinopoulou *et al.* (2020) that *Rhodotorula* genera have inhibition effect reduced mycelial growth rate, colony size and toxin production aflatoxigenic *Aspergillus carbonarius*. Organic acids produced by lactic acid bacteria in yogurt reduced aflatoxin M_1 in milk was reported by Seyedjafarri (2021). Whereas Zhu *et al.* (2022) and Zhang *et al.*, 2019) reported that *Kloeckera apiculata* reduced colony diameter and mycelial weight of *Penicillium expansum* and *Monilinia fruticola* respectively. The presence of yellow pigment to detect qualitative aflatoxin production of aflatoxigenic *A. flavus* has been previously described by Lin and Dianese (1976) and Davis *et al.* (1987). The absence of yellow pigment treated with yeast organic acids indicate no aflatoxin was observed by aflatoxigenic *A. flavus*. Therefore, endophytic yeast and their organic acids as secondary metabolites were potential as biological control capacity of *A. flavus*.

CONCLUSION

The obtained results from the study revealed that fresh ripe soursop fruit contain abundance of endophytic yeasts that provide dissolve phosphate, inhibit mycelial growth and showed capacity to reduce aflatoxin of aflatoxigenic *A. flavus*. Therefore, the yeast might be exploited as a biofertilizer and biocontrol agents.

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Conflict of interest

The authors declare no conflict of interest in the submission of this manuscript.

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