

## Selection of Yeasts Antagonists as Biocontrol Agent of Mango Fruit Rot caused by *Botryodiplodia theobromae*

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Fruit rot caused by *Botryodiplodia theobromae* is one of the most important post harvest disease of mango in Indonesia. Study on biological control on the disease is required to develop environmentally-sound control technology. The research objectives were to study the potency of yeasts in controlling post harvest mango disease i.e. fruit rot caused by *B. theobromae* and mechanism involve in the biocontrol. Total yeast isolates used for screening were twenty one, four from collection of Plant Clinic of Institut Pertanian Bogor, and twenty one isolated from healthy mango skin. All of yeast isolates were characterized and identified using BIOLOG. Bioassay on antagonistic activity of yeasts against fruit rot, in-vitro dual culture test and chitinolytic activity were carried out. *Cryptococcus albidus* var. *aerius* WSW1, *Pichia guilliermondii* K1, and *Debaryomyces hansenii* K12 were the three most effective antagonistic yeasts against *B. theobromae* with effectiveness of 70.83, 45.83, 37.50% respectively. In vitro bio-assay showed that *C. albidus* var. *aerius* WSW1, *C. albidus* WSW2, *C. albidus* K6, *C. terreus* YSW1, *Candida edax* OSW1, *Candida edax* K13, and *Cryptococcus luteolus* K2 had high antibiosis activity. Biocontrol activity of tested yeasts against fruit rot of mango did not correlate to its antibiosis and chitinolytic activity.

Key words: biocontrol, *Botryodiplodia theobromae*, *Cryptococcus albidus*, mango, yeast

Busuk buah yang disebabkan *Botryodiplodia theobromae* merupakan salah satu penyakit pasca panen mangga terpenting di Indonesia. Penelitian tentang pengendalian hayati penyakit tersebut diperlukan untuk mengembangkan teknologi pengendalian yang ramah lingkungan. Penelitian ini bertujuan mengkaji potensi khamir antagonis dalam pengendalian busuk buah mangga yang disebabkan *B. theobromae* dan beberapa mekanisme yang terlibat. Isolat khamir diperoleh dari koleksi Klinik Tanaman Institut Pertanian Bogor (IPB), yang diisolasi oleh salah seorang penulis dan diisolasi dari buah mangga sehat. Isolasi khamir dilakukan dengan pencucian kulit buah yang dilanjutkan dengan penanaman pada media PDA pH 5.5. Jumlah isolat khamir yang diseleksi semuanya ada 21, 4 dari koleksi Klinik Tanaman IPB dan 17 dari hasil isolasi penulis. Semua isolat khamir identifikasi dengan BIOLOG. Semua isolat khamir dikaji kemampuan antagonisnya terhadap busuk buah, uji koloni ganda in-vitro dan uji aktivitas kitinase. *Cryptococcus albidus* var. *aerius* WSW1, *Pichia guilliermondii* K1, dan *Debaryomyces hansenii* K12 adalah tiga isolat khamir antagonis paling efektif terhadap *B. theobromae* dengan tingkat penekanan secara berturut-turut 70.83, 45.83, dan 37.50%. Uji koloni ganda in-vitro menunjukkan bahwa *C. albidus* var. *aerius* WSW1, *C. albidus* WSW2, *C. albidus* K6, *C. terreus* YSW1, *Candida edax* OSW1, *Candida edax* K13, dan *C. luteolus* K2 mempunyai aktivitas antibiosis yang tinggi. Keefektifan pengendalian hayati khamir yang diuji terhadap busuk buah mangga tidak berkorelasi dengan aktivitas antibiosis dan kitinase khamir tersebut.

Kata kunci: *Cryptococcus albidus*, *Botryodiplodia theobromae*, khamir, mangga, pengendalian hayati

Mango is one of important tropical fruit commodity for many tropical countries for domestic trade and -also export. One major post harvest disease in Indonesia is fruit rot caused by *Botryodiplodia theobromae*, causing serious damage in storage and shipping. Infection rate of disease on mango fruit *cv.* Arumanis was average 54% (Yulianingsih *et al.* 1990). There are no effective control measures against this disease. Existing control measures is the application of fungicide after harvest. The fungicide use has low consumer's acceptance due to environment and health issues.

Biological control using yeasts is a promising alternative to control post harvest diseases of fruits and

vegetables. The advantages using yeast for biocontrol agent *i.e.* it grows fast, dry tolerance; do not produce mycotoxin and allergenic spores (Droby and Chalutz 1994). Previous research showed that yeasts can be applied to delay fruit ripening process and disease control as well (Janisiewicz and Korsten 2002). Delay of fruit ripening by yeast is through inhibition of ethylene production (Droby *et al.* 1997). Some research showed that some yeasts are effective biocontrol agent of post harvest diseases (Fan and Tian 2001; Janisiewicz and Korsten 2002) The use of *Pichia guilliermondii* was effective to control anthracnose of chili caused by *Colletotrichum gloeosporioides* in storage (Chanchaichaovivat *et al.* 2007). In addition, *Candida membranifaciens* was also effective biocontrol agents against anthracnose, a post harvest disease of mango (Kefalew and Ayalew 2008).

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Information on the use of yeast to control fruit rot of mango is not available. The objectives of this research were to investigate the potency of yeasts as biocontrol agent of fruit rot of mango caused by *B. theobromae* and to examine mechanism involved in the biocontrol.

## MATERIALS AND METHODS

**Yeast Used in This Study.** Twenty one yeasts isolates were used in this study. Four yeasts isolates (WSW1, WSW2, OSW1, and YSW1) were obtained from Plant Clinic of Institut Pertanian Bogor. Seventeen other yeast isolates were obtained from healthy mango fruit originated from Cirebon, West Java.

Yeast isolation from fresh fruit of mango was done by skin washing than serial dilution technique up to 10 000 and continued by plating onto potato dextrose agar (PDA) pH 5.5. The isolated yeasts then purified and identified physiologically up to species level by using BIOLOG TM (Micro Log TM System, release 4.2). The pathogen *B. theobromae* was isolated from infected mango fruit by tissue plating technique and cultivated on PDA pH 5.5 and identified morphologically by based on Watanabe (2002).

**Biocontrol Assay by *in-vivo* Test.** Selection of yeast isolates for biocontrol effectiveness was conducted according to modified technique of Kefialew and Ayalew (2008). All of yeast were cultured on potato dextrose broth (PDB) pH 5.5 and shaken at 100 rpm for 3 days. Mango *cv.* "Gedong Gincu" were obtained from farmer's field in Cirebon, West Java and kept under cool box and stored in refrigerator before handled. Mango fruits were disinfected by sodium hypochloride 1% for 1 min and rinsing by sterilized water and air dried. Thereafter mango fruit was dipped by yeast suspension at density of  $10^6$  cells  $\text{mL}^{-1}$  for 1 min, then air dried. Skin fruit was taken up by knife up to size of 8 cm x 3 cm, put and placed into moistened 30 cm x 20 cm x 4 cm plastic pans. Preliminary research shows that disease severity produced by artificial inoculation of *B. theobromae* on mango fruit skin highly correlated to whole fruit inoculation. Then conidia of 7-day old of *B. theobromae* grown on PDA at the rate of  $10^6$   $\text{mL}^{-1}$  as 50  $\mu\text{L}$  was pipetted on the yeast-treated mango skin, then mangos skin was incubated under dark for 24 h then incubated for 5 days. Disease severity was assessed by necrotic percentage of skin (Kefialew and Ayalew 2008). Biocontrol efficacy (BE) of yeasts was calculated using formulae of Chanchaichaovivat *et al.* (2007) as follow:

$$\text{BE} = (\text{dc} - \text{dt}) / \text{dc} \times 100\%;$$

where BE, biocontrol efficacy (%); dc, disease severity of control; dt, disease severity of treatment.

Fruits dipping with sterilized water and fungicide with active ingredients thiram (Tiflo 80 WP) at the rate of 0.1% (w/v) for 1min were applied for negative and positive control respectively.

The experiment was carried out using randomized complete designed with 4 replications.

**Dual Culture Assay.** Dual culture assay was performed to determine if biocontrol mechanism is antibiosis (Wisniewski *et al.* 2007). All of yeasts isolates were tested against fruit rot fungi *B. theobromae*. Yeast was streaked forming line in the middle of 9-mm petridish containing PDA. Agar plug  $\varnothing$  3 mm-7- day old *B. theobromae* was seeded on the right and left of yeast streak, therefore line connecting two fungal colony centers was perpendicular to the yeast streak. Inhibition zone was assessed after 3 days of incubation and expressed in cm. The size of inhibition zone indicated antibiosis of yeasts against tested fungus (Spadaro 2002; Indriatmi 2008). All of treatments was arranged in randomized complete design and three times replicated.

**Chitinolytic Activity.** All yeast isolates were grown on chitin agar, containing 0.5% colloidal chitin for 3 days (Shanmugaiah *et al.* 2008). Chitinolytic activity was indicated by formation of clear zone surrounding yeast colony.

## RESULTS

Identification using BIOLOG resulted that there was variability of yeast originated from fructoplane of mango and Plant Clinic of IPB collection (from shallot's leaves). Four yeast isolates from plant clinic collection were identified as three species *i.e.* *Cryptococcus albidus* var. *aerius* WSW1, *C. albidus* var. *aerius* WSW2, *Candida edax* OSW 1. Only one isolate of plant clinic was a different species as from mango fructoplane *i.e.* *C. terreus* YSW1. Moreover, seventeen yeast isolates from mango consisted of 11 species *i.e.* *Candida edax*, *Candida mexicana*, *Candida terestre*, *Bulleromyces albus*, *C. albidus* var. *aerius*, *Cryptococcus amyloolentus*, *Cryptococcus luteolus*, *Debaryomyces hansenii*, *Pichia guilliermondii*, *Rhodotorula aurentica*, and *R. glutinis*.

Bioassay on biocontrol effectiveness showed that some yeasts isolates had ability to suppress fruit rot caused by *B. theobromae* (Table 2). Three isolates had disease biocontrol effectiveness rate over 35% which were *C. albidus* var. *aerius* WSW1, *P. guilliermondii* K1, *D. hansenii* K 12 with biocontrol effectiveness as 70.83, 45.83 and 37.5%, respectively. Such effectiveness rate was comparable, even higher than standard fungicide Tiflo 80 WP with active ingredient thiram that had 35% (Table 2, Fig 1). Other important

Table 1 Identification of yeasts isolates using BIOLOG TM

Isolates	Origins	Identified species	Probability (%)
WSW1	Plant Clinic (Shallot, Brebes)	<i>Cryptococcus albidus</i> var. <i>aerius</i>	100
WSW2	Plant Clinic (Shallot, Brebes)	<i>Cryptococcus albidus</i> var. <i>aerius</i>	100
OSW1	Plant Clinic (Shallot, Brebes)	<i>Candida edax</i>	100
YSW1	Plant Clinic (Shallot, Brebes)	<i>Cryptococcus terreus</i>	84
K1	Mango, Cirebon	<i>Pichia guilliermondii</i>	94
K2	Mango, Cirebon	<i>Cryptococcus luteolus</i>	91
K3	Mango, Cirebon	<i>Debaryomyces hansenii</i>	99
K4	Mango, Cirebon	<i>Candida terestre</i>	54.7
K5	Mango, Cirebon	<i>Candida edax</i>	94
K6	Mango, Cirebon	<i>Cryptococcus albidus</i> var. <i>aerius</i>	99
K7	Mango, Cirebon	<i>Bulleromyces albus</i>	99
K8	Mango, Cirebon	<i>Rhodotorula glutinis</i>	100
K9	Mango, Cirebon	<i>Pichia guilliermondii</i>	99
K10	Mango, Cirebon	<i>Cryptococcus albidus</i> var. <i>aerius</i>	99
K11	Mango, Cirebon	<i>Rhodotorula aurantica</i>	99
K12	Mango, Cirebon	<i>Debaryomyces hansenii</i>	98
K13	Mango, Cirebon	<i>Candida edax</i>	99
K14	Mango, Cirebon	<i>Pichia guilliermondii</i>	99
K15	Mango, Cirebon	<i>Candida mexicana</i>	99
K16	Mango, Cirebon	<i>Cryptococcus amyloolentus</i>	84
K17	Mango, Cirebon	<i>Debaromyces hansenii</i>	99

Table 2 Biocontrol effectiveness of yeasts against fruit rots of mango

Isolate Codes	Yeast species	Disease severity (%)	Biocontrol efficacy (%)
WSW1	<i>Cryptococcus albidus</i> var. <i>aerius</i>	29.17 ± 5.13 g	70.83
WSW2	<i>Cryptococcus albidus</i> var. <i>aerius</i>	87.50 ± 4.54 b	12.50
OSW1	<i>Candida edax</i>	91.67 ± 4.37b	8.33
YSW1	<i>Cryptococcus terreus</i>	75.00 ± 5.81 cd	25.00
K1	<i>Pichia guilliermondii</i>	54.17 ± 4.69 f	45.83
K2	<i>Cryptococcus luteolus</i>	91.67 ± 6.53 b	8.33
K3	<i>Debaryomyces hansenii</i>	91.67 ± 6.53 b	8.33
K4	<i>Candida terestre</i>	95.83 ± 4.46 ab	4.17
K5	<i>Candida edax</i>	100.00 ± 0.00 a	0.00
K6	<i>Cryptococcus albidus</i> var. <i>aerius</i>	95.83 ± 4.46 ab	4.17
K7	<i>Bulleromyces albus</i>	95.83 ± 4.46 ab	4.17
K8	<i>Rhodotorula glutinis</i>	70.83 ± 6.72 d	29.17
K9	<i>Pichia guilliermondii</i>	75.00 ± 4.85 cd	25.00
K10	<i>Cryptococcus albidus</i> var. <i>aerius</i>	79.17 ± 4.48 c	20.83
K11	<i>Rhodotorula aurentica</i>	75.00 ± 4.35 cd	25.00
K12	<i>Debaryomyces hansenii</i>	62.51 ± 6.53 e	37.50
K13	<i>Candida edax</i>	70.83 ± 5.72 d	29.17
K14	<i>Pichia guilliermondii</i>	91.67 ± 6.53 b	6.33
K15	<i>Candida mexicana</i>	87.50 ± 7.44 b	12.5
K16	<i>Cryptococcus amyloolentus</i>	70.83 ± 6.53 d	29.17
K17	<i>Debaryomyces hansenii</i>	100.00 ± 0.00 a	0.00
	Untreated (water)	100.00 ± 0.00 a	-
	Fungicide <i>a.i.</i> Thiram	65.00 ± 7.11 e	35.00

Note: numbers followed by same symbols are not significantly different according DMRT test at  $\alpha=0.05$

finding was even though some isolates belong to the same species they had very different antagonistic activities. For instance, species *C. albidus* var. *aerius* only isolates WSW1 had high antagonistic activity (70.83%), other isolates *i.e.* WSW2, K6, and K 10 which had no antagonistic activity.

The yeasts had variability in antibiosis indicating by the size of inhibition zone (Table 3). Some yeasts had strong antibiosis *in-vitro* against *B. theobromae* such as *C. albidus* var. *aerius*. *In-vitro*-dual culture test showed that *C. albidus* var. *aerius* WSW1, *C. albidus*

WSW2, *Ca. edax* OSW1, *C. terreus* YSW1, *C. luteolus* K2, *Ca. edax* K13, *C. albidus* K6 had high antibiosis activity. Furthermore, other yeast isolates had lower antibiosis activity. Interestingly, there was no correlation between antibiosis activity *in vitro* and biocontrol efficacy. Among yeasts having high efficacy rate against fruit rot disease, only *C. albidus* var. *aerius* WSW1 was effective yeasts and had high antibiosis activity. Other effective yeasts *P. guilliermondii* K1 and *D. hansenii* K7 had low antibiosis activity (Table 3).

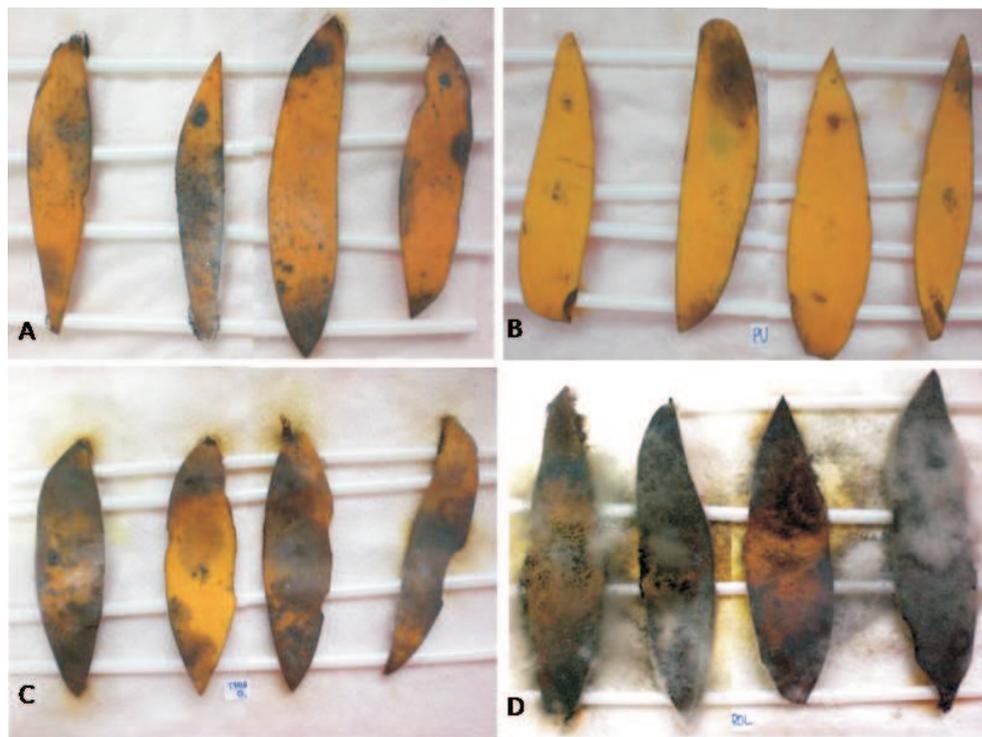


Fig 1 Effectiveness of antagonistic yeasts against fruit rot disease of mango caused by *Botryodiplodia theobromae* in bioassay using mango skin. Note: A. *Pichia guilliermondii* K1, B. *Cryptococcus albidus* var. *aerius* WSW1, C. Fungicide (thiram), D. Untreated

Table 3 Antibiosis of yeasts against *Botryodiplodia theobromae* and their chitinolytic activity

Code	Species	Inhibition zone (cm)	Chitinolytic activity
WSW1	<i>Cryptococcus albidus</i> var. <i>aerius</i>	2.10 ± 0.23a	-
WSW2	<i>Cryptococcus albidus</i> var. <i>aerius</i>	1.97 ± 0.16ab	-
OSW1	<i>Candida edax</i>	1.80 ± 0.12bc	-
YSW1	<i>Cryptococcus terreus</i>	1.83 ± 0.12bc	-
K1	<i>Pichia guilliermondii</i>	0.37 ± 0.11ij	-
K2	<i>Cryptococcus luteolus</i>	1.67 ± 0.12 c	-
K3	<i>Debaryomyces hansenii</i>	0.50 ± 0.21i	-
K4	<i>Candida terestre</i>	1.20 ± 0.26 de	-
K5	<i>Candida edax</i>	0.70 ± 0.33 j	-
K6	<i>Cryptococcus albidus</i> var. <i>aerius</i>	1.47 ± 0.18 d	-
K7	<i>Bulleromyces albus</i>	0.50 ± 0.18 i	+
K8	<i>Rhodotorula glutinis</i>	0.47 ± 0.19i	-
K9	<i>Pichia guilliermondii</i>	1.00 ± 0.10 fg	-
K10	<i>Cryptococcus albidus</i> var. <i>aerius</i>	1.17 ± 0.15 ef	-
K11	<i>Rhodotorula aurentica</i>	0.50 ± 0.20 i	-
K12	<i>Debaryomyces hansenii</i>	0.40 ± 0.22 i	-
K13	<i>Candida edax</i>	1.43 ± 0.21d	-
K14	<i>Pichia guilliermondii</i>	1.03 ± 0.89 fg	+
K15	<i>Candida mexicana</i>	1.07 ± 0.25f	-
K16	<i>Cryptococcus amyloolentus</i>	0.97 ± 0.14 fg	-
K17	<i>Debaryomyces hansenii</i>	0.57 ± 0.14 i	-

Note: + forming clear zone, numbers followed by same symbols are not significantly different according DMRT test at  $\alpha=0.05$

Among yeasts tested, only *Bulleromyces albus* K7 and *P. guilliermondii* K 14 had chitinolytic activity (Table 3). Those two yeasts had low biocontrol effectiveness.

## DISCUSSION

Fructoplane and phylloplane are rich source of microbes, in which yeast is dominant group followed

by bacteria and filamentous fungi (Elmer and Reglinski 2002). The number of isolated yeasts from fructoplane of mango in this study was 11 species *i.e.* *Ca. edax*, *Ca. mexicana*, *Ca. terestre*, *B. albus*, *C. albidus* var. *aerius*, *C. amyloolentus*, *C. luteolus*, *D. hansenii*, *P. guilliermondii*, *R. aurentica*, and *R. glutinis*. Some of isolated yeasts were potential as biocontrol agent *i.e.* *C. albidus* var. *aerius* WSW1, *P.*

*guilliermondii* K1, and *D. hansenii* K12 indicated by having biocontrol efficacy rate over 35%. Yeasts belong to the genus *Pichia*, such as *P. anomala*, *P. membranifaciens*, and *P. guilliermondii* have been reported as effective biocontrol agents against various post harvest diseases (Elmer and Reglinski 2001; Fan and Shiping 2002; Chancaichivat *et al.* 2008). Moreover *Debaryomyces* sp. is also reported by lesser extent as biocontrol agent of post harvest diseases (McLaughlin *et al.* 1990).

The most effective yeast antagonist obtained in the study is *C. albidus* var. *aerius* WSW1. Even though there are not many report on biocontrol using *Cryptococcus*, some species of *Cryptococcus* were reported as biocontrol agent, e.g., *C. infirmo-miniatus* and *C. laurentii* against pear rot disease (Benbow and Sugar 1999), *C. laurentii* against blue mold of peach (Zhang *et al.* 2007), and *C. albidus* against blue and grey mold of apple (Fan and Tian 2010). It is interesting that one yeast isolate, *C. albidus* var. *aerius* WSW1 was very effective against fruit rot of mango (70.83% of biocontrol effectiveness) (Fig 1, Table 2), it was even higher than fungicide Tiflo 80 WP with active ingredients thiram that provide 35%. As comparison, research of Kefialew and Ayalew (2008) showed that the use *Candida membranifaciens* as biocontrol yeasts on mango anthracnose provided efficacy rate of 82%, *Ca. membranifaciens* on grey mold 61 % - 81 % (Gholamnejad *et al.* 2010). This effectiveness of *C. albidus* var. *aerius* WSW1 used in the study was very high and promising, because it was achieved under extreme disease conducive environment, in which diseases pressure is very high (conidia density  $10^6 \text{ mL}^{-1}$ ), and incubated in moistened (100 % relative humidity) and darkened environment. In standard storage condition, the effectiveness is expected to be higher. Further test on combination of the yeast and real storage condition is necessary Other technique to increase efficacy are combining with other species, and or application and formulation optimization (Janisiewicz 1996).

Based on data obtained in this experiment, even isolates WSW1, WSW2, K6, and K10 referred to species *C. albidus* var. *aerius*, they were very different in their antagonistic activity, only WSW1 had effective biocontrol activity. This shows that biocontrol effectiveness of tested yeasts is isolate-base and not species-base. Even though yeast isolates belong to same species, may have different antagonistic activity, therefore biocontrol activity test is necessary for each isolates.

Mechanism of biocontrol is necessary to be recognized, therefore a biocontrol agent can further

developed and optimized. There are various mechanism involve in biocontrol using yeasts - competition, antibiosis, lysis and resistance induction (Janisiewicz and Korsten 2001; De Ingeniis 2004; Wisniewski *et al.* 2007). Weak correlation of antibiosis activity *in-vitro* and biocontrol effectiveness such as depicted in Table 2 and Table 3, show that antibiosis is not main mechanism underlying biocontrol of yeasts. In this research, antibiosis is valid only for biocontrol mechanism of *C. albidus* var. *aerius*.

Even though other previous researcher reported that chitinolytic and other lytic enzyme activity involve in mechanism of biocontrol using yeast (Spadaro 2002; Masih and Paul 2002), this study showed no relation between chitinolytic activity and biocontrol effectiveness of yeasts. Nutrients competition and induced resistance which involve in biological control using yeast (Janisiewicz and Korsten 2001; El-Tarabily 2004; Yao and Tian 2005) were not investigated in this study. Further study to examine the role of induced resistance and competition of the three antagonistic yeasts is required.

The study yielded three potential antagonistic yeasts effective against fruit rot of mango. Some of mechanism of antagonism has also been investigated. Thus one of important initial step in developing biocontrol agent has been carried out. Further study is needed to develop them as biocontrol agents: other mechanism involved, efficacy with real storage condition, environment and nutritional affecting factors, mass production and formulation technology.

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