Evaulation of effective antagonistic yeasts in Kalasin province, a biocontrol agent against the fungal *Colletotrichum capsici* caused anthracnose diseases in chili fruits

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ABSTRACT

Primary screening of epiphytic and endophytic yeasts, the majority yeast was found from rhizosphere soil (21 isolates, 31.81%), and 5 antagonistic yeasts can have inhibited the growth of mycelia of *C. capsici* up to 60%. Efficacy of five antagonistic yeasts were screened under plant nurseries conditions. The result found all isolates were tested and compared with control and chemical plots. The result of plant height showed that, yeast isolate KS34 had highest of plant height at 45.80 cm. For the result of branch per plant of chili plant found isolate KS37 showed highest of branch per plant at 16.60 branches and the flower of chili per plant presented that isolate KS37 had highest of flower at 18.60 flowers. The result of anthracnose disease control by antagonistic yeasts in disease incidence and category mean as plant resistance. These results found that, Isolates KS46 had a great disease incidence at 7.80% that mean this isolate prove chili plant to resistance to *C. capsisci*. Addition, isolate KS46 had highest of activity of chitinase enzyme at 170.72 U/ml. Isolate KS46 identified by D1/D2 domain of 26S ribosomal DNA sequencing. The sequence analysis showed maximum identity of 100% with *Candida haemulonii*. This assumed *C. haemulonii* was showed the best to control *C. capsici*. For confirmed this result, all isolates were determined the mode of action by studied in relationship between antagonist isolates. The action of yeast isolate could competition the nutrient with mycelia of pathogen. This mode effect to *C. capsici* loss nutrient caused the pathogen get stuck to braked and died later.

Keywords: Antagonistic yeasts, Biocontrol, Chili fruits, Anthracnose disease, *Colletotrichum capsici*, *Candida haemulonii*

1. INTRODUCTION

Chili (*Capsicum* spp.) is considered an important tropical and subtropical crop on the basis of its high consumption, nutritional value, and economic value to farmers [1]. Thailand is the largest producer and exporter of chili in the international market and exports to United State of America, Australia, England, Philippines, Japan, Singapore, Taiwan and the Netherlands, amongst others. In the Northeast of Thailand chili production is distributed and exported to the market. One of the most common diseases in chili plants and a lot of other tropical vegetables is anthracnose and it is caused by *Colletotrichum acutatum,* Simmonds ex Simmonds, *C. capsici* (Syd) Butl & Bisby, *C. coccodes* (wallr.) Hughes and *C. gloeosporioides* Penz & Sacc., which is

widespread during the rainy season [2]. Anthracnose disease can damage to mature fruits in the field as well as during transit and storage. Typically, symptoms first appear on mature fruits as small, water soaked, sunken lesions that rapidly expand. The lesion can increase to 2-3 cm in diameter on large fruits. The appressoria from germination conidia then penetrate into the chili epidermal cells. Mycelial growth inside host tissues produces a thermostable toxin that causes injury, particularly to the protoplasmic content of the host tissues [3]. Control of the disease on chili fruits still relies mainly on the use of synthetic fungicides such as Carboxin, Synap, Benomyl and Carbendaxin, etc. This may result in fungicide resistant pathogens such as those that occur in copper fungicides. Biocontrol yeast can prevent mold spoilage during the postharvest storage of fruits and vegetables. Yeasts have several important properties that make them useful for biocontrol purposes, most are non-pathogens and do not produce mycotoxins or allergenic spores, most can utilize a broad range of nutrients, and many can grow at low water activity and oxygen levels. The yeast can inhibit the growth of pathogen on fruits and stimulate plant resistance the pathogen [4]. The mechanisms included parasitism, competition for space water or food by using secondary metabolite that harm the target pathogen. To support an advantage of yeast to control anthracnose disease can have described with previously research, Veeranee *et al*. (2016) [5] reported the mechanisms of metabolites from yeast *Aureobasidium pullulans* TISTR 3389 for controlling anthracnose diseases of banana cv. Hom Thong, caused by *Colletotrichum musae* (BerK&Curtis). The metabolite from yeast *A. pullulans* TISTR 3389 can reduce the diseases severity by up to 81.2%. These metabolites also inhibit the mycelia growth and spore germination of pathogens at the EC_{50} value of 190.4 and 149.0 mg/L, respectively. Suangsan (2006) [6] reported eleven yeast isolates were evaluated for their antagonistic properties in controlling green mold rot, caused by *P. digitatum*. *Candida utilis, C. tropicalis*, *Debaryomyces hansenii* and *Pichia* sp., were promising antagonistic yeasts. They inhibited spore germination and germ tube elongation without any antibiotic production. This research is related to Druvefors et al. (2005) [2] found that the *Pichia* species, especially *P. anomala*, is the best yeast among 60 different yeast species that were tested with regard to the inhibition of *Penicillium* sp. grown in the test tube version of airtight grain silos. The mechanism by which biocontrol agents target pathogens is poorly carried out as it extremely difficult to construct experiments that can exclude all other possible mechanisms in the complex biocontrol environment. Several possible yeast biocontrol mechanisms have been suggested, such as enzymes secretion, nutrient competition and killer toxin effect with pathogens [3]. Competition for nutrients has been suggested as the mode of action of several biocontrol organisms, *e.g*. *Pichia guilliermondii* against *Penicillium digitatum*, *Candida guilliermondii*, *Cryptococcus laurentii* and *Metschnikowia pulcherima* against *Botrytis cinerea* and *Penicillium expansum* [7]; [8]. Several enzymes can have used as biocontrol agents; such as β-1, 3-glucanase and chitinase, especially, chitinase can degraded chitin that the major composition in pathogen fungal cell wall. These advantages, both enzymes could apply to control the pathogen in several plants. Grevess et al. (2003) [9] reported the exo-β-1, 3-glucanase from yeast *Pichia anomala* could destroyed mycelia of pathogen *Botrytis cinerea* on apple fruit and inhibited spore germination. In addition, yeast *Aureobasidium pullulans* can applied to control pathogen in post-harvest time and promoted to yeast cell adhere on apple fruit. For chitinase enzyme can used in direct by viable cells and indirect way by purification protein and stimulate gene that regulated the expression of protein enzyme. Bar-Shimon et al. (2004) [10] presented β-1, 3-glucanase and chitinase enzymes secretion from *Candida oleophila* show high amount of both enzymes when cultivated in cell wall preparation substrate from *Penicillium guilliermondii*. This research related with Chan and Tian (2005) [11] demonstrated both enzymes showed high activities in Lilly-Barnett media enriched with CWP and role to promote antagonistic yeast adhere to mycelia of pathogen. This result as an important role to control the pathogen in plant [12]. So, we were objects to screening, selection, the mechanism of hydrolytic enzyme from antagonistic yeast, identification of antagonistic yeast and postharvest disease control on chili fruit tested.

2. material and methods

- **Collection for yeast antagonists**

Yeast antagonists were collected during from chili plantation, Kalasin Province, Northeast of Thailand. Fruits, leaves and soils were sampled from a chili plantation. The symptoms of the chili diseases were collected by random sampling. Fungal extraction took place within 2 days. Samples of (i) fruits and leaves that had not been treated with any fungal were used for the control group (ii) fruits and leaves that displayed anthracnose disease were collected and (iii) soil was collected from around the base of the chili plant to a depth of between 0-15 cm. Yeasts isolates were kept at 4°C for further study. The pathogen *C. capsici*, was sourced from chili field in Kalasin province and proved the disease again follow Koch's postulation method.

2.2 Screening for effective yeast to control C. capsici

Epiphytic and endophytic yeasts were isolated from the surface of fruit, leaves and soil of chili plants. Ten grams of sample were suspended in 100 ml sterile distilled water and shaken vigorously for a few minutes. Serial dilutions for yeast sample suspension were made in sterile distilled water. A suspension of 0.1 ml of each dilution was placed on yeast malt extract agar (YM agar) and the culture plates were incubated at 30 C for 48 h. Yeast colonies were examined under the microscope and colonies from fruits, leaves and soil were chosen on the basis of their different visual characteristics. The yeast isolates were re-streaked on YM agar to obtain pure cultures and they were maintained on nutrient yeast dextrose agar. The cultures were stored at 4 C until further study on antagonistic activity. The colonies were counted by colony counter.

2.3 Screening for yeast antagonists by dual culture method

Screening for yeast antagonist was conducted using the dual culture method. Pathogen *C. capsici* was cultured on PDA agar media then cut by cork border number 3 (diameter = 5 mm). This pathogen was then put into the center of the PDA plate. The plate was incubated at 28 C for 2 days. Next, single yeast colony was streaked 2 cm inside the pathogen. Sample was incubated at 28 C for 5 days. The radius of the pathogen mycelium was measured and compared with the control. Finally, each isolate was done in four replicates. The percentage of inhibition was measured from the radius of the effective yeast inhibit mycelia pathogen and compared with control. Yeast isolates that completely inhibited fungal mycelia growth were used for further studies.

2.4 Postharvest disease control by antagonistic yeasts in vivo test

To confirm the presence of yeast isolates for biocontrol efficacy was carried out using *in vivo* tests. Chili fruits (no wound or scar on the surface) from untreated orchards were selected for the experiments. They were surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air-drying, the chili fruits were treated with 70% ethanol. Each fruit was wounded using a sterile cork-borer (5 mm in diameter and 1 mm in depth), one wound per fruit. Yeast isolates with high biocontrol efficacies, selected based on data from the *in vitro* tests, were cultured in a yeast dextrose broth (NYDB). Yeast cells were collected by centrifugation at 3,000 rpm for 20 min, washed twice with sterile distilled water and re suspended in sterile distilled water. Then 20 μl of cell suspension of each isolate at a concentration of $5x10^8$ cells/ml was added to a wound on chili fruits. After air drying, 20 μl of *C. capsici*, $5x10^4$ cells/ml was added to the wound. The chili fruits were put on a plastic tray and stored at 28 ^{O}C. Disease severities, as indicated by increased wound diameter, and counted after 5 days of inoculation. The ability to reduce disease incidence of each yeast isolate was observed and compared. For control, wounded chili fruits were inoculated with *C. capsici* only. There were three replicates of 30 wounded chili fruits per treatment. The whole experiment was conducted three times. The most effective yeast strain was selected for further studies [3].

2.5 Anthracnose disease control by antagonistic yeasts in plant nurseries studies

Efficacy of antagonistic yeasts were screened under plant nurseries conditions by randomized block design with three replications. Healthy chili plants were used 60 days of chili plant age with 20 plant per plots. Antagonistic yeast was cultured in YMB at 25 °C, 48h. Cells and culture filtrate were collected by centrifugation at 12,000 rpm for 20 min. One group of chili plant plot samples were thoroughly spray with 300 ml of cell suspension in distilled water ($5x10^8$ cells/ml). The mycelium of *C. capsici* pathogen was grown in nutrient and collected the spore ($5x10^5$ conidia/ml) and mixed with 300 ml of sterile distilled water. These microbes were spray in to chili plant by using hand sprayer in plant field. Un-inoculate plots served as control. All plants were watered with two times per day. When 60 days of plant age sprayed with spore of *C. capsici* and then covered with black plastic for 24 h. Next day, chili plants were spray with cell suspension of antagonistic yeasts. All plots were replicated spray every 15 days until 60 days and collected the results when 120 days of chili plant age. Compared treatment plot with watered plot, biocontrol agent plots (*Bacillus* PK) and chemical agent plot. Finally, the percentage of incidences of anthracnose, plant height (cm), branch/plant, fruits/plant, fruit length (cm) and fruit weight (g) in each plot were calculated. Data analysis was done by using one-Way ANOVA and the least significant difference (LSD) test at $P<0.05$ was made for mean comparison. The plants were rated as resistance and susceptible based on the range of lesion area (mm) or the disease incidence (%) (Susheela, 2012) [13] given as:

Range (%)	Category Immune
0.1-5.0	highly resistance
5.1-10.0	resistant
10.1-50.0	tolerant
50.1-90.0	susceptible
>90.0	highly susceptible

2.6 Mode of action of antagonistic yeast to control C. capsici on chili fruits

The relationship between antagonistic yeast and the pathogen on the surface of chili fruits. Chili fruit was wounded using the cork borer (diameter as 5 mm). Each wounded was inoculated with 20 l of *C. capsici* ($5x10^4$ cells/ml) for 36 h before applying 20 l of cell suspension ($5x10^8$ cells/ml). Fruit was incubated at 28 ^OC on enclosed plastic tray for 24 h. The interaction of yeast and pathogen was observed under the light microscope. The experiment was performed in triplicate with 30 fruits each treatment.

2.7 Determination of hydrolytic enzymes; chitinase activities

Antagonistic yeasts were cultured in YMB medium by adding 0.1 g/l of yeast extract and 0.5 g/l of colloidal chitin as a carbon source. The culture flasks were incubated on a rotary shaker (200 rpm) at 28°C for 120 h. The culture was centrifuged for 10 min at 3000 *xg* and collected culture filtrate collected and tested for enzyme activity. Chitinase activity was tested according to the method of Zheng et al. (2008) [14] using colloidal chitin azure as a substrate. Using 1 ml of 1 mg/ml colloidal chitin azure in 0.1 mM sodium acetate buffer (pH=5), was incubated with 1 ml of yeast culture filtrate for 1 h at 37°C. The reaction was stopped with 50 µl of 1M HCl, left to cool on ice for 10 min and centrifuged at 15000 *xg* for 5 min. The supernatant was measured for optical density at 550 nm with spectrophotometer. So, the chitinase activity was measured for reducing sugar content using 3, 5-dinitrosalicylic acid (DNS) method. The unit of enzyme activity was determined from a standard curve of chitinase from *Streptomyces griseus* (Sigma C-6137). The chitinase activity was defined in a unit of enzyme per mg protein. Protein content was measured according to Bradford's method (1976) [15] using bovine serum albumin as a standard.

2.8 Yeasts identification

The nucleotide sequences of D1/D2 domain of 26S rDNA were directly determined using PCR products according to Kurtzman and Robnett (1998) [16] method. For direct sequencing was carried out by National Center of Genetic Engineering and Biotechnology, Bangkok,

Thailand. The sequence of D1/D2 domain of 26S rDNA was compared by BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast) and the phylogenetic tree was constructed by Neighbor-joining.

2.9 Statistical analysis

Data analyses for activity of chitinase enzymes, disease incidence, plant height (cm), branch/plant, fruits/plant, fruit length (cm) and fruit weight (g) were completed using comparative means with One-Way ANOVA of SPSS program. Normally distributed data were compared using a one-way analysis of variance (ANOVA). Differences between the treatments were analyzed by least significant difference (LSD). Treatment effects were revealed and Duncan multiple range test was employed significant at $P<0.05$.

3. results and discussion

3.1 Collection and screening for yeast antagonists

Yeast antagonists were collected from rhizosphere soil, normal leaves, disease leaves, normal fruits and disease fruits. All samples were kept and 3 replicated from 5 chili plantations at Buangwichai village, Buangwichai district, Muang, Kalasin province. These samples were shown on figure 1. Primary screening of epiphytic and endophytic yeasts, 66 isolates were isolated from the surface of fruit, leaves and soil of chili plants. The majority isolated yeast was found from rhizosphere soil (21 isolates, 31.81%), because this area had a complex environment and high microbial diversity [3]. Addition results followed by disease fruits (19 isolates, 28.78%), normal leaves (11 isolates, 16.66%), normal fruit (9 isolates, 13.63%) and disease leaves (6 isolates, 9.12%). The reason support, because yeast can growth rapidly and tolerant to drought [17]; [18] .The total of screening show as in Fig 2.

Figure 1. The antagonistic microbes separated from chill (a) chili plantation (b) normal leaves (c) disease leaves (d) rhizosphere soil (e) fruits (f) disease fruits

Figure 2. Percentage of total yeasts isolated from chili plantation.

The best 66 isolates of yeast were tested for against the pathogen *C. capsici* on PDA agar plate. The percentage of inhibition was measured from the radius of the effective yeast inhibit mycelia pathogen and compared with control (Fig 3). The results found 13 isolates were able to inhibit mycelia of *C. capsici* that showed in Table 1. All antagonistic yeasts can have inhibited the growth of mycelia the pathogen up to 60%. These could separate from rhizosphere soil (7 isolates), normal fruits (4 isolates) and normal leaves (2 isolates). The percentage of inhibition from Table 1, found isolate KS20 had highest of inhibition as 67.74%, followed by isolate KS18 (67.06%) and KS21 (65.78%) respectively. Several researches reported many yeast species that separated from chili plant could inhibited the mycelia of pathogen such as Chanchaichaovivat et al. (2007) presented 4 yeast species as *Pichia guillermondii* R13, *Candida musae* R6, *Issatchenkia orientalis* ER1 and *Candida quercitrusa* L2 to tested with pathogen *Colletotrichum gloeosporioides*, caused anthracnose disease. The result found, yeast strain *P. guillermondii* R13 had highest of inhibition (85.84%). Dual culture method is a standard basic method for screening, because this convenient and accurate result [18]. All 13 isolates were shown significant difference ($P<0.05$).

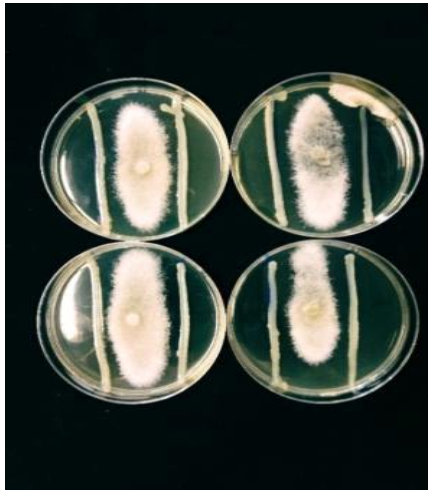

Figure 3. Yeast isolate inhibited mycelia of *C. capsici* by dual culture method

Table 1. Percentage inhibition of yeasts isolates inhibited mycelia of *C. capsici* by dual culture method.

No	Isolate	Inhibition (%)
1	KS20	67.74g
2	KS18	67.06g
3	KS21	65.78f
4	KS34	65.23f
5	KS47	64.83ef
6	KS44	64.04de
7	KS19	63.20cd
8	KS29	62.53bc
9	KS02	61.96b
10	KS46	61.73b
11	KS37	61.46ab
12	KS03	60.60a
13	KS42	60.58a
P-value		0.00

The value of a, b in percentage of inhibition column by a different letter indicate significant differences ($P<0.05$) according to Duncan test.

- **Postharvest disease control by antagonistic yeasts in vivo test**

13 antagonistic yeasts were used for confirm the efficacy of them *in vivo* tests. The disease severities, as indicated by increased wound diameter, and counted after 5 days of inoculation. The ability to reduce disease incidence of each yeast isolate was observed and compared (Fig. 4). The ability to reduce disease incidence of each yeast isolate at 5 days showed as in Fig. 4 and Table 2. The result found 9 isolates can reduce disease incidence or disease inhibition, while 4 isolates cannot inhibit when compared with control. Isolate 18 and isolate 37 showed greatest to inhibit *C. capsici* on chili fruits at 28.79 percentage of inhibition (Table 2). From this incident result, we selected 5 isolates that present the inhibition more than 20% as KS18, KS37, KS42, KS34 and KS46 for control anthracnose disease caused by *C. capsici* in chili plant nurseries. Antagonistic yeast isolate KS37 and KS18 were had high of inhibition at 28.79% at 10 days. This result related with the report of Chanchaichaovivat et al. (2007) [19], they used yeast *P. guillermondii* R13 to control pathogen *C. gloeosporioides* on chili fruit and used yeast strain *P. guillermondii* R13, *C. musae* R6, *Issatchenkia orientalis* ER1 and *C. quercitrusa* L2 at $5x10^8$ cell/ml sprayed on chili fruit. This found all yeast strains can have showed great inhibition at 66.4-93.3%. However, concentration of yeast cell is the main factor to inhibited mycelia of pathogen [20]. Mechanism of yeast to control *C. capsici* under the light microscope, found yeast cell can growth rapidly and attached to mycelia of pathogen. In addition, this mechanism can describe yeast cell can have competed nutrient and area that

caused *C. capsici* slow dead. In general, yeast can grow rapid by budding more than fungi in the same condition [19]. Competition process is the main mechanism of antagonistic yeast to control pathogen [21]; [20]. Saravanakumar et al. (2008) [22] showed that yeast *M. pulcherrima* control pathogen *B. cinerea* on apple fruit by competed mineral in nutrient and similar study with Nantawanit et al. (2010) [23] used yeast strain *P. guillermondii* R13 to control *C. capsici* on chili fruit. This study found the diameter of lesion by several mechanisms such as nutrient competition, secreted enzyme to digest mycelia, especially chitinase and β-1,3-glucanase enzyme [24].

Figure 4. Effective of yeast antagonists to control *C. capsici* on chili fruit at 5 days.

Table 2. Percentage of disease inhibition by antagonistic yeasts on chili fruits at 5 days.

No	Isolate	Disease Inhibition (%)
1	control	-
2	KS20	5.00
3	KS18	28.79
4	KS21	8.00
5	KS34	21.00
6	KS47	-
7	KS44	-

8	KS19	-
9	KS29	-
10	KS02	9.00
11	KS46	20.00
12	KS37	28.79
13	KS03	15.00
14	KS42	24.00

- **Anthracnose disease control by antagonistic yeasts in plant nurseries studies**

Efficacy of five antagonistic yeasts (KS18, KS37, KS42, KS34 and KS46) were screened under plant nurseries conditions by randomized block design with three replications. Five isolates were tested and compared with control and chemical plots. All plots were sprayed in every 15 days until 60 days and collected the results when 120 days of chili plant age. The average of plant height (cm), flower/plant and branch/plant in each plot were calculated. The plants were rated as resistance and susceptible based on the range of lesion area (mm) or the disease incidence (%). The results of plant growth as measured in plant height, branch per plant and flower per plant. The result of plant height showed that, yeast isolate KS34 had highest of plant height at 45.80 cm, followed by KS37 (44.00 cm) and control plot (43.40 cm). For the result of branch per plant of chili plant found isolate KS37 showed highest of branch per plant at 16.60 branches, followed by isolate KS18 (12.80 branches) and KS42 had 11.80 branches. The flower of chili per plant presented that isolate KS37 had highest of flower at 18.60 flowers, in addition, the control group showed flower at 16.20 flowers and KS18 had flower per plant at 15.40 flowers. The results of plant growth showed significant difference ($P<0.05$) in each parameter (Table 3). The result of anthracnose disease control by antagonistic yeasts in disease incidence and category mean as plant resistance shown in Table 4. These results found that, Isolates KS46 had a great disease incidence at 7.80% that mean this isolate prove chili plant to resistance to *C. capsisci*. Addition result of another isolates were had high the percentage incidence of disease (10.1-50.0%) and these mean to plant were tolerant to the pathogen. However, isolate KS46 was showed plant growth less than another isolates, but this test was mainly to select the best isolate that control anthracnose disease in chili plant. This assumed KS46 was showed the best to control *C. capsici*.

Table 3. Efficacy of antagonistic yeasts to anthracnose disease control *C. capsici* in chili plant nurseries.

Treatment	Height (cm)	Branch	Flower
1. Control	43.40b	10.00abc	16.20bc
2. Chili + *C. capsici*	30.00a	5.60ab	1.20a
3. Chili + *C. capsici* + KS18	43.20b	12.80c	15.40bc
4. Chili + *C. capsici* + KS37	44.00b	16.60c	18.60c
5. Chili + *C. capsici* + KS42	43.00b	11.80bc	7.00abc
6. Chili + *C. capsici* + KS34	45.80b	10.40abc	10.60abc
7. Chili + *C. capsici* + KS46	35.60ab	5.00ab	1.80a
8. Chili + *C. capsici* + mancozeb	38.60ab	4.60a	5.60ab
p value	0.148	0.003	0.010

The value of a, b, c and d in the average of plat height, branch and flower in each column by a different letter indicate significant differences (*P<0.05*) according to Duncan test.

Table 4. Disease incidence and category of plant resistance in chili plant nurseries.

Treatment	Disease Incidence (%)	Category of resistance
1. Control	22.60bc	Tolerant
2. Chili + *C. capsici*	23.40bcd	Tolerant
3. Chili + *C. capsici* + KS18	21.80bc	Tolerant
4. Chili + *C. capsici* + KS37	28.60cd	Tolerant
5. Chili + *C. capsici* + KS42	31.80d	Tolerant
6. Chili + *C. capsici* + KS34	17.60b	Tolerant
7. Chili + *C. capsici* + KS46	7.80a	Resistant
8. Chili + *C. capsici* + mancozeb	19.80bc	Tolerant
p value	0.000	

The value of a, b, c and d in the average of plat height, branch and flower in each column by a different letter indicate significant differences (*P<0.05*) according to Duncan test.

- **Mode of action of antagonistic yeast to control C. capsici on chili fruits**

A symptom on chili fruit found the white mycelia of *C. capsici* could attach at the terminal of fruit, next, appear on mature fruits as small, water soaked, sunken lesions that rapidly expand. The lesion can increase to 2-3 cm in diameter on large fruits. The appressoria from germination conidia then penetrate into the chili epidermal cells. In addition, the pathogen can have created acervulus, setae and conidia spores on the fruit surface (Fig. 5). The relationship between antagonistic yeast and the pathogen on the surface of chili fruits was studied under the light microscope. These relationships found yeast cell can division rapidly and habitat on the mycelia of *C. capsici* (Fig. 5). The action of yeast isolate could competition the nutrient with mycelia of pathogen. This mode effect to *C. capsici* loss nutrient caused the pathogen get stuck to braked and died later. Several possible yeast biocontrol mechanisms have been suggested to nutrient competition and killer toxin effect with pathogens [3]. Competition for nutrients has been suggested as the mode of action of several biocontrol organisms, *e.g. Pichia guilliermondii* against *Penicillium digitatum*, *Candida guilliermondii*, *Cryptococcus laurentii* and *Metschnikowia pulcherima* against *Botrytis cinerea* and *Penicillium expansum* [7]; [8]. For confirmed this result, all isolates were determined the mode of action by studied in relationship between antagonist isolates and measured the chitinase enzyme. The real efficacy of antagonistic yeast could have tested in green house condition, this research found, yeast isolate KS46 had greatest of disease incident (7.80%) and showed plant resistance. Nevertheless, this isolate presented plant growth rate in plant height, braches/plant and flower/plant less than another isolates in plant growth promoting result.

Figure 5. (A) symptom of anthracnose disease by *Colletotrichum capsici* on chili fruit,

(B) The relationship between antagonistic yeast and *C. capsici*

3.5 Determination of chitinase enzyme activities

To confirmed and selected the best of yeast isolate, the mode chitinase enzyme activity was measured. This enzyme is the most hydrolytic enzyme in several yeast cell that secreted to hydrolyze the mycelia of *C. capsici*. Five yeast isolates as KS18, KS37, KS42, KS34 and KS46 were determined. The result found isolate KS46 had highest of activity of chitinase enzyme at 170.72 U/ml, follow by KS42 was presented chitinase enzyme at 169.36 U/ml and isolate KS18 showed activity of enzyme at 166.96 U/ml. While isolate KS37 and KS34 showed nearly activity of chitinase enzyme at 165.28 U/ml and 165.20 U/ml respectively (Table 5). To confirm efficacy of KS46, activity of chitinase enzyme was tested. The result found, yeast isolate KS46 had highest of chitinase activity at 170.72 U/ml. This result confirmed KS46 was the best antagonist to control anthracnose disease caused by *C. capsici*. Mycoparasitism of antagonist is associated with the production of a cell wall degrading enzyme and inducement of host defense. Concurrent, induction of chitinase has been described in plants as a response to infection by microbial pathogen. Previously, the research on lytic enzyme produced by yeast antagonists of postharvest pathogen of fruits and vegetables was mainly focused on chitinase. Wisniewski et al. (1991) [25] found that the yeast exhibited high levels of chitinase activity; *P. membranefaciens* and *C.guilliermondii* significantly inhibited *R. stolonifer*. The antagonistic yeast isolate KS46 exhibited chitinase, which was relative with growth inhibition of *C. capsici*. The chitinase from yeast could be significantly induced by CWP substrate. Chan and Tian (2005) [11] reported the endo chitinase of *P. membranefaciens* and *C. albidus*with high activity in CWP as the sole carbon source. This is the first report of chitinase from yeast strain *C. haemulonii*. The chitinase hydrolyse fungal cell wall and inhibit *in vitro* growth of several pathogenic fungi. It is possible that these hydrolyse enzymes play an important role in degradation of the *C. capsici* cell wall, especially when yeast attaches to the pathogen hypha. Chitinase may help to induce resistance of plants to pathogenic fungi [26].

Table 5. Chitinase enzyme activity of five yeast isolates.

Isolate	Chitinase (U/ml)
KS18	166.96b
KS37	165.28a
KS42	169.36c
KS34	165.20a
KS46	170.72d

The value of a, b, c and d in the average of chitinase enzyme activity in the column by a different letter indicate significant differences (*P<0.05*) according to Duncan test.

3.6 Antagonistic yeasts identification

From all results, KS46 presented the best isolate to control anthracnose disease. Identified by D1/D2 domain of 26S ribosomal DNA sequencing. The nucleotide sequence was compared using the basic local alignment search tool (BLAST), with the difference sequences in the nucleotide sequence database of NCBI. The sequence analysis showed maximum identity of 100% with *Candida haemulonii* as shown in Fig 6. The antagonistic yeasts KS46 has been successfully identified *Candida haemulonii* by using 26S ribosomal DNA. The strain was presented the morphological characteristic similarly *Candida haemulonii* Type II (Van Uden&Kolipinski) S.A. Meyer&Yarrow that located at *Metschnikowia* clade.

4. Conclusion

These antagonistic yeasts can control *C. capsici* mycelium in disease incidence and postharvest disease control on chili fruits. Several antagonistic yeasts in *Candida* have previously been used as biocontrol agents to control the anthracnose disease caused by *C. capsici* in postharvest crops such as *C. tropicalis*, *C. famata* and *C. membranifaciens* [27]. However, there is no report on use of *C. haemulonii* to control *C. capsici* in chili fruits. These studies presented the first evidence of antagonistic yeast *C. haemulonii* to control anthracnose disease. This strain can have reduced disease incidence and can preserved chili fruits from the pathogen at 10 days.

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