Screening and activity of yeast-associated with cocoa-bean fermentation against phytopathogenic yeast and fungi

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Abstract. Cocoa-bean fermentation has been associated with the involvement of diverse microbial assemblages which consist of a wide array of bacteria and yeast. We attempted to screen and to identify the potential antifungal yeast from this assemblage against phytopathogenic fungi. We employed in-vitro antagonism assay using agar plug methods to perform preliminary screening from 35 yeast isolates followed by total protein production and measurement with Bradford methods. We found three yeast strains that were effective against Trichoderma sp. T009, and two moulds associated with cocoa (Penicillium sp. Cocoa 2 and Fusarium sp. Cocoa 1). The three bioactive yeast strains were identified as Saccharomyces cerevisiae IDI-002, Hanseniaspora uvarum IDE-056 and Hanseniaspora uvarum IDE-271 based on molecular identification and phylogenetic analysis. The production of antifungal protein from pure cultures on YEPG media resulted in total protein concentration between 6.20 - 8.17 mg/L. Cell suspension showed higher inhibitory activity compared to the cell-free supernatant gave indication that antifungal proteins in the bulk fermentation was below the minimal inhibitory concentration to cause the inhibitory effect. Further characterization, purification, and optimization are still needed before the up-scale production of antifungal metabolites and its biological control application.

1. Introduction
About 25% of the harvested fruits worldwide have been reported to loss due to postharvest decay which can be directly associated to decay fungi. Theobroma cacao L., amongst many functional and economically significant plants, has been known to possess a set of defense mechanisms against pathogenic fungi. They produce an antifungal peptide/protein referred to as pathogenesis-related protein (PR) [2]. Among many functional parts of a plant, the seed part is widely reported to contain such antifungal protein [5, 6]. Once the seed is harvested for cocoa production purpose, it will undergo spontaneous fermentation driven by postharvest period by different microorganisms [7]. It was reported that spontaneous aerobic fermentation containing indigenous microbial assemblages of cocoa husks yields a crude husk extract (CHE) with antimicrobial activity composed by phenols, steroids or terpenes, amino acids and alkaloids [4].

Compare to the ecologically unfriendly chemical fungicides, the use of biological control from bacteria and yeasts is gained attention nowadays. However, as the main inhibitory action of bacteria is by producing antibiotics that cause problematic antimicrobial resistance phenomenon, yeast with...
antagonist activity may be a safer option as it does not produce allergenic spores and toxins as many mycelial fungi [1]. Yeast also able to colonize the dry surfaces for longer periods with only simple nutritional requirements and their potential as biocontrol agents has been investigated widely[16]. This yeast antimicrobial protein is termed as 'killer protein'. This protein was first isolated in Saccharomyces cerevisiae and now has been found in a wide range of genera such as Candida, Hanseniaspora, Kluyveromyces, and Pichia[1]. This killer-protein producer has been demonstrated to be applicable as a biocontrol and preservation agent. For example, to inhibit the growth of pathogenic yeast during wine fermentation [10].

In cocoa itself, fungal or mould attacks and overgrowth are not only found during fermentation process, but also during sun-drying and bean storage. Aspergillus and Penicillium genera are among the spoilage fungi reported to be isolated from fermented and dried cocoa beans [21] which has ability to produce mycotoxins. These mycotoxins can lead to a food safety concerns and obviously cause economic losses for the producer and consumer levels. Fungal metabolism can also reduce organoleptic properties of the infected cocoa pods and beans [22]. Fusarium is also one of the fungal organisms that can appear as saprophytes as well as parasites in various plants. For example, Fusarium oxysporum is well known to cause most serious disease in plants [23].

In Indonesia, there is not much study has been done about the exploration of antifungal protein from yeasts associated with cocoa-bean fermentation. In addition, a considerable antifungal from microorganisms-associated with cocoa-bean fermentation have only been reported from acetic acid and lactic acid bacteria and Bacillus species [9, 7, 21]. Therefore, this is the first study about investigation of the potential indigenous yeast strains, which have good bioactivity, especially to control the growth of pathogenic fungi in cocoa plants.

2. Material and Methods

2.1. Yeast strains and media

Thirty-five (35) yeast strains were previously isolated from cocoa beans in the context of commercial operations using box fermentations at PTPN XII, Jember, East Java, Indonesia. The beans were sampled for isolations at 24 h intervals for 4 days natural fermentations and were taken approximately 15 cm from the surface. Ten grams of the beans and the adhering pulp were added to 90 ml Ringer’s solutions (Merck) and mixed for 2 min in laboratory blender Stomacher 400 (Seward Medical, UK) at medium speed. From this, 10-folds dilutions were made for yeast isolation. Yeast were grown and enumerated on Malt extract agar media containing 100 mg/L chloramphenicol and incubated for 3 days at 28°C. These 35 yeast strains were being purified, characterized (i.e. heat, acid and ethanol tolerance) then stored at of the culture collection of Applied Microbiology Laboratory, Indonesian Institute of Sciences (LIPI) for 2 years (2015 - 2017) before used for this study. Malt extract agar (MEA) (Difco) media was used for isolate maintenance that consists of (per Litre): 3 g Peptone, 30 g malt extract and 15 g Bacto agar. Malt extract broth (MEB) media was made from the same components without addition of Bacto agar. Potato Dextrose Agar or PDA (Difco) was also used for fungal media. Small scale production/fermentation of antifungal metabolites (protein) was done using YEPC (yeast extract-peptone-glucose) media consists of (per Litre): 10 g yeast extract, 20 g peptone and 20 g glucose.

2.2. Preliminary screening

Preliminary screening was conducted by agar plug methods[11] against Saccharomyces cerevisiae, Candida albicans and Aspergillus niger (test isolates) for all 35 yeast strains. The results from this assessment is qualitative and described as positive and negative based on the size of inhibition zone formed compared to the positive control. The two former yeasts were grown, each, until they reach the late exponential phase in a liquid media and then diluted using physiological solutions (NaCl 0.85%) to get the reach the turbidity of 0.5 McFarland (1 x 10^6 CFU/ml) standard solutions. Meanwhile, for A. niger, the spore was taken from 48 h culture with an addition of physiological solutions and diluted to reach the same turbidity standard solutions. The 0.5 McFarland equal of test strain solution was then
spread into sterile MEA plate using a sterile cotton swab. All yeast isolates with 24-48 h inoculum age grown in another MEA was then cut using a sterile plastic borer with a diameter size of 0.5 cm and then put on the test media already contained the test strains. Sterile plug ME agar was used as negative control while cycloheximide 0.1% in a sterile disk paper was used as a positive control. An apparent (inhibition) zone formed around the yeast isolates against test isolates was then measured after 24-48 h incubation.

2.3. In-vitro antagonism assay
Antagonism assay against moulds was performed using the same principle as preliminary screening with a few modifications. The three moulds were used as a test strain namely Fusarium sp. Cocoa 1, Penicillium sp. Cocoa 2 (both are previously isolated from fungal-infected Cocoa pods) and Trichoderma sp. T009 from BTCC (LIPI). The test moulds were grown for 48-96 h and then cut into 1 cm² square in size and wereput at the centre of sterile MEA media. The potential yeast isolates were then also cut into 0.5 cm in diameter and put at the same fixed distance from the test moulds. The reduction of fungal hyphae growth was then measured as the inhibition zone after 7 days incubation [12].

2.4. Metabolites production and total protein measurement
Lab-scale production using pure culture of the three yeast isolates was done in YEPG media. Fresh plate culture (24-48 h) on MEA was picked (3-4oose) and inoculated into 5 ml activation I media (YEPG) and incubated overnight at room temperature, 200 rpm. This activation culture was further used in 100 ml production media and incubated 5 days at room temperature, 200 rpm. At the end of incubation period, 50 ml of whole broth culture was centrifuged 7500 rpm for 15 min at 4°C to separate the cell from supernatant containing antifungal protein (designated as the crude extract). For total protein measurement, the cell-free supernatant was further precipitated using cold acetone (1:1 v/v) overnight followed by centrifugation at 13000 rpm for 10 minutes to obtain protein pellet. Both crude extract and protein pellet (after dilution in sterile MilliQ water) was used following in-vitro antagonism procedure. Measurement of the total protein produced by yeast isolates was performed using UV spectrophotometer by reading absorbance detected at wavelength 562 nm[13] using Bradford Reagent Kit (Thermo Scientific). Procedure for protein measurement was done according to the manufacturer's instructions.

2.5. Molecular identification of yeast strains
For identification of the potential yeast isolates, peqGOLD fungal DNA Kit (PEQLAB Biotechnologie, Germany) was used. 5 ml volume of pure culture was used, and cell was harvested after centrifugation at 13000 g for 5 min. DNA extraction was performed according to the manufacturer's protocols. PCR was then performed using 24-48 h grown pure culture to amplify the target marker DNA using the primers pair NL1-forward (5'-GCA TAT CAA TAA GCG GAG GAA CAG) and NL4-reverse (5'-GGT CCG TGT TTC AAG ACG G) (Operon Biotechnologies, Germany). KAPA2G Robust PCR Kit (PEQLAB Biotechnologie, Germany) was used for the PCR. Each 25 μL PCR reaction consisted of: 14.3 μL PCR grade water, 5x KAPA2G Buffer B (1x final concentration), 0.5 μL dNTP mix (0.2 mM for each dNTP), 1.25 μL forward primer (0.5 μM) and reverse primer (0.5 μM) each, 2.5 μL template DNA and KAPA2G Polymerase 0.2 μL. Thermocycler was set as follows: 95°C initial denaturation for 5 min, followed by 35 cycles of 95°C denaturation for 1 min, 52°C annealing for 15 sec and 72°C extension for 2 min, ended with 72°C of final extension for 7 min. DNA quantity and purity was then measured using a spectrophotometer (Biorad, US) and DNA visualization was done using agarose gel electrophoresis methods using Ethidium bromide solution staining. PCR product was then purified using GeneJET PCR Purification kit (Fermentas, Germany) according to the manufacturer's protocols before sent for sequencing based on Sanger technique to Eurofins MWG Operon (Germany). The resulted sequences were then matched against Ribosomal Database Project.
(RDP) for identification and the similarity and identity was determined using BlastN. Multiple sequence alignment and phylogenetic tree was constructed using MEGA7 Software.

3. Results and Discussion

3.1. Screening of potential yeast isolates against phytopathogenic fungi and yeast

We conducted initial screening from the previously isolated and well-characterized yeast strains from Cocoa-bean fermentation to identify the potential antifungal isolates. From 35 strains that were tested against *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus niger*, only four isolates showed a positive inhibitory activity to one or more tested yeasts and moulds (table 1). *Candida albicans* was not inhibited by all the yeast strains that were tested. Three yeasts were able to inhibit the fungal growth of *A. niger*, namely IDE-271, IDI-002, IDE-056. Meanwhile, there are four isolates that showed inhibition against *Saccharomyces cerevisiae*, namely IDE-271, IDE-056, IDI-002, and IDI-005. Based upon the result, the three yeast isolates were chosen for the next antagonism assay against phytopathogenic fungi which are IDE-271 and subsequent antifungal-containing protein fermentation.

Following the preliminary screening results, we continued with the three most potential yeast strains that showed narrow-range inhibition activity namely IDI-002, IDE-056 and IDE-271 (table 1) by testing their ability to inhibit phytopathogenic fungi isolated from deteriorated cocoa pods. As depicted in figure 1, the three isolates were able to inhibit *Trichoderma* sp. T009 and *Fusarium* sp. cocoa 1. In addition, IDE-056 and IDE-271 were also active against *Penicillium* sp. cocoa2. The strongest inhibitory activity, i.e. based on the number of test strains that were inhibited and the diameter zone produced, was shown by IDE-271, followed by IDE-056 and IDI-002 as the least bioactive strain.

![Figure 1. Bioactivity results of three yeast isolates against phytopathogenic fungal strains.](image)

Based on in-vitro assessment, the reduction of fungal mycelia indicates the existence of bioactivity from the potential yeast strains. The three yeast isolates exhibited inhibitory activity against the tested pathogenic fungal. *S. cerevisiae* IDI-002 was highly effective against *Trichoderma* sp. T009. On one hand, some of the *Trichoderma* species associated with cacao are known to possess antagonistic activity against plant pathogens and can produce antifungal compounds [24] and have been studied as biofungicides such as *T. virens*, *T. harzianum* and *T. asperellum* [25]. However, according to the de Souza et al (2008) *Trichoderma stromaticum*, a mycoparasite of the cacao witches' broom pathogen *Monilithophora pernicios*, was studied for their endophytic potential in cacao and bean seedlings but the results showed no plant growth promotion nor induced resistance against the broom pathogen [27]. Some of the *Trichoderma* species indeed have poor competitive ability with fungi and bacteria that invaded cacao seedlings, reflecting the complex mechanisms of interaction between plants, pathogen and the mycoparasites itself.
### Table 1. List of 35 yeast strains used in preliminary screening and their inhibition potential

| No. | Isolates | *Saccharomyces cerevisiae* | *Aspergillus niger* | *Candida albicans* |
|-----|----------|---------------------------|--------------------|-------------------|
| 1   | IDE-271  | +                         | ++                 | -                 |
| 2   | IDI-003  | -                         | -                  | -                 |
| 3   | IDI-043  | -                         | -                  | -                 |
| 4   | IDI-051  | -                         | -                  | -                 |
| 5   | IDI-059  | -                         | -                  | -                 |
| 6   | IDE-025  | -                         | -                  | -                 |
| 7   | IDE-062  | -                         | -                  | -                 |
| 8   | IDE-015  | -                         | -                  | -                 |
| 9   | IDE-007  | -                         | -                  | -                 |
| 10  | IDE-091  | -                         | -                  | -                 |
| 11  | IDT-004  | -                         | -                  | -                 |
| 12  | IDT-013  | -                         | -                  | -                 |
| 13  | IDI-019  | -                         | -                  | -                 |
| 14  | IDI-057  | -                         | -                  | -                 |
| 15  | IDI-058  | -                         | -                  | -                 |
| 16  | IDI-060  | -                         | -                  | -                 |
| 17  | IDE-056  | +                         | +                  | -                 |
| 18  | IDI-002  | +                         | ++                 | -                 |
| 19  | IDI-004  | -                         | -                  | -                 |
| 20  | IDI-026  | -                         | -                  | -                 |
| 21  | IDI-027  | -                         | -                  | -                 |
| 22  | IDI-047  | -                         | -                  | -                 |
| 23  | IDI-048  | -                         | -                  | -                 |
| 24  | IDI-001  | -                         | -                  | -                 |
| 25  | IDI-005  | +                         | -                  | -                 |
| 26  | IDI-011  | -                         | -                  | -                 |
| 27  | IDI-015  | -                         | -                  | -                 |
| 28  | IDI-032  | -                         | -                  | -                 |
| 29  | IDI-042  | -                         | -                  | -                 |
| 30  | IDI-044  | -                         | -                  | -                 |
| 31  | IDI-045  | -                         | -                  | -                 |
| 32  | IDI-046  | -                         | -                  | -                 |
| 33  | IDI-049  | -                         | -                  | -                 |
| 34  | IDI-050  | -                         | -                  | -                 |
| 35  | IDE-001  | -                         | -                  | -                 |

**Control** (Cycloheximide 0.1%) | +++ | + | + |

**Notes:** Isolates typed in bold showed at least inhibition against one of the tested test strains.
On the other hand, other species of *Trichoderma* such as *T. reesei* is being known to be pathogenic as a dead wood fungus. A cysteine inhibitor from pearl millet seeds had been reported to reduce the growth of this parasitic fungi [26]. Therefore, to the best of author's knowledge, this is the first study that reported the antifungal protein potential from yeast-associated with cacao that can inhibit the growth of pathogenic model *Trichoderma* sp. T009 used as the test strain.

Based on the results, *S. cerevisiae* IDI-002 showed more sensitivity towards *Trichoderma* sp. T009 compared to the other two fungal test isolates probably due to the different in susceptibility (target receptor, fungal cell-wall structure etc) against the produced antifungal protein [3]. The variation in susceptibility, as reflected by the size of the inhibition zone formed, indicating that some defense mechanisms to counteract the action of antifungal metabolites through a series of signaling pathways/cascade or using cell-wall modification and over production of chitin [18] may happen.

Moreover, the two yeast strains *Hanseniaspora uvarum* IDE-056 and *H. uvarum* IDE-271 also showed a wide range spectrum in their inhibitory activity. *H. uvarum* species has been reported to reduce the infection rate of grey mould by inhibiting its spore germination and mycelial growth [15]. In the same report, the cell density of *H. uvarum* was also increased and therefore contributes to its biocontrol activity.

### 3.2. Live-cell suspension inhibitory activity was more effective than cell-free supernatant

Antifungal protein production was done using YEPG media. As shown in figure 2, all three isolates produced total protein in high concentration. IDE-271 has the highest protein concentration (8168 μg/ml) followed by IDE-056 (7284 μg/ml) and the IDI-002 (6204 μg/ml) respectively. The crude extract from this protein production, however, did not show any inhibition towards test strains (whether yeast or moulds). In contrast, the living-cell suspension demonstrated the inhibitory activity in agreement with in vitro bioassay.

![Figure 2](image-url)

*Figure 2. Total protein content of the three yeast isolates measured by Bradford methods.*

The production of antifungal protein in YEPG media showed that *H. uvarum*, both IDE-056, and IDE-271, produced higher concentration of total protein compare to the *S. cerevisiae* IDI-002, in agreement with their wide range activity and sensitivity against the tested fungal strains.

However, the cell-free supernatant or the crude extract used for confirmation on inhibitory activity showed negative results for all three yeast isolates, as well as the protein pellet extract. This is in contrast with live-cell suspension that showed antifungal activity in concurrence with semi qualitative in-vitro test. This finding suggests that:(i) the antifungal protein produced was very low (below the required minimum inhibitory concentration) relative to the total protein produced, and therefore no
inhibition effect is shown [19]; (ii) the antifungal protein produced as a secondary metabolite and not associated with their cell growth. Therefore, the production of such secondary metabolites could be hindered when there is no stress present in the environment or no specific inducer was added to the media [20]. Liu [1] mentioned that NaCl addition is needed for killer protein production in yeast and can enhance the killing activity and its spectra as the salt concentration increased. In fact, it may be easier and relatively cheaper when biocontrol application is using the live-cell suspension because extensive downstream process such as purification for the antifungal compound is not required.

This result agrees with the previous report from [17] in which he demonstrated that the filtrate culture of *Pichia membranefaciens* was not effective against deterioration process of citrus caused by *Penicillium italicum* and *P. digitatum* while the previous soaking of the fruit in live-cell suspension can reduce the percentage of disease incident and lesion diameter. More recently, a live and viable cell of *W. anomalus* (*P. anomala*) incorporated inside bio-based films of sodium alginate and locus bean gum coated in Valencia oranges during storage can reduce the green mould by more than 73% after 13 days [8].

### 3.3 Identification and phylogenetic analysis of potential yeast isolates

The colony of IDI-002 has a white color, smooth and round-shape in appearance on malt extract agar media. Meanwhile, another two isolates, IDE-056 and IDE-271 have smooth and round with a dark color and soft pink color appearance, respectively (Fig. 3). The sequencing of 26s rRNA gene from the three isolates was able to acquire 563, 575 and 1154 bp length for IDI-002, IDE-056, and IDE-271 respectively. Identification was done by NCBI BlastN and RDP. The results from RDP match showed that all three isolates were classified as Ascomycota phylum within Saccharomycetae family and *Saccharomyces* genus for IDI-002 and *Hanseniaspora* genus for IDE-056 and IDE-271. The blastN confirmed 100% identity with species *Saccharomyces cerevisiae* strain SE11 (Accession number MK907998.1) for IDI-002; 99.8% identity with *Hanseniaspora uvarum* strain B-NC-13-F25 (Accession number KJ794666.1) for IDE-056; and 99.5% identity with *Hanseniaspora uvarum* isolate Soil120 (Accession number MG707675.1) for IDE-271. Further analysis by reconstructing phylogenetic (Fig. 4) tree revealed that IDI-002 is included as one cluster group with *Saccharomyces* genus while IDE-056 and IDE-271 is clustered together with *Hanseniaspora* genus. In accordance to their 26s rRNA sequences and phylogenetic analysis, the three yeast isolates named as *Saccharomyces cerevisiae* IDI-002, *Hanseniaspora uvarum* IDE-056 and *Hanseniaspora uvarum* IDE-271.

![Figure 3](image_url)

*Figure 3.* The macroscopic appearance of *Saccharomyces cerevisiae* IDI-002(A), *Hanseniaspora uvarum* IDE-271 (B), and *H. uvarum* IDE-056 (C) in MEA plate.

Based on the results of this study, we suggest the following work to be done in the near future: (a) optimization of antifungal protein production. As the production and action of many killer toxins depend on the presence of NaCl, it may be possible to add and find optimal salt concentration needed to produce the antifungal protein. It is known that most of the killer proteins are stable and function only at acidic pH values and low temperatures; (b) bioactive protein identification and its further characterization. From microbiology and chemistry point of view, it may be interesting to explore the structure of killer toxins and how this structure resulted in a killing activity and binding to target receptors on the sensitive cells. This can employ tandem mass spectrometry and liquid chromatography such as HPLC-ICP-MS. These two recommendations are probably more possible to be elucidated in the future in comparison to the cloning and cell-factory production approach as it is
known that many genes encoding the killer toxins have not been cloned so far [1]. Moreover, it may be interesting to assess the biocontrol activity of these three yeast isolates in reducing disease incidence in the cocoapods and bean itself.

**Figure 4.** Phylogenetic tree of the tree yeast isolates based on 26S rRNA sequencing analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1578.19) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Pichia* genus is the outgroup.
4. Conclusion
Recently, the use of biological agents as a green biocide (biocontrol agent) has been widely investigated to reduce the negative impacts on the chemical biocides. The use of antifungal-producer microbes is foreseen to be a promising solution in the future, particularly to prevent and delay fruit/pods deterioration from phytopathogenic fungal attack. From this study, we established the potential bioactive of the yeast strains isolated from cocoa-bean fermentation. *Hanseniaspora varum IDE-271* was identified to have the most potential inhibitory capacity against *Fusarium* sp. cocoa 1 and *Penicillium* sp. cocoa 2 and *Trichoderma* sp. T009. Besides, the use of live-cell suspension in comparison to the crude or pellet extract of the protein itself has shown to be more effective in inhibiting the growth of the phytopathogenic fungi. Further studies are still needed to optimize and characterize those antifungal compounds.

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