Optimization Production of Antifungal Substance from a Sponge-associated Trichoderma harzianum cultivated in the Tofu Dregs and Rice Bran

Agus Trianto, Agus Sabdono, Ocky Karna Radjasa, Rini Pramesti, Nur Taufiq Samsudin Putrajaya, Muhammad Syaifudien Bahry, Desy Wulan Triningsih, Sulistiowati and Rachmat Afriyanto

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Optimization Production of Antifungal Substance from a Sponge-associated *Trichoderma harzianum* cultivated in the Tofu Dregs and Rice Bran

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Abstract

This study aimed to evaluate the production of the crude extract Trichoderma harzianum and its antifungal activity and toxicity. T. harzianum was cultured in tofu dregs and rice bran media both in saline and non-saline media. The fungi were cultured for 15 days at 24-25 ^{O}C. The media and the mycelia were extracted with methanol. All the extracts were tested against Candida albicans, Malassezia furfur and the Trichophyton sp. The extract was also tested to the human cells for the toxicity assessment. T. harzianum cultured in rice bran produced the extract significantly higher than those in the tofu dregs either in saline or non-saline media.

However, the activity of the extract of T. harzianum cultured in the tofu dregs has higher activity than the extract of those cultured in rice bran. However, the use of seawater gave significant effect to the antifungal compounds production of the fungus cultured in rice bran media. The thin layer chromatography test showed that the fermentation process converts the compounds from media into new derivatives that are active against the pathogenic fungi. The extract of T. harzianum cultured in tofu dregs (saline and non-saline) and saline rice bran did not become toxic to the human cells. In conclusion, the antifungal activity of the T. harzianum extract was influenced by media type. Tofu dregs is a promising media for the large-scale production of non-toxic antifungal compounds.

Keywords: Antifungal compounds, marine-derived fungus, salinity, tofu dregs, *Trichoderma harzianum*.

Introduction

Since the last decades, the increased incidence of invasive fungal infections was reported and nowadays fungi are one of the ten most frequently isolated pathogens among patients. Fungi are able to grow in any condition and develop the immune system against modern drugs.[6] The superficial fungal infection is the most common disease that attaches almost 1.7 billion people worldwide. The fatal fungal infection is also to be incorporated with other diseases and other health treatment such as HIV/AIDS, leukemia, asthma and transplant patients.[4] Some well-known genera pathogenic fungi are Candida, Aspergillus, Malazzesia and Trichophyton that cause candidiasis, aspergillosis and superficial fungal infections respectively.[13,15]

Marine sponges and their associated fungi are potential sources of antifungal compounds.[8] Some antifungal compounds isolated from marine sponges include callipeltins, crambecin, theonellamide G, tetramic acid glycoside aurantoside K, plakortide F, bromopyrole and many others that are active against various species of Candida.[8,29] However, most of the sponges contain the antifungal compounds in low concentration, so extracting the compound from the natural sponge will cause a high ecological impact. Some fungi have been used in biosynthetic for producing the structurally complex medicines and life-enhancing drugs. Biosynthesis using the marine derive fungi is a more feasible method for producing the bioactive compounds.[2]

To develop the antifungal drug, large amounts of the compounds are needed for further study such as toxicological and efficacy tests. One of the major obstacles in natural product drug development is the problem of supply. The compounds must be produced continuously in sufficient quantities and of good quality to overcome the problem. Utilizing the bye-product as alternative culture media can be expected to suppress the production cost without sacrificing the production scale and quality. However, the alternative media must be available in good quantity, cheap, contains nutrient for fungal growth and the production of the compounds target.

Rice bran is a bye-product of raw rice that is continuously available in the agricultural area with low price. The production of rice bran is approximately 70 MMT worldwide.[10] Rice bran is potential as a basic medium for fungal culture as it contains fat (15.85–18.80%), protein (12.07–13.66%), carbohydrate (40.63–45.06%), fiber (11.77–12.68%) and ash (9.72–11.41%). Rice bran is also rich in phenolic compounds, phytic acid, γ-oryzanols, α-tocopherol (μg/g) and γ-tocopherol(μg/g)[17].

Another cheap medium tofu dreg is a bye-product from tofu production. Tofu, one of the most popular food in Indonesia, consumes almost 2.56 million tons of soybeans every year. The tofu industry produces about 1,024 million tons of solid waste called tofu dreg that is usually used for animal feed.[9] Damanik et al[5] reported that tofu dregs contain energy

(107,32 ± 0,58 kcal), moisture (77,22 ± 0,35 %), ash (0,8 ± 0,01 %), fat (3,88 ± 0,07 %), protein (3,7 ± 0,02 %) and carbohydrate (14,4 ± 0,00 %).[5]

In this work, we evaluate effectivity of the tofu dregs and rice bran as solid media culture of marine fungus *T. harzianum* for production of the antifungal metabolites. We would also like to demonstrate the effect of the presence of seawater on the production of crude extracts and their bioactivity and toxicity.

Material and Methods

Fungal isolate: The fungus *Trichoderma harzianum* JX473716.1 was isolated from a sponge *Dysidea* sp. collected off Wakatobi Island, Indonesia. In the previous test, the fungus showed strong activity against the pathogenic fungi *Malatzesia furfur, Trichophyton sp. and Candida albicans.*[25] The fungus was cultured on malt extract agar (MEA) plates for seven days at 25°C as the starter.

Culture Media: Then, the fungus was cultured in four different media i.e. saline tofu dregs (TD-S), non-saline tofu dregs (TD-NS), saline rice bran (RB-S) and non-saline rice bran (RB-NS). The TD-S and TD-NS were made of 400 g tofu dregs, 20 g honey, 4 g yeast extract, 2.55 g peptone, 100 mL salt water or tap water for saline medium and non-saline medium respectively. The RB-S and RB-NS were made of 400 g rice bran, 20 g honey, 4 g yeast extract, 2.55 g peptone, 100 mL salt water or tap water for saline medium and non-saline medium respectively. All cultures were grown for 15 days at 25°C with four replications. Two plates media were not inoculated with the fungal as controls for possible contamination. All the media were sterilized on 121°C for 20 minutes in an autoclave.

Fungal extraction: Each cultivated fungal was extracted with 400 ml methanol for 24 h at room temperature with triplication. After filtration, the methanol extract was concentrated with a rotary evaporator under vacuum at 35-40°C.

Antifungal assay: The antifungal assay was conducted with disk diffusion agar method against pathogenic fungi i.e. *Trichophyton* sp., *Candida albicans* and *Malassezia furfur* obtained from the Diponegoro National Hospital. The pathogenic fungal stocks were cultured on potato dextrose agar (PDA) plates for 24 h. Fungal density was adjusted by using 0.5 M McFarland standard solution on malt extract broth (MEB) medium. The fungal isolates extracts were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions with a concentration of 50 µg/disc. Then, a 10 µL of the extract solution was transferred into the paper disc to provide the concentration of 500 µg/disc.

The discs were placed on the PDA plates previously swabbed with the pathogenic fungi. The fungi were incubated for 24 h and the inhibition zone was measured. Nystatin (30 units) was used as positive control, while the media extract and DMSO were used as negative controls.

Toxicity test against human cell: The extracts were tested to the human cell to measure the toxicity using the MTT tetrazolium method with concentration 1 mg/ml in two replicates. The assay was conducted in The Center for Connective Tissue research, VU University medical center, Amsterdam, The Netherlands. The general procedure of assay is as follows: The cell was incubated in the media for 16-18 hours in CO_2 incubator at 36 ^{0}C; then the media was removed and replaced with MTT solution. The cells were incubated in a 96-well plate with approximate cell density 10^4 cells/ml in FBS – Bovine Serum (FBS), F-10 Nutrient Mixture with Penicillin 1%. After 18 hours incubation, the media were replaced with 20 ml of 5 g/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in phosphate buffered saline (PBS) and the cells were incubated for 3.5 h. After removal of the PBS solution, 150 ml of dimethyl sulfoxide was added and the cells were incubated again for 15 min prior to measurement with OD 540 nm.[7,8]

Results and Discussion

Crude extracts of *Trichoderma harzianum*: *T. harzianum* fungus cultured for 15 days on different media provides the various amount of extract as shown in figure 1. The fungi cultured in the non-saline rice bran (T-RB-NS) produces the highest amount of the crude extract. On the other hand, the fungi cultured in saline tofu dregs (T-TD-S) produced the smallest amount of extract. The tofu dreg has less density than rice bran that provides bigger volume for fungal growth and gives the opportunity to the fungus to produce a higher amount of mycelia and the extract. Rice bran contains protein and carbohydrates that serve as nitrogen and carbon source for the fungal growth and production of the bioactive compounds.[5,24]

The salinity of the media gave a negative effect on the production of the extract, although the fungus *T. harzianum* is originally from the marine environment. Masuma and collaborators[16] reported that the salinity did not influence the antimicrobial activity of the marine *Aspergillus*. However, other research showed that the hight salinity media had lowered the growth rate of *Phlyctochytrium* sp., a marine origin fungus.[14] Salinity has also lowered the production of methanolic extract of *Leptosphaeria oraemaris.*[18] Although this recent work did not apply a variety of salinity concentration, the result indicated that the absence of salt in the medium might take a role in the fungal metabolites production.

Antifungal activity of the *T. harzianum* crude extracts: Culture media gave a significant effect on the production of antifungal compounds of the fungus *T. harzianum* cultured as shown in figure 2. Tofu dregs is an appropriate media culture for the fungus *T. harzianum* to produce the antifungal compounds. The fungus cultured in T-TD-S media provides

the highest activity against the *Trichophyton* sp followed with the T-TD-NS and T-RB-S media while the T-TD-NS is the best media for production of the anti-*C. albicans* and *M. furfur* compounds. Rice bran is not a suitable media for production of the compounds.

The non-saline media gave better result to the production of anti-*C. albicans* and *M. furfur* compounds. In contrast, the fungus cultured on the non-saline media produces the extract with higher activity against the *Trichophyton* sp (Figure 2). Even though the fungus was isolated from the marine environment, the presence of salt did not have significant effect on the production of the secondary metabolites. Many researchers showed the various effect of the salinity on the production of bioactive compounds from marine origin fungi. The antioxidant activity of *Aspergillus elegans* cultured in non-saline media expressed a higher value compared to those cultured on higher salinity media.[1] Salinity has also reported notable impacts on the total of fungal chemical substances.[17] The presence of NaCl and KCl in the media significantly enhanced the mycelial growth but did not affect the antifungal activity.[12]

Wang et al[27] have also reported that high salinity media decreased the production of antioxidant compounds from *Aspergillus elegans*, associated seaweed fungus. The marine-derived fungi produce prominent secondary metabolites against the salinity stress that generates distinct chemical constituents.[20] The TLC profile showed the different chemical profiles of extracts from fungi cultured in various media (Figure 3). TLC also indicated that the original compound in both tofu dregs and rice bran had been converted to new compounds that may be responsible for the antifungal property. Riciputi et al[22] reported that fermentation of soybean changes the composition of fatty acids, sterols, phospholipids, tocopherols and isoflavones.

The investigation of the tofu dreg as the culture media was rarely done. Despite using the bye-product, most of the studies utilized soybean products such as soy meat or soy flour as the fungal growth medium.[22] A study about the soybean milk bye-product, named okara, was done by Naeem to culture *T. harzianum* previously isolated from the okara and *Aspergillus oryzae* as an agent in the soy sauce production.[3] The significant antioxidant activity was evaluated from these fungal extracts. The antifungal activity on the recent evaluation displayed notable results, especially against *Trichophyton* sp. The current results convince the efficacy of tofu dregs as an alternative medium to produce the antifungal substances of marine *T. harzianum*.

TLC test indicated that the fermentation products are different among the media and conditions. The TLC has also shown that the fungus was able to convert the compounds in the media become other compounds (Figure 3).

Toxicity test against human cell: The assay showed that extract of T-TD-S, T-TD-NS and T-RB-S is not toxic to the human cells up to 1 mg/ml. On the other hand, the extract of T-TD-NS exhibited remarkably toxicity to the human cells at 1 mg/ml (Table 1). Our previous work indicated that the fungus is able to convert the fatty acid in the rice bran with different end-product between saline and non-saline condition.[26]

Toxicity is one of the problems in drug development. Some drug candidates with strong potential have to be dropped due to the toxicity to the human cells. Several toxicities and safety assessments are needed at many steps so that the earlier toxicity test will save a big expenditure, time and other resources in the drug development.[11]

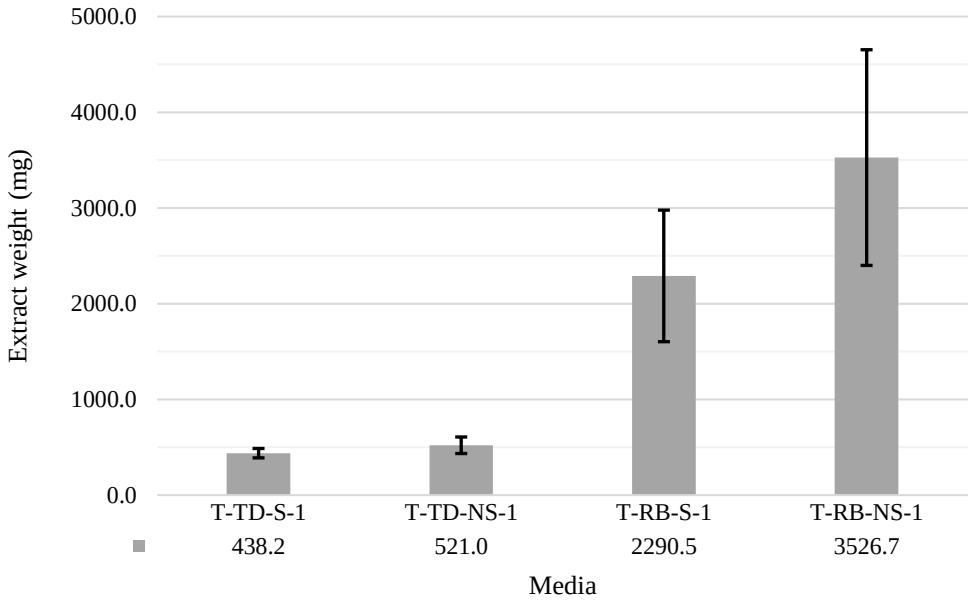


Figure 1: The amount of crude extracts of the fungal culture in different media

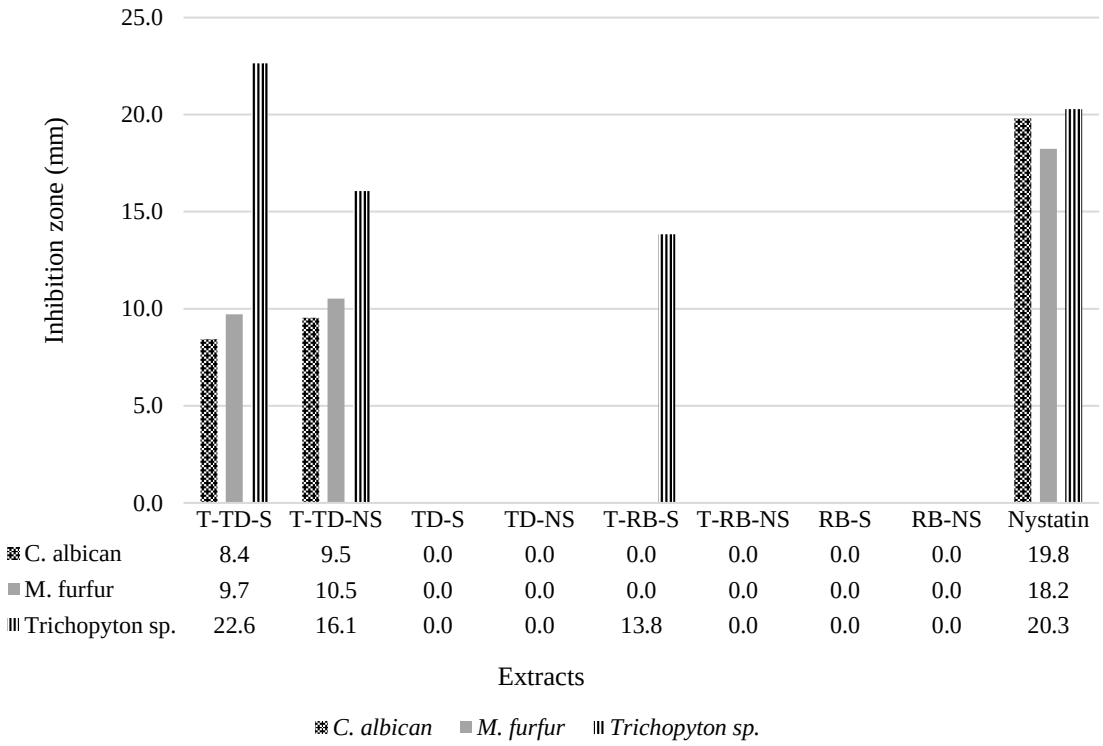
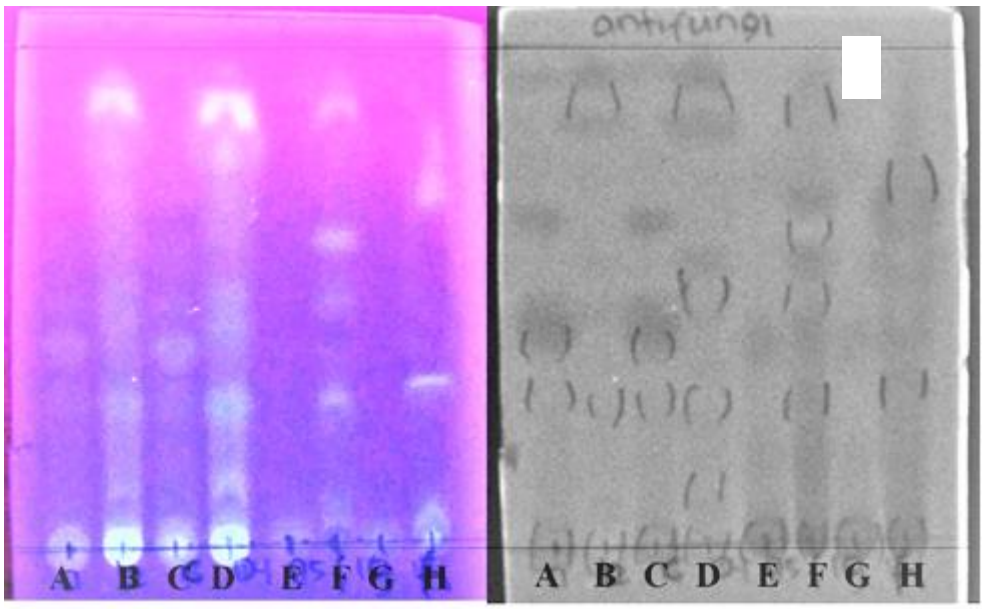


Figure 2: The antifungal activity of crude extracts of the fungal culture in different media against pathogenic fungi. Note: T-TD-S: fermented saline tofu dreg, T-TD-NS: non-fermented non-saline tofu dreg, TD-S: unfermented saline tofu dreg, TD-NS: fermented non-saline tofu dreg, T-RB-S: unfermented saline rice bran, RB-S: unfermented saline rice bran, RB-NS: unfermented non-saline rice bran, T-RB-NS: unfermented non-saline rice bran, Nystatin: positive control.



a. **b.**

Figure 3: The TLC profile (*n*-hexane:ethyl acetate=4:6) of the crude extracts of the fungal culture in different media visualized with UV light (a) and vaniline-sulfuric acid in methanol. A (TD-S: unfermented saline tofu dreg), B (T-TD-S: fermented saline tofu dreg), C (T-TD-NS: non-fermented non-saline tofu dreg), D (TD-NS: fermented non-saline tofu dreg), E (RB-S: unfermented saline rice bran), F (T-RB-S: unfermented saline rice bran), G (RB-NS: unfermented non-saline rice bran), H (T-RB-NS: unfermented non-saline rice bran)

Table 1
The UV absorbance and growth inhibition of the extract to the human cell at 1 mg/mL.

Extract		Absorbance		Number of living cell	
	1	2	Average		Inhibition (%)
T-TD-S	34.124	24.796	29.460	105	-11,9
T-TD-NS	31.794	28.607	30.201	100	-7,4
T-RB-S	33.422	31.706	32.564	109	-16,3
T-TD-NS	2.327	2.470	2.399	2	97,9
DCM	28.543	27.971	28.257	93	0,0
MCP	27.293	27.362	27.328	90	0,0
M	1.720	1.852	1.852	nc	nc

Note: Negative inhibition value indicate the cell number is higher than control. nc: not calculated. DCM=DMSO + Cell + Medium, MCP= Medium+ Cell + Penicillin, M= Medium only

Conclusion

The salinity affected the production of the antifungal compounds by *T. harzianum*, either on the tofu dregs or rice bran. The tofu dreg can be used as a medium for sponge-associated fungus *T. hazianum*. In a large scale production of antifungal compounds, the products have strong activity against the pathogenic fungi without toxic effect to the human cells.

On the other hand, rice bran is not suitable medium for the production of antifungal compounds, even though this medium supports the production of the crude extract.

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Dear Editor in Chief,

Thanks for the acceptance. Herein, I enclosed the revised manuscript.

Best regards,

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