

POTENCY OF BIOCONTROL AGENTS ISOLATED FROM COMPOST AND PEAT SOIL OF TROPICAL PEAT SWAMP FOREST IN KALAMPANGAN ZONE, CENTRAL KALIMANTAN

Yuliar^{1,2}, Zaenal Abidin³ and Wibowo Mangunwardoyo³

ABSTRACT

Rhizoctonia solani is a soil pathogen that causes diseases in wide range of hosts of agricultural, horticultural and flower crops. Biological control is the most promising way for the diseases management and it is environment friendly too. The objective of this study was to isolate and screen the potency of soil bacteria as biological control from various local compost and peat soil of tropical peat swamp forest in Kalampangan Zone, Central Kalimantan. Forty seven isolates from peat soil and compost were screened for biocontrol agent of *Rhizoctonia solani*. Seven out of thirteen peat soil isolates, and six out of thirty three compost isolates showed antagonistic activity against *R. solani* in Potato Dextrose Agar. The cultivation of the antagonistic isolates in Trypticase Soy Broth (TSB) was extracted and analysed by high performance liquid chromatography (HPLC) column. The HPLC analyzes indicated that the antagonistic isolates produce an antifungal iturin A. Macroscopic observation of isolates colonies showed that form of their colonies were amuboid, myceloid, curled, circular, rhizoid, irregular and filamentous. These achievement indicate peat swamp forest not only offer a potential biocontrol agents of damping off but also provide a new source for production of antibiotics.

Keywords: Soil bacteria, peat swamp forest, compost, *Rhizoctonia solani*, iturin A

I. INTRODUCTION

Damping off disease not only causes problem on horticultural crop, but also often makes predicament on forest plant nurseries. As reported by Hood *et al.* (2004), that *Milicia regia* seedlings had a higher probability of dying due to damping-off disease in low-light conditions characteristic of tropical forest under storey as opposed to higher light conditions that may be found in light gaps. Moreover, Lee *et al.* (2008) stated that damping off caused by *Rhizoctonia solani* resulted in yield losses in more than 200 crops globally. The use of chemical pesticides is becoming restricted because of concerns for the environment and health. Biological control is therefore a promising strategy for disease management of damping off and it is also environmental friendly.

¹Microbiology Division, Research Center for Biology, The Indonesian Institute of Science, Bogor 16002

²Corresponding author: Yrivaie@yahoo.com

³Dept. of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia

Different mechanisms are involved in the interaction between bacteria, used as a biocontrol agents, and fungal plant pathogen, such as a parasitism, cross protection, antibiosis and competition. The antibiotic mechanisms are said to operate when the metabolic products (antibiotics) produced by one species inhibits or suppresses the growth of another species (Shoda, 2000).

The main sources of microbial antibiotics are *Streptomyces* (Actinomycetes), *Bacillus* (bacteria) and *Penicillium* (fungi) (Madigan *et al.*, 1997). These bacteria are used commercially and intensively studied. Bacteria having the ability to form antifungal metabolites can be isolated easily from soil samples. Lievens *et al.* (1989) and Leyns *et al.* (1990) found about 30% of all bacteria isolated from soils were able to produce antifungal inhibition zones *in vitro*. Soil of peat swamp forest and compost are good samples for searching of bacteria for biocontrol agents. As organic material degrades, composts are able to stimulate bacteria activities (Aryantha *et al.*, 2000). Therefore, compost may contain various genus or species of bacteria; while soil of peat swamp forest that is acidic, may contain acidophilic bacteria. This research will explore the importance of peat swamp forest as source of bacterial control agents of damping off and a new source of peptide antibiotic producer. More specifically, members of the genus *Bacillus* produce a variety of antifungal peptide antibiotics (Katz and Demain, 1977). Strains of *Bacillus subtilis* have also been studied as biological control agents of plant pathogens. But, only a few of them were isolated and identified. Indonesia is a mega biodiversity country, and there is a great potential to utilize many antifungal agents especially from genus *Bacillus*. Increasing concern regarding food safety, environmental pollution and detrimental effects of agrochemical on a variety of non-target organisms, reducing the number of approved active pesticide ingredients has generated an interest in biological control agents to prevent and control plant diseases. The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides. It has been tested for control of a variety of fungi in pure cultures and during composting (Phae *et al.*, 1990). The objective of this study was to isolate and screen the potency of soil bacteria as biocontrol agents from compost and peat soil of tropical peat swamp forest especially *Bacillus* spp. These bacteria produce antifungals (iturin A). This antibiotic has a strong antifungal activity on large variety of yeast and fungi, but its activity is limited to a few bacteria specially *Micrococcus luteus* (Besson *et al.*, 1978 as cited in Yuliar, 2002).

II. MATERIALS AND METHODS

A. Samples for bacteria isolation

Samples were used for the isolation of the bacteria are as follows:

1. Peat soils from Kalampangan, Palangkaraya (2°04'51.21"S 114°02' 04.18"E) were collected in different plots (plots number; A1 (pH=6.5), E6 (pH=6.0), G5 (pH=5.4), J1 (pH=4.4), and J5 (pH=3.2) (Figure 1).

2. Various brands of composts (Ratna Cibedug compost (KA), microbiology section, Biology Research Center (KB), Enka Saritani, Bogor (KC), Depok Compost (KD), Sinar Katel Perkasa Bogor (KE) and Anonymous compost (KF).

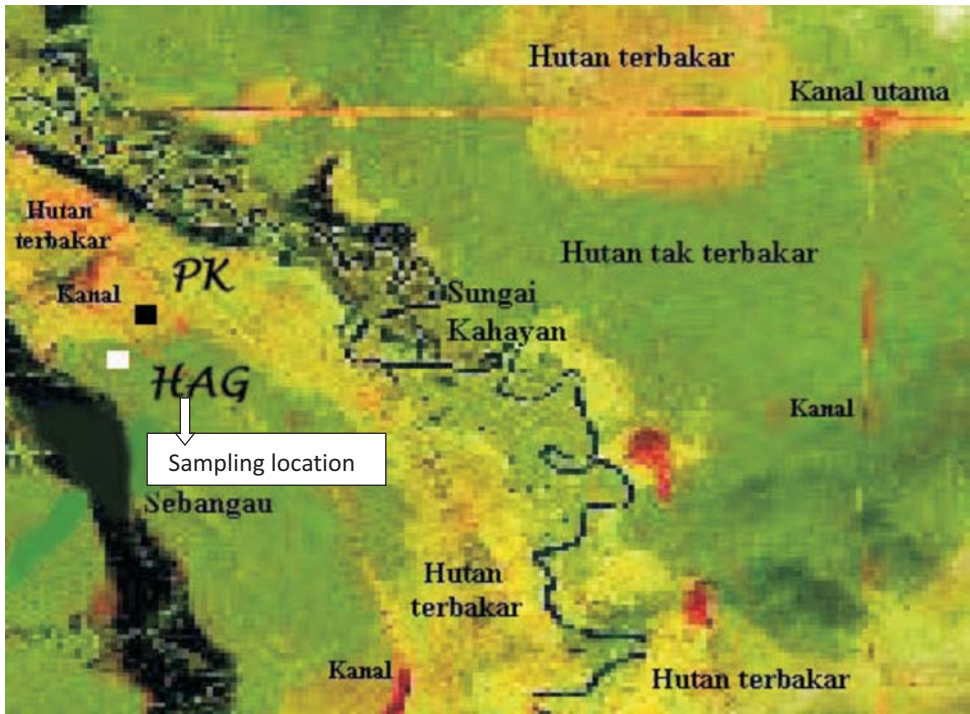


Figure1. Sampling was performed in the forest area around the station JSPS-LIPI

Research cooperation, Kalamangpan, Central Kalimantan. This area located between Kahayan and Sebangau river, approximately 40 km Southeast Palangkaraya (HAG: unburnt natural forest, plots in which sampling has been done, where A1, E6 G5, J1 and J5).

B. Bacteria isolation procedure

Bacteria isolation was carried out by the method described by Steubing (1993). Two grams of each sample (peat soil or compost) were heated at 100°C for 15 minutes to kill non-spore-forming mesophilic bacteria. After the heating, the sample was diluted with 2 ml of 0.85% NaCl and mixed thoroughly. One hundred µl of 1/4000 dilution sample was spread over the sterilized Nutrient Agar (NA) medium on petridish, and incubated at room temperature until day fifth incubation time.

C. Purification of bacteria isolates

A single colony of bacteria was streaked into sterilized NA medium plate. Then, it was incubated for two days (Steubing, 1993).

D. Precultivation

Five mL sterilized LB medium in test tube were inoculated with one loop bacteria isolate. Then, it was incubated in an incubator shaker at 37°C, 124 rpm for about 16 hours (Yuliar, 2002).

E. Cultivation

Fifty mL sterilized TSB medium in Erlenmeyer flask were inoculated with 500 μ l of pre-cultivated isolate, then it was incubated in the incubator shaker at room temperature for seven days (Yuliar, 2002).

F. Antagonistic test (*in vitro* test)

Rhizoctonia solani (sized of 5 x 5 mm) was inoculated onto the center of sterile PDA medium in petridish. After that, four holes in the PDA medium were made using cork borer (the position of four holes were at the same distance from the center of the medium, where the *R. solani* plug was placed). One hundred μ l of a 7- day cultivated isolates was put into each hole. For the negative control was distilled water. Finally, the plates were incubated for five days and growth inhibition area was observed (Yuliar, 2002).

G. Extraction and measurement of iturin A

One mL of the cultivated isolates for each of these three samples (KB6, KC3 and A13) was transferred to sterile eppendorf 1.5 mL tube and it was acidified with 2 N HCl to pH around two. After that, the samples were kept overnight at 4°C and centrifuged at 4,000 rpm for 15 minutes. Subsequently, the samples were re-suspended and extracted with one mL methanol for about one hr at room temperature. Then, the samples were centrifuged at 4,000 rpm for 15 min, and the supernatant was filtered with 0.20 μ m PTFE membrane filter (Albet-JPT 020, Hahnemuhle company, Barcelona, Spain). Twenty μ l of the filtrate was injected into HPLC (WATER, WATER cooperation, Milford, USA) and was monitored by the UV detector at λ 205 nm. Condition of HPLC was as follows: Mobile phase; acetonitrile: ammonium acetate (35:65), column C18, flow rate 2 mL/min.

H. Measurement of cell concentration and pH

Cell concentration was measured by optical density using a spectrophotometer

(Perkin Elmer, Perkin Elmer company, Cambridge, USA) at OD 660 nm. pH was measured with a pH meter (Horiba, Horiba Ltd, Kyoto, Japan).

III. RESULTS AND DISCUSSION

A. Number of Bacterial Isolates

Isolations were conducted on 14 bacterial from peat soil and 33 isolates from composts (Table 1). The result showed that soil of peat swamp forest contains less number of bacterial isolates than compost. It was probably because the pH of soil of peat swamp forest was lower than the compost pH. The pH range of peat soil samples were 3.2 to 6.5 and compost samples pH were about 6.8.

Macroscopic observation showed that the colony of isolates were effuse, low convex, raised and raised with concave beaded edge (Table 2). Edges of the colony form were: verrucose, wave and irregular. A single colony form was; circular, amuboid, myceloid, curled, rhizoid, filamentous and irregular. The colors of colonies were varied from white to yellow.

B. Cell concentration and pH of isolates cultivation in Tryptic Soy Broth medium

Isolates A11, KB6 and KC3 entered to the end of exponential growth phase on days two and days three of incubation times and the end of stationary phase occurred on days five of incubation time (data not shown). pH of cultivation medium increased from seven on days three to nine on days five of incubation time (data was not shown). The pH increased of the medium occurred because the isolates produced the secondary of metabolite compounds namely iturin A (see HPLC analysis). Huang *et al.* (1993) described the chemical structure of iturin A is a cyclic peptidolipid, that contains L-amino acids (Asparagine and Glutamine) (Figure 2). These amino acids are bases, so that the pH of medium increased from seven to nine.

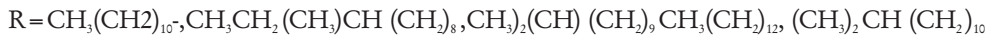
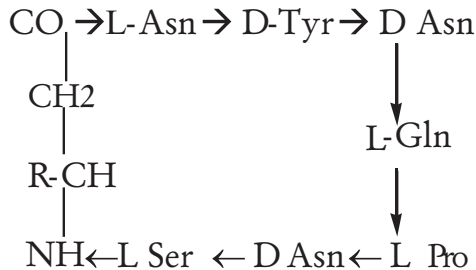


Figure 2. Chemical structure of iturin A

C. *In vitro* test

In vitro test indicated that a seventh 7-day cultivation time of 13 isolates (A12, A13, A14, J11, J13, J51, J52, KB2, KB4, KB6, KC2, KC3, and KC) inhibited *R. solani* growth (Figure 3 A). The ability of the isolate to inhibit *R. solani*, was because the isolates produce an antifungal of iturin A. Iturin A belongs to polypeptide antibiotic that is secreted by *Bacillus* spp. to the cultivation medium (Shoda, 2000; Yuliar, 2002). Phae *et al.* (1990) also reported that four isolates (*Bacillus* spp.) out of 204 isolates inhibited *R. solani* growth. Some others possible inhibition mechanisms of biocontrol agent of the plant diseases *in vitro* test are chitinase (Huang *et al.*, 2005) and protease production (Olajuyigbe and Ajele, 2005) and siderophore production (de Boer *et al.*, 2003). Mechanisms of biological control agents to antagonize plant pathogens use multiple actions. For instance, *Pseudomonas* known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses (Lavicoli *et al.*, 2003). Additionally, DAPG producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients (Raaijmakers and Weller, 2001). There have been many researcher reported a potential use of biocontrol agent microorganisms as an agent stimulating of plant growth (Bottini *et al.*, 2004), fixing nitrogen (Khan and Doty, 2009) and solubilizing phosphate (Malboobi *et al.*, 2009). These reports implied that biocontrol agents not only important for agronomic crops but also applicative and necessary for plant forest. Furthermore application of biocontrol agent A12, A13, A14, J11, J13, J51, J52 to plant of peat swamp forest will get adaptation process easier to environment, because their source are from soil of peat swamp forest. Additionally their application results may be better than with another biocontrol agent that are isolated from other than peat soil. The reason is to achieve an optimal function of them, they should be in optimal growth condition as well as an optimal growth can be reach when they can adapt well to their environment.

D. Detection of Iturin A

Iturin A fraction was detected by peaks of retention times of 4.317, 5.642, 5.892 and 9.092 minutes (Figure 4). Yuliar (2002) reported that iturin A fraction of *Bacillus subtilis* strain RB14-CS has chromatogram peaks pattern with five peaks. This is in an agreement with Phae *et al.* (1990) and Yu *et al.* (2002) that detected iturin A with the same peaks pattern. In this experiment, we found only four peaks of the iturin A, with number areas of: 699684, 267647, 365363 and 109323. HPLC analysis was performed on three samples: KB6, KC3 and A13, their concentration were about 3.7 ppm for KB 6 and KC3 and 3.1 ppm for A13 (Figure 5, 6 and 7). The iturin A production of the three isolates was lower than iturin A production by *Bacillus subtilis* S499 (140 mg/l) that was reported by Jacques *et al.* (1999). The highest iturin A production was 3,300 mg/L that was produced by *B. subtilis* RB14-CS in soybean meal medium (Yuliar, 2002). The

lowest of iturin A production by isolate KB6, KC3 and A13, it was probably because of medium production (TSB) was not a good medium for iturin A production. As Theobald *et al.* (2000) declared that antibiotic production depend on the medium composition especially on the carbon and nitrogen source and the fermentation condition. Antibiotic production is also dependent on culture condition like temperature, pH, aeration, agitation and cultivation methods. Lynch and Bushell (1995) reported that erythromycin production was significantly enhanced in cyclic feed batch culture compared to batch culture. Furthermore, micro elements also influence the antibiotics production. Wei and Cu (1998) were successful on enhancement of surfactin productivity by addition of 2-4 mM iron to the medium cultivation. The supplementation of iron to the culture highly improved the production of surfactin as high as 3,500 mg/L which was almost ten times of the control.

Table 1. Bacteria isolates (*Bacillus* spp.) from peat soil and composts

No.	Soil samples	Samples codes	pH	Isolates codes
1.	Soil peat swamp forest	A1	6.5	A11
2.				A12
3.				A13
4.				A14
5.		E6	6.0	E61
6.				E62
7.		G5	5.4	G51
8.				G52
9.		J1	4.4	J11
10.				J12
11.				J13
12.		J5	3.2	J51
13.				J52
14.				J53
15.	Compost Pondok Ratna Cibebug	KA	6.8	KA1
16.				KA2
17.				KA3
18.	Compost Microbiology, Research Center Biology, LIPI	KB	6.8	KB1
19.				KB2
20.				KB3
21.				KB4
22.				KB5
23.				KB6
24.				KB7
25.				KB8

Table 1. Continued

No.	Soil samples	Samples codes	pH	Isolates codes
26.	Compost Enkaritani Bogor	KC	6.8	KC1
27.				KC2
28.				KC3
29.				KC4
30.				KC5
31.				KC6
32.	Compost Depok	KD	6.8	KD1
33.				KD2
34.	Compost Sinar Katel Perkasa Bogor	KE	6.8	KE1
35.				KE2
36.				KE3
37.				KE4
38.				KE5
39.				KE6
40.	Compost Bogor (unlabelled)	KF	6.8	KF1
41.				KF2
42.				KF3
43.				KF4
44.				KF5
45.				KF6
46.				KF7
47.				KF8

Table 2. Macroscopic observation of *Bacillus* spp. colonies

<i>Bacillus</i> spp.	Elevationedge	Surface	Form	Colour	Codes
A11	low convex	undulate	irregular	amuboid	cream
A12	low convex	lobate	Irregular	amuboid	cream
A13	low convex	lobate	Irregular	amuboid	cream
A14	effuse	lobate	irregular	amuboid	cream
E61	low convex	erose	smooth	myceloid	cream
E62	low convex	undulate	irregular	myceloid	cream
G51	convex	erose	smooth	circular	light yellow
G52	effuse	lobate	verrucoser	myceloid	white
J11	convex	undulate	undulate	curled	light cream
J12	raised	erose	smooth	myceloid	light cream
J13	effused	entire	smooth	circular	light yellow
J51	low convex	erose	smooth	circular	light cream
J52	raised	lobate	smooth	circular	cream
J53	effuse	crenate	irregular	rhizoid	light yellow

Table 2. Continued

<i>Bacillus</i> spp.	Elevationedge	Surface	Form	Colour	Codes
KA1	raised	undulate	smooth	circular	light cream
KA2	effuse	lobate	smooth	amuboid	cream
KA3	raised with concave beaded edge	erose	smooth	circular	light yellow
KB1	raised	crenate	verrucose	amuboid	cream
KB2	convex	lacerate	undulate	rhizoid	cream
KB3	low convex	undulate	smooth	curled	light brown
KB4	low convex	entire	verrucose	irregular	white
KB5	low convex	undulate	smooth	irregular	light cream
KB6	low convex	entire	smooth	circular	light brown
KB7	low convex	undulate	verrucose	circular	cream
KB8	low convex	entire	verrucose	circular	cream
KC1	effuse	ramose	smooth	rhizoid	cream
KC2	raised	entire	smooth	circular	light cream
KC3	low convex	erose	smooth	circular	cream
KC4	low convex	crenate	smooth	irregular	cream
KC5	low convex	entire	smooth	circular	cream
KC6	effuse	lacerate	smooth	myceloid	cream
KD1	effuse	crenate	irregular	amuboid	light cram
KD2	low convex	lobate	verrucose	rhizoid	light cram
KE1	low convex	crenate	smooth	circular	light cream
KE2	low convex	lobate	verrucose	irregular	cream
KE3	low convex	undulate	smooth	irregular	white
KE4	effuse	erose	smooth	filamentous	white
KE5	raised	entire	smooth	cueled	light cream
KE6	low convex	lobate	smooth	circular	white
KF1	effuse	lobate	smooth	rhizoid	dark cream
KF2	effuse	entire	irregular	amuboid	cream
KF3	low convex	entire	smooth	circular	white
KF4	effuse	erose	smooth	amuboid	dark yellow
KF5	effuse	lobate	smooth	amuboid	light cream
KF6	low convex	undulate	undulate	curled	white
KF7	low convex	smooth	smooth	amuboid	light cream
KF8	effuse	smooth	smooth	curled	light cream

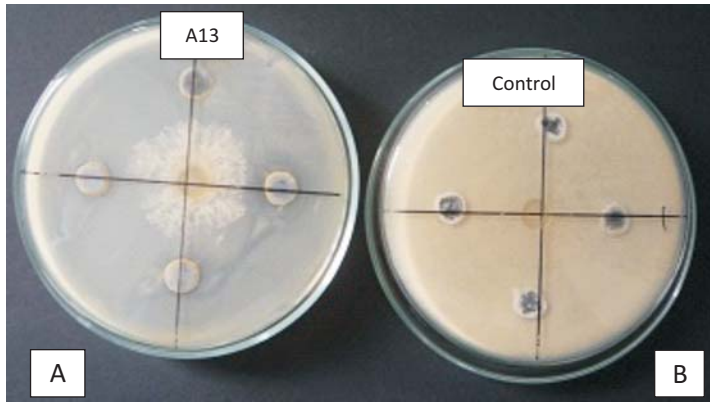


Figure 3. (A) Culture broth of the isolates A13 inhibited *Rhizoctonia solani* growth, the inhibition area were observed around of the four holes (*R. solani* mycelia keep away from the holes). (B) Sterilized distilled water as control did not inhibit *R. solani* growth, its mycelia bypass the holes.

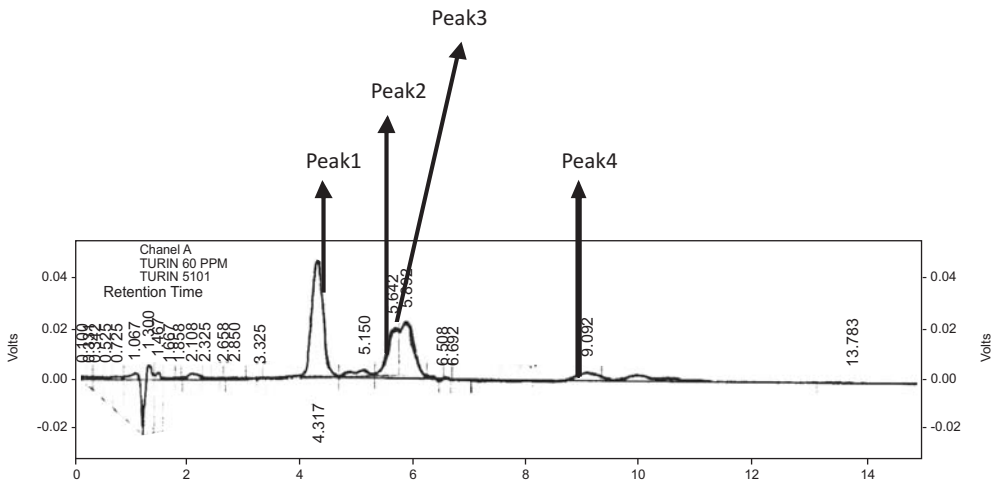


Figure 4. HPLC pattern of iturin A standard (50 ppm), detector A-1 (205 nm)

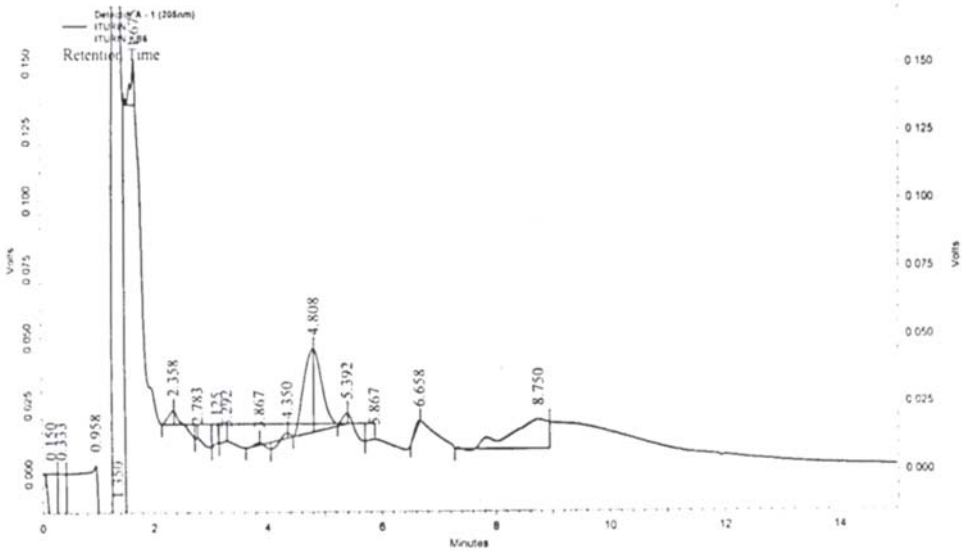


Figure 5. HPLC pattern of iturin A of KB6 isolate, detector A-1 (205 nm)

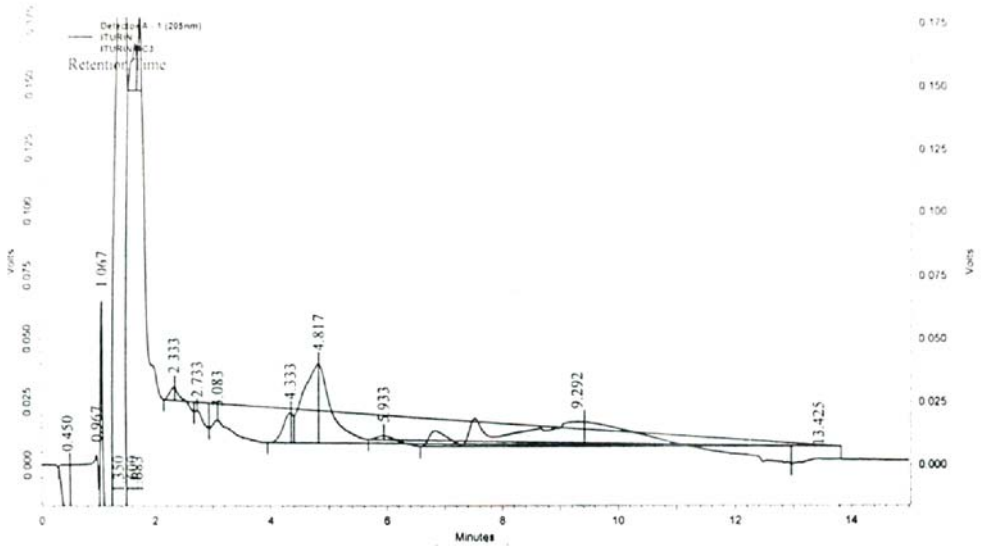


Figure 6. HPLC pattern of iturin A of KC3 isolate, detector A-1 (205 nm)

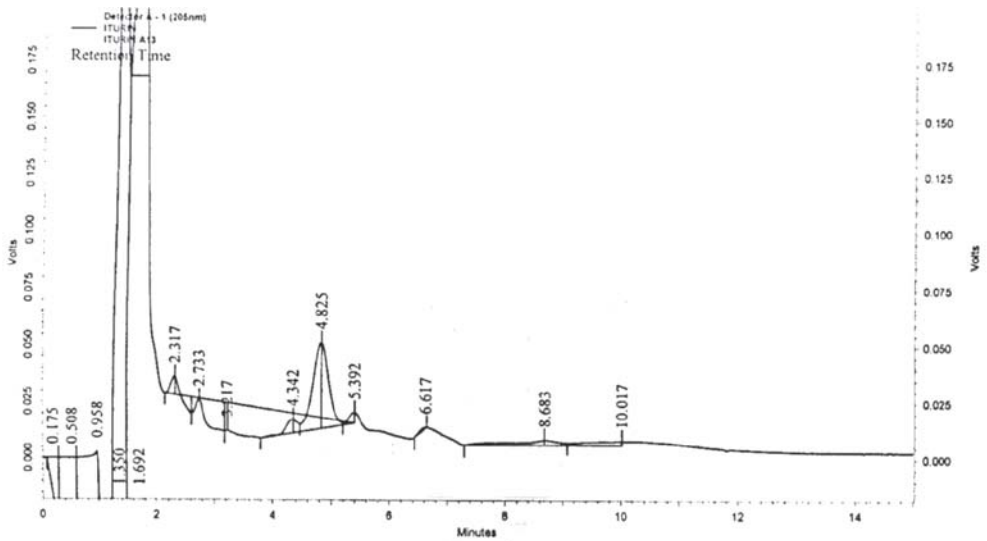


Figure 7. HPLC pattern of iturin A of A13 isolate, detector A-1 (205 nm).

IV. CONCLUSION

This study shows that seven out of 13 peat soil isolates, and six out of 33 compost isolates showed antagonistic activity against *R. solani* in PDA. Thirteen isolates (A12, A13, A14, J11, J13, J51, J52, KB2, KB4, KB6, KC2, KC3, and KC) are potential isolates to inhibit *R. Solani* growth. The highest inhibition zone was observed for isolates number KB6, with 32 milliliter the clear zone diameter. The antagonistic isolates produce an antifungal iturin A in TSB medium. It is also need futher research, especially in greenhouse test of the potential isolates to suppress damping off on agricultural crops and plantation forest.

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