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Optimization of Bioactive Metabolite Production From *Fusarium oxysporum* LBKURCC41

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Abstract. *Fusarium oxysporum* is one of endophyte microbes isolated from dahlia tuber (*Dahlia variabilis*) which has the ability to produce secondary metabolites. In this research, optimization of chemichal and physical fermentation conditions were carried out. Corn, potato, and sweet potato with particle size of 80 mesh were used as corbon sources to produce secondary metabolite for 5, 10, 15, 20, and 25 days respectively with ethyl acetate was used to extract secondary metabolites. The assessment of antimicrobial activity was then performed against *Candida albican*, *Escherichia coli* and *Staphylacoccus aureus*. A serial of metabolite concentrations 5.7 mg/ml, 3.8 mg/ml, and 1.9 mg/ml were used. The optimum inhibition against microbial pathogen growth was corn for 15 day fementation (C15). The inhibition zone against *Candida albicans*, *E.coli*, and *S. aureus* were 7.62±0.32, 14.15±0.09, and 15.24±0.24 mm respectively at 5.7 mg/ml metabolite concentration. The result of metabolites screening showed overall extract secondary metabolites comprised terpenoid group. The separation of the active ingredients was performed using both Thin Layer Chromatoghraphy and High Performance Liquid Chromatoghraphy (HPLC) depicted 4 component.

Keywords: *Fusarium oxysporum*, optimization, antimicrobial, secondary metabolite and terpenoid.

1. Introduction

Endophytic fungi is defined as the fungi which spend the whole or part of their lifecycle colonizining inter and/or intracellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease [1]. In the past decade, many valuable bioactive compounds have been successfully discovered from endophytes. During the long period of co-evolution, a friendly relationship was gradually set up between each endophyte and its host plant. Therefore, endopyhtes show abilty to produce same or similar bioactive subtances as those originated from their host plants [2].

Endophytic fungi have also been reported to possess bioactive secondary metabolites and enzymes such as inulinase, chitinase, amylase, and cellulase [3-4]. Endophyte microbial could be classified as bacteria, fungi, and actinomycetes [5]. *Fusarium* sp LBKURCC73 produced cellulase [6], while *Fusarium oxysporum* (LBKURCC6) isolated from pink-flowered dahlia tuber have ability to produce inulinase [7].

Fusarium oxysporum (LBKURCC41) is an endophytic fungi isolated from red-flowered dahlia tuber collected from Bukit tinggi, Sumatera barat [8], it was indentified based on the gene sequenced analyse according to a molecular biological protocol by DNA amplification and sequencing of the ITS region [9]. On the other hand, it has been found ability to produce saponin which produced in Huang medium

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production with antimicrobial activity [10-11]. The production of secondary metabolite from the *Fusarium oxysporum* can be influenced by medium and time of fermentation. The aim of this research was to discover the best natural carbon resources and time fermentation in order to produce optimum secondary metabolites through corn, potato, and sweet potato.

2. Materials and Methods

Sample Preparation

Corn, potato and sweet potato bought at traditional market in Pekanbaru, Riau, Indonesia were washed and dried at sunlight for 8 h and 40°C for 48h. All sample were blendered separately and sieved for 80 mesh. The powder was stored saparately at room temperature.

Cultivation and preparation of *Fusarium oxysporum* LBKURCC41

Isolate LBKURCC41 *Fusarium oxysporum* was cultivated in potato dextrose agar asecptically and incubated for 4 days at room temperature. Fresh isolate rinsed with NaCl 0.8%, scraped and filtered using sterile glasswoll. Suspencion of spores inoculated into potato dextrose broth at ~7 x 10^{12} spore/ml was determined optical density using spectrophotometer Ultra Viloet-Visible (UV-Vis) Genesis 10 S (*Thermo Scienstific)* then incubated for 4 days at room temperature using *rotary shaker shaking incubator* model LSI 301 6R (Daihan Lab Tech Co. LTD) with150 rpm.

Fermentation

Inoculum starter was placed to Huang production medium with modification in carbon source (corn, potato, and sweet potato) and fementation time 5, 10, 15, 20, and 25 days. After fermentation filtrate was saparated by Buchner funnel through filter paper Whatman No.1. The filtrate was transferred aseptically into a conical flask and stored at 4°C for futher assay. The culture filtrate was extracted 3 times with ethyl acetate. The ethyl acetate phase was saparated from aquos using saparating funnel. Ethyl acetate layer concentrate was evaporated using *vacuum rotary evaporator* Heidolph WB 2000, and stored at room temperature for futher assay.

Antimicrobial assesment

Crude extract was tested for antimicrobial activity using agar well difussion method against *Candida albicans*, Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*). Extract was solved in methanol with a series of concentration of 5.7, 3.8, and 1.9 mg/ml. ketokenazole and amoxan 3.8 mg/ml were used as positive control while methanol as negative control.

Screening of secondary metabolites

Secondary metabolites were screening for their content of terpenoid/steroid, alkaloid, phenolic, flavonoid dan saponin.

Separation Using TLC and HPLC

Extract possessing optimum antimicrobial activity was analyzed further with TLC and HPLC. Aluminum plates pre-coated with silica gel as stationary phase and ethyl acetate 100% as a mobile phase. Chromatograms were observed under UV light, and calculated the retention factor. On the other hand, a number 20 µl extract was analyzed by HPLC Shim-pack VP-ODS (250 x 4,6 mm) using water:methanol sovent with gradient elution system. UV detector was used to determine its maximum wavelenght.

3. Result and Discussion

Fusarium oxysporum growth and fermentation observation

Fusarium oxysporum grew at the second day, produced white mycelia on PDA and purple spore at third day. This inoculum was removed to modified production medium after being incubated for 96 hours. Secondary metabolites produced by *Fusarium oxysporum* were assayed against 3 microbial pathogens. Inhibition zone resulted against *Candida albicans* was showed in Table 1.

Table 1. Ethyl acetate extract from *Fusarium oxysporum* metabolites and inhibition zone against *Candida albicans*

Carbon source	Fermentation Day	Concentration	Inhibition zone diameter (mm)
Corn	5	Extract 5.7 mg/ml	6.74±0.34[b]
		Extract 3.8 mg/ml	0.00±0.00[c]
		Extract 1.9 mg/ml	0.00±0.00[c]
		Ketoconazole	8.31±0.32[c]
		Methanol	0.00±0.00[c]
Corn	10	Extract 5.7 mg/ml	6.81±0.12[b]
		Extract 3.8 mg/ml	6.14±0.04[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	8.28±0.27[a]
		Methanol	0.00±0.00[d]
Corn	15	Extract 5.7 mg/ml	7.62±0.00[a,b]
		Extract 3.8 mg/ml	7.37±0.15[b]
		Extract 1.9 mg/ml	6.29±0.05[c]
		Ketoconazole	7.87±0.13[a]
		Methanol	0.00±0.00[d]
Corn	20	Extract 5.7 mg/ml	7.22±0.03[b]
		Extract 3.8 mg/ml	6.73±0.00[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.37±0.15[a]
		Methanol	0.00±0.00[d]
Corn	25	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Ketoconazole	7.39±0.16[a]
		Methanol	0.00±0.00[b]
Potato	5	Extract 5.7 mg/ml	6.54±0.05[b]
		Extract 3.8 mg/ml	0.00±0.00[c]
		Extract 1.9 mg/ml	0.00±0.00[c]
		Ketoconazole	7.54±0.24[a]
		Methanol	0.00±0.00[c]
Potato	10	Extract 5.7 mg/ml	6.91±0.11[b]
		Extract 3.8 mg/ml	6.26±0.01[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.09±0.17[a]
		Methanol	0.00±0.00[d]
Potato	15	Extract 5.7 mg/ml	7.39±0.10[b]
		Extract 3.8 mg/ml	6.44±0.03[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.76±0.07[a]
		Methanol	0.00±0.00[d]
Potato	20	Extract 5.7 mg/ml	7.18±0.11[b]
		Extract 3.8 mg/ml	6.53±0.40[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.88±0.16[a]
		Methanol	0.00±0.00[d]
Potato	25	Extract 5.7 mg/ml	6.56±0.18[b]
		Extract 3.8 mg/ml	0.00±0.00[c]
		Extract 1.9 mg/ml	0.00±0.00[c]
		Ketoconazole	7.43±0.22[a]
		Methanol	0.00±0.00[c]

Sweet potato	5	Extract 5.7 mg/ml	7.42±0.08[b]
		Extract 3.8 mg/ml	6.27±0.06[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.63±0.15[a]
		Methanol	0.00±0.00[d]
Sweet potato	10	Extract 5.7 mg/ml	7.77±0.03[b]
		Extract 3.8 mg/ml	6.52±0.04[c]
		Extract 1.9 mg/ml	6.23±0.08[d]
		Ketoconazole	8.09±0.16[a]
		Methanol	0.00±0.00[e]
Sweet potato	15	Extract 5.7 mg/ml	8.01±0.06[a]
		Extract 3.8 mg/ml	7.00±0.13[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	7.76±0.03[a]
		Methanol	0.00±0.00[d]
Sweet potato	20	Extract 5.7 mg/ml	7.20±0.06[b]
		Extract 3.8 mg/ml	6.68±0.03[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Ketoconazole	8,41±0,22[a]
		Methanol	0.00±0.00[d]
Sweet potato	25	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Ketoconazole	8.41±0.21[a]
		Methanol	0.00±0.00[b]

Means were calculated by one-way ANOVA and separated by Duncan least Significicant Difference at 5% level. Value with same alphabets does not differ significantly. Ketoconaloze as positive control, methanol as negative control.

Based on antifungal activity from secondary metabolites obtained from *Fusarium oxysporum* against *Candida albicans* showed sweet potato for 15 day fermentation possessed highest antifungal activity with inhibition zone diameter 8.01 mm at 5.7 mg/ml.

Inhibition zone diameters of ethyl acetate extract from *Fusarium oxysporum* against *Escherichia coli* is showed in Table 2.

Table 2. Ethyl acetate extract from *Fusarium oxysporum* metabolite and inhibition zone diameters against *Escherichia coli*

Carbon source	**Fermentation (Day)**	**Concentration assay**	**Averages**
Corn	5	Extract 5.7 mg/ml	13.70±0.22[b]
		Extract 3.8 mg/ml	7.79±0.22[c]
		Extract 1.9 mg/ml	7.02±0.23[d]
		Amoxan	14.68±0.25[a]
		Methanol	0.00±0.00[e]
Corn	10	Extract 5.7 mg/ml	13.72±0.36[a]
		Extract 3.8 mg/ml	8.22±0.26[b]
		Extract 1.9 mg/ml	7.24±0.18[c]
		Amoxan	14.04±0.76[a]
		Methanol	0.00±0.00[d]
Corn	15	Extract 5.7 mg/ml	14.15±0.09[a]
		Extract 3.8 mg/ml	10.08±0.62[b]
		Extract 1.9 mg/ml	7.31±0.18[c]
		Amoxan	14.24±0.36[a]
		Methanol	0.00±0.00[d]
Corn	20	Extract 5.7 mg/ml	11.55±0.21[b]

		Extract 3.8 mg/ml	9.97±0.51[c]
		Extract 1.9 mg/ml	8.37±0.51[d]
		Amoxan	13.83±0.15[a]
		Methanol	0.00±0.00[e]
Corn	25	Extract 5.7 mg/ml	11.16±0.39[b]
		Extract 3.8 mg/ml	8.66±0.21[c]
		Extract 1.9 mg/ml	7.37±0.31[d]
		Amoxan	12.80±0.14[a]
		Methanol	0.00±0.00[e]
Potato	5	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Amoxan	7.65±0.20[a]
		Methanol	0.00±0.00[b]
Potato	10	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Amoxan	7.63±0.27[a]
		Methanol	0.00±0.00[b]
Potato	15	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Amoxan	7.87±0.12[a]
		Methanol	0.00±0.00[b]
Potato	20	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Amoxan	7.56±0.26[a]
		Methanol	0.00±0.00[b]
Potato	25	Extract 5.7 mg/ml	0.00±0.00[b]
		Extract 3.8 mg/ml	0.00±0.00[b]
		Extract 1.9 mg/ml	0.00±0.00[b]
		Amoxan	7.67±0.16[a]
		Methanol	0.00±0.00[b]
Sweet potato	5	Extract 5.7 mg/ml	7.56±0.33[b]
		Extract 3.8 mg/ml	6.60±0.22[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Amoxan	11.49±0.53[a]
		Methanol	0.00±0.00[d]
Sweet potato	10	Extract 5.7 mg/ml	9.96±0.21[b]
		Extract 3.8 mg/ml	7.71±0.29[c]
		Extract 1.9 mg/ml	6.45±0.21[d]
		Amoxan	12.77±0.13[a]
		Methanol	0.00±0.00[e]
Sweet potato	15	Extract 5.7 mg/ml	10.54±0.32[b]
		Extract 3.8 mg/ml	8.45±0.40[c]
		Extract 1.9 mg/ml	6.71±0,06[d]
		Amoxan	11.77±0.21[a]
		Methanol	0.00±0.00[e]
Sweet potato	20	Extract 5.7 mg/ml	8.65±0,33[b]
		Extract 3.8 mg/ml	7.06±0.69[c]
		Extract 1.9 mg/ml	6.56±0.31[c]
		Amoxan	11.37±0.46[a]
		Methanol	0.00±0.00[d]
Sweet potato	25	Extract 5.7 mg/ml	7.42±0.36[b]
		Extract 3.8 mg/ml	6.44±0.21[c]

	Extract 1.9 mg/ml	6.35±0.21[c]
	Amoxan	10.37±0.48[a]
	Methanol	0.00±0.00[d]

Means were calculated by one-way ANOVA and separated by Duncan least Significicant Difference at 5% level. Value with same alphabets does not differ significantly. Amoxsan as positive control, and methanol as negative control.

Based on antibacterial activity from overall extract metabolites isolated from *Fusarium oxysporum* against *E.coli* showed that corn as carbon source for 15 day fermentation indicated highest antibacterial activity with inhibition zone diameter 10.54 mm at 5.7 mg/ml. Inhibition zone diameters against *Staphylococcus aureus* is showed in Table 3.

Table 3. Ethyl acetate extract from *Fusarium oxysporum* metabolite and inhibition zone diameters against *S. aureus*

Carbon source	Fermentation (Day)	Concentration assay	Averages
Corn	5	Extract 5.7 mg/ml	7.09±0.09[b]
		Extract 3.8 mg/ml	6.68±0.22[c]
		Extract 1.9 mg/ml	0.00±0.00[d]
		Amoxan	17.51±0.42[a]
		Methanol	0.00±0.00[d]
Corn	10	Extract 5.7 mg/ml	8.68±0.29[b]
		Extract 3.8 mg/ml	7.56±0.32[c]
		Extract 1.9 mg/ml	6.72±0.25[d]
		Amoxan	17.44±0.32[a]
		Methanol	0.00±0.00[e]
Corn	15	Extract 5.7 mg/ml	15.24±0.42[b]
		Extract 3.8 mg/ml	11.78±0.29[c]
		Extract 1.9 mg/ml	10.86±0.13[d]
		Amoxan	16.09±0.39[a]
		Methanol	0.00±0.00[e]
Corn	20	Extract 5.7 mg/ml	12.54±0.29[b]
		Extract 3.8 mg/ml	10.26±0.29[c]
		Extract 1.9 mg/ml	9.03±0.24[d]
		Amoxan	15.53±0.38[a]
		Methanol	0.00±0.00[e]
Corn	25	Extract 5.7 mg/ml	9.98±0.53[b]
		Extract 3.8 mg/ml	8.01±0.21[c]
		Extract 1.9 mg/ml	6.77±0.25[d]
		Amoxan	16.63±0.29[a]
		Methanol	0.00±0.00[e]
Potato	5	Extract 5.7 mg/ml	11.34±0.49[b]
		Extract 3.8 mg/ml	9.57±0.09[c]
		Extract 1.9 mg/ml	7.93±0.08[d]
		Amoxan	15.83±0.14[a]
		Methanol	0.00±0.00[e]
Potato	10	Extract 5.7 mg/ml	11.63±0.34[a]
		Extract 3.8 mg/ml	9.64±0.39[c]
		Extract 1.9 mg/ml	8.18±0.20[d]
		Amoxan	15.76±0.36[a]
		Methanol	0.00±0.00[e]
Potato	15	Extract 5.7 mg/ml	12.20±0.57[b]
		Extract 3.8 mg/ml	8.74±0.27[c]
		Extract 1.9 mg/ml	7.36±0.19[d]
		Amoxan	15.64±0.34[a]

		Methanol	0.00±0.00[e]
Potato	20	Extract 5.7 mg/ml	8.57±0.31[b]
		Extract 3.8 mg/ml	7.45±0.27[c]
		Extract 1.9 mg/ml	7.08±0.15[c]
		Amoxan	14.27±0.28[a]
		Methanol	0.00±0.00[e]
Potato	25	Extract 5.7 mg/ml	7.40±0.29[b]
		Extract 3.8 mg/ml	6.98±0.19[b,c]
		Extract 1.9 mg/ml	6.62±0.33[c]
		Amoxan	13.64±0.29[a]
		Methanol	0.00±0.00[d]
Sweet potato	5	Extract 5.7 mg/ml	7.54±0.44[b]
		Extract 3.8 mg/ml	6.97±0.52[b,c]
		Extract 1.9 mg/ml	6.59±0.51[c]
		Amoxan	13.30±0.13[a]
		Methanol	0.00±0.00[d]
Sweet potato	10	Extract 5.7 mg/ml	8.69±0.31[b]
		Extract 3.8 mg/ml	7.66±0.26[c]
		Extract 1.9 mg/ml	7.57±0.07[c]
		Amoxan	14.44±0.10[a]
		Methanol	0.00±0.00[d]
Sweet potato	15	Extract 5.7 mg/ml	8.88±0.35[b]
		Extract 3.8 mg/ml	7.95±0.25[c]
		Extract 1.9 mg/ml	7.68±0.41[c]
		Amoxan	14.60±0.57[a]
		Methanol	0.00±0.00[d]
Sweet potato	20	Extract 5.7 mg/ml	9.05±0.35[b]
		Extract 3.8 mg/ml	8.37±0.54[b,c]
		Extract 1.9 mg/ml	7.75±0.09[c]
		Amoxan	14.78±0.56[a]
		Methanol	0.00±0.00[d]
Sweet potato	25	Extract 5.7 mg/ml	7.06±0.51[b]
		Extract 3.8 mg/ml	6.81±0.08[b]
		Extract 1.9 mg/ml	0.00±0.00[c]
		Amoxan	13.18±0.52[a]
		Methanol	0.00±0.00[c]

Means were calculated by one-way ANOVA and separated by Duncan least Significicant Difference at 5% level. Value with same alphabets does not differ significantly. Amoxsan as positive control, and methanol as negative control.

Based on antimicrobial activity from overall extract metabolites were isolated from *Fusarium oxysporum* toward *S. aureus*, corn as carbon source for 15 day fermentation posses highest antibacterial activity with inhibition zone diameter 15.24 mm at 5.7 mg/ml..

The result of metabolite screening was carried out toward overall ethyl acetate extract isolated from *Fusarium oxysporum* (Table 4).

Table 4. Screening metabolit test of ethyl acetate extract isolated from *Fusarium oxysporum*

Carbon source	Fermentation (day)	Metabolites test				
		Alkaloid	Phenolic	Flavonoid	Saponin	Terpenoid
Corn	5	–	–	–	–	+
	10	–	–	–	–	+
	15	–	–	–	–	+
	20	–	–	–	–	+
	25	–	–	–	–	+
Potato	5	–	–	–	–	+

	10	–	–	–	–	+
	15	–	–	–	–	+
	20	–	–	–	–	+
	25	–	–	–	–	+
Sweet potato	5	–	–	–	–	+
	10	–	–	–		+
	15	–	–	–	–	+
	20	–	–	–	–	+
	25	–	–	–	–	+

Metabolites isolated from *Fusarium oxysporum* achieved maximum production at 15 day fermentation and decreased at 20 and 25 day fermentation, which was assumed to have started death phase [12]. Production of secondary metabolites by microorganisms was often influenced by primary metabolism. The composition and medium concentration were closely linked with the metabolic producing capacities by microorganism and greatly influenced the biosynthesis of bioactive molecules. There were many factors playing important to produce secondary metabolite such as carbon source and fermentation time [13]. Overally, metabolite extract with corn as carbon source and 15 day fermentation (C15) showed better activity than other extracts. Inhibition zone diameter lower than triterpenoid saponin was isolated from endophytic fungi *Fusarium* sp from *Panax notogingseng* plant [14].

Based on screening metabolite *Fusarium oxysporum* have the ability to produce secondary metabolite, overally comprise terpenoid group through mevalonate pathway. Mostly, crude extract from *Fusarium oxysporum* showed antimicrobial activty againts Gram positive bacteria of genera *Staphylococcus aureus*. Previously Ibrahim et al (2016) reported endhopytic fungus *Fusarium* sp isolated from the roots *Mentha longifolia* have the ablity to produce two new tetracyclic terpenoid, and antibacterial acitvity [15]. *Fusarium chlamydosporium* isolated from leaves *Anvillea garcinii* produced aminobenzamide derivative with potent cytotoxic and antimicrobial agent (Ibrahim *et al*., 2018) [16].

C15 metabolite has been analyzed both thin layer chromatography and high performance liquid chromatography and depicted 4 component with Rf 0,3 ; 0,7; 0,84 ; dan 0,93 respectively using ethyl acetate 100% as mobile phase for TLC and water and methanol for HPLC with t_R 16.86, 17.62, 18.23 and 21. 54 min. Every peak comfirm one compound, it was showed at Fig 1.

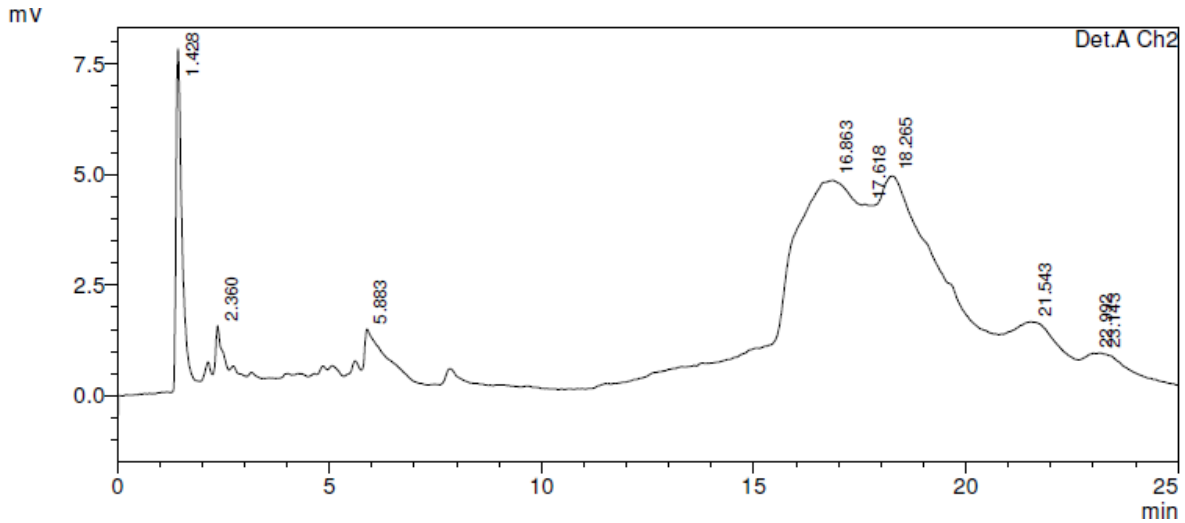


Figure 1. Chromatogram HPLC C15.

4. Conclusion

Fusarium oxysporum had ability to produce secondary metabolites. Based onoptimization of carbon source and time of fermentation, corn is better carbon source than other medium for 15 day fermentation. Screening metabolite showed that C15 comprised terpenoid. Chromatogram analyzed using TLC and HPLC achieved 4 component.

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