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An antifungal phenazine pigment obtained from *Pseudomonas aeruginosa* inhibits the growth of *Aspergillus ochraceus*

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Abstract

Aspergillus ochraceus, a pathogenic mold frequently found in grains, soil, and dried food products. This mold can produce several toxins especially Ochratoxin A and B on infected crops. These toxins are associated with food intoxications both in humans and animals. This study aimed to evaluate the antifungal potentiality of a pigment produced by *Pseudomonas aeruginosa,* against *A. ochraceus in-vitro*. Primarily, the cross streak method showed *P. aeruginosa* (isolate PU_8 and PU_{10}) inhibits the growth of *A. ochraceus*. Then in the quantitative assay using crude extract of the isolate showed significant inhibitory ($p<0.05$) activities which were up to 72% inhibition by PU_8 and 59% by PU_{10}. Then the pigment was extracted, purified, and characterized. The UV spectrophotometry, FT-IR (Fourier Transform Infrared Spectrometry), TLC (Thin-Layer Chromatography) and microscopic analysis proved that the crude extract contained pyocyanin as a potent antifungal phenazine pigment. The adverse effects of chemical fungicides necessitated the use of eco-friendly biological control agents against fungi. Fortunately, from this study, we can infer that *P. aeruginosa* can produce an antifungal phenazine pigment pyocyanin that inhibits the growth of *A. ochraceus* and be used as a potential bio-control agent.

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Introduction

Fungi can produce various detrimental mycotoxins, which are potent causes of many diseases of humans, plants and cattle (Bouhet and Oswald 2005). Besides, spoilage of tons of crops and vegetables due to fungal infection is a worldwide alarming issue for agriculture rendering huge economic losses. Common fungal spoilage agents of fresh vegetables and fruits are various species of the genera *Alternaria, Aspergillus, Cladosporium, Colletotrichum, Fusarium, Penicillium, Phytophthora, Pythium* and *Rhizopus spp* (Tournas 2005 and Moss 2008). Out of these, a few organisms show a substrate preference whereas others such as *Botrytis cinerea, Colletotrichum, Alternaria, Cladosporium, Phytophthora,* and *Rhizopus spp*., can infect a wide variety of vegetables causing devastating losses (Tournas 2005). One of the early interventions which prevents mycotic infection is inhibition of fungal colonization by chemical fungicides or antimycotic agents. However, most of the time these fungicides are not eco-friendly and hazardous to human and cattle health. To tackle this problem in an eco-friendly way biological inhibition of those fungi using biocontrol agents opens a safe path to follow (Gupta 2016, 2018). One of the best actions of microorganisms in our environment is that they can kill each other in such a way which will not cause any diverse effect on the environment. Fortunately, the widely found bacteria named *Pseudomonas aeruginosa*, can kill fungi very incredibly by producing several antimicrobial coloring agents (Jayaseelan *et al.,* 2014). They can inhibit a wide range of plant pathogenic fungi and control the spreading of various plant diseases (Morales *et al.,* 2013). Previously it has been studied that phenazine pigment of *P. aeruginosa* named pyocyanin can inhibit the growth of aflatoxin producing *A. flavus and* other fungus species such as *A. niger*, *Rhizoctonia solani* and *Candida albicans* (Dharni *et al.,* 2012; Morales *et al.,* 2013).

To focus on the biological fungicidal activity of the bacteria, pathogenic fungi named *A. ochraceus* will be used in this study. *A. ochraceus* is a clinically important pathogen, which can produce several mycotoxins, such as ochratoxins, penicillic acid, xanthomegnin and viomellin (Pitt *et al.,* 2009). Ochratoxin A (OTA) is a carcinogenic mycotoxin, generally a secondary metabolite produced by several *A. ochraceus* that contaminate grains, legumes, coffee, dried fruits, beer, wine, and meats (Bayman *et al.,* 2002). The mode of action of this toxins are very complex but researches revealed that OTA inhibits protein synthesis of the infected cell by inhibiting the phenylalanine t-RNA synthetase and isocoumarin, and also enhances the production of oxygen free-radicals, apoptosis or necrosis to exert its toxic action (Kőszegi and Poór 2016; Dirheimer and Creppy 1991). In particular, this study aimed to biologically control the growth of *A. ochraceus* by phenazine pigment-producing *P. aeruginosa*.

Materials and Methods

Isolation and identification

P. aeruginosa was isolated from various clinical samples (blood, urine and pus) using brain-heart infusion (BHI) broth (Oxoid™) as enrichment medium and cetrimide agar (Oxoid™) as selective media for the bacterial growth. Finally, two isolates (PU_8 and PU_{10}) were selected for further study.

Their identification as *P. aeruginosa* was confirmed based on their microscopic features, physiological and biochemical characteristics following Bergey's manual (ninth edition) (Holt *et al.,* 1994).

Determination of antifungal activity with crude extract

To determine the primary antifungal activity of the selected pigment-producing isolates, Glycerol-alanine solidified media (Glycerol 1ml, $MgCl_2$ 0.14g, K_2SO4 1g, Asparagine 0.1%, Peptone 2g, Agar 1.5g Distilled water 100 ml) was used. In cross streak method, at first, fresh bacterial culture was streaked on the one end of the petriplate, then after overnight incubation (which helps diffusion of pyocyanin pigment in the media) fresh fungal culture (cut by a cork borer) was placed on the center of the same petriplate (Fig.1. Treatment).

The crude extract of selected isolates was used in pour plate technique to observe antifungal activity with different concentrations (10-50 µL/ml). The selected *P. aeruginosa* isolates were grown first in the Glycerol alanine pseudomonas broth (Glycerol 1ml, $MgCl_2$ 0.14g, K_2SO_4 1g, Asparagine 0.1%, Peptone 2g, Distilled water 100 ml) at 37°C for 48 hours for better pigment production. Pigment rich broth culture was then centrifuged at 10,000 rpm for 15 minutes and then the supernatant was collected, filtered through a membrane filter (0.45 µm pore size) and used as a crude extract. *A. ochraceus* fresh culture was grown on Potato Dextrose Agar (PDA) media.

Agar cultures of selected fungus were taken with a cork borer and placed in the center of the PDA media plates containing different concentrations of 100 of µL crude extract. A plate without any concentration of crude extract pyocyanin was used as a control. Plates were incubated at 27°C for 5 days. Radial growth of the fungus was measured and growth inhibition of the isolates was calculated by dividing colony diameter in amended plates by that in the non-amended control plates and expressed in percentage.

$$\text{Percentage inhibition: } \frac{\text{Control (C)} - \text{Treatment (T)} \times 100\%}{\text{Control (C)}}$$

Extraction and purification of the pigment produced by Pseudomonas isolates

The extraction of pigment from the crude extract was done using chloroform and HCl (Essar *et al.*, 1990 and Devnath *et al.*, 2017). After extraction, the amount of pyocyanin in the pink acidified layer was quantified by measuring its optical density (absorbance) at 520 nm wavelength using a UV-1800 UV-VIS Spectrophotometer (Shimadzu). Pyocyanin concentration (µg/mL) was determined by multiplying the optical density value (OD_{520}) with 17.072 (Essar *et al.*, 1990 and Wu *et al.*, 2014). Extracted pyocyanin pigment was purified by column chromatography according to Ohfuji *et al.*, (2004) using the ratio of 1:1 (chloroform: methanol) as a mobile phase. The fraction of the eluted sample was crystallized following the previous method of Devnath *et al.* (2017). The crystals were kept as dried purified pigment.

Characterization of the pigment

After column chromatography, the eluted sample was further characterized by TLC (Thin layer chromatography) using a chloroform-methanol solvent.

Then microscopic analysis was done to observe fine crystals. Extracted pigment from the two efficient isolates of *P. aeruginosa* was subjected to a UV-visible spectrophotometer (Watson *et al.,* 1986) and a maximum absorbance was recorded. Fourier Transform-Infrared spectrophotometry (FT-IR) analysis of the pyocyanin pigment was also done using potassium bromide (KBr) as a window material and pyocyanin solution was dried at 60°C to make a powder (Stuart 2005).

Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) and Duncan post-hoc test, using Statistical Package for Social Sciences (SPSS) software trial version (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Determination of antifungal activity

After fungal culture inoculation on glycerol alanine medium, we observed the plate up to 4-5 days and recorded no spread of fungal mycelia on the plate compared to control (Fig.1.Control). The visual deposition of pyocyanin on the media plate was clear. Control plates without *P. aeruginosa* streaked line showed profuse growth of fungal mycelia (Fig.1. Treatment).

Further antifungal activity was confirmed using the crude extract. Different concentrations of crude extract gave different patterns of inhibition and control with no inhibition gave a zone diameter of 52mm.

Table 1. Percentage inhibition of *A. ochraceus* by different concentration of crude extract.

Conc. (µl/ml)	Inhibition by PU_{10}*	(%)**	Inhibition by PU_8*	(%)**
10	43 ±1.0	17%	40.6±1.5	22%
20	33 ±3.0	37%	31.3±1.5	40%
30	30 ±3.0	42%	30.33±2.5	41%
40	26.67±06	49%	26.67±0.6	49%
50	21±1.0	59%	14.33±0.6	72%

*Mean ± Standard Deviation (three replicate)

**Percentage inhibition was calculated using (C) control (mm) =52mm and using mean inhibitory zone diameter (T) for all the concentrations.

It was noticeable that inhibition of fungal mycelial spread up to 72% and 59% by 50µl/ml of PU_8 and PU_{10} respectively (Table 1). All the concentrations of PU_{10} and PU_8 showed decent evidence of antifungal activity of crude extract, the inhibitory activities were found statistically significant using one way ANOVA (P = 0.00). Furthermore, Post-Hoc analysis using Tukey HSD exhibited a statistically significant difference between the groups except concentrations between 20, 30 and 40 µL/mL (Table 2).

However, non-significant (P>0.05) data between all concentration was found using Duncan Post-Hoc analysis.

Table 2. Multiple Comparisons of Zone of inhibition between different groups.

Conc. (µL/mL) PU10		Mean Difference (I-J)	sig	Conc. (µL/mL) PU8		Mean Difference (I-J)	Sig.
Conc. (I)	Compared Conc. (J)			Conc. (I)	Compared Conc. (J)		
10	20	10.000*	.001	10	20	9.333*	.000
	30	13.000*	.000		30	10.333*	.000
	40	16.333*	.000		40	14.000*	.000
	50	22.000*	.000		50	26.333*	.000
20	10	-10.000*	.001	20	10	-9.333*	.000
	30	3.000	.413		30	1.000	.924
	40	6.333*	.021		40	4.667*	.025
	50	12.000*	.000		50	17.000*	.000
30	10	-13.000*	.000	30	10	-10.333*	.000
	20	-3.000	.413		20	-1.000	.924
	40	3.333	.321		40	3.667	.086
	50	9.000*	.002		50	16.000*	.000
40	10	-16.333*	.000	40	10	-14.000*	.000
	20	-6.333*	.021		20	-4.667*	.025
	30	-3.333	.321		30	-3.667	.086
	50	5.667*	.040		50	12.333*	.000
50	10	-22.000*	.000	50	10	-26.333*	.000
	20	-12.000*	.000		20	-17.000*	.000
	30	-9.000*	.002		30	-16.000*	.000
	40	-5.667*	.040		40	-12.333*	.000

*The mean difference is significant at the 0.05 level.

Characterization of pigment

For further characterization, the pigment was then subjected to UV visible spectrophotometer (T-1800) for spectral analysis and an analyte peak was observed at a maximum range of 270-271 nm (Fig.2.). TLC analysis of all isolates showed a blue spot with Rf value around 0.8. Infra-Red spectrum showed characteristic absorption bands at 3429 cm^{-1}, 1620 cm^{-1}, 1604 cm^{-1}, 1384 cm^{-1} and 1320 cm^{-1} (Fig.3).

Discussion

Fungi from *Aspergillus* species are among the molds considered to be most pathogenic, due to their toxicity (Gniadek, 2009). Mycotoxins (e.g., aflatoxin,

ochratoxin, etc.,) produced as a metabolite by the *Aspergillus* group, which are carcinogenic, teratogenic, mutagenic, hepatotoxic and nephrotoxic in action (Fischer and Dott, 2003). To prevent mycoses, the use of efficient fungicides is in urgent need. Adverse effects of chemical fungicides on the environment supported the need for substitute ways for curbing fungal growth on crops and grains. For this reason, experiments on antimicrobial metabolites extracted from microbes and plants, deriving greater impulse in the agricultural economy. *P. aeruginosa* has recently been a focus of interest as bio-control agents and a source of bioactive metabolites (Someya *et al.,* 2006).

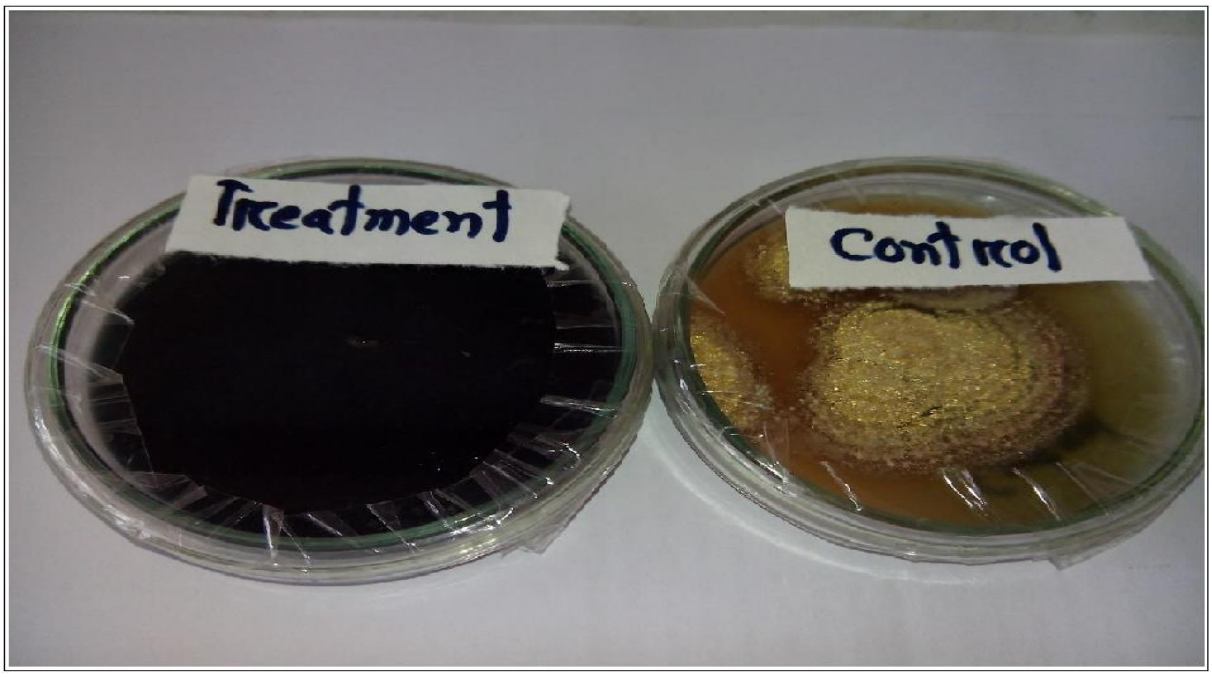

Fig. 1. Inhibition of fungal growth by culture of *P. aeruginosa* in cross streak method after 5 days of incubation. [Here in treatment, *P. aeruginosa* line was streaked on one side of the petriplate, deposition of pyocyanin on the media turned the media dark bluish. In control no inoculation was done].

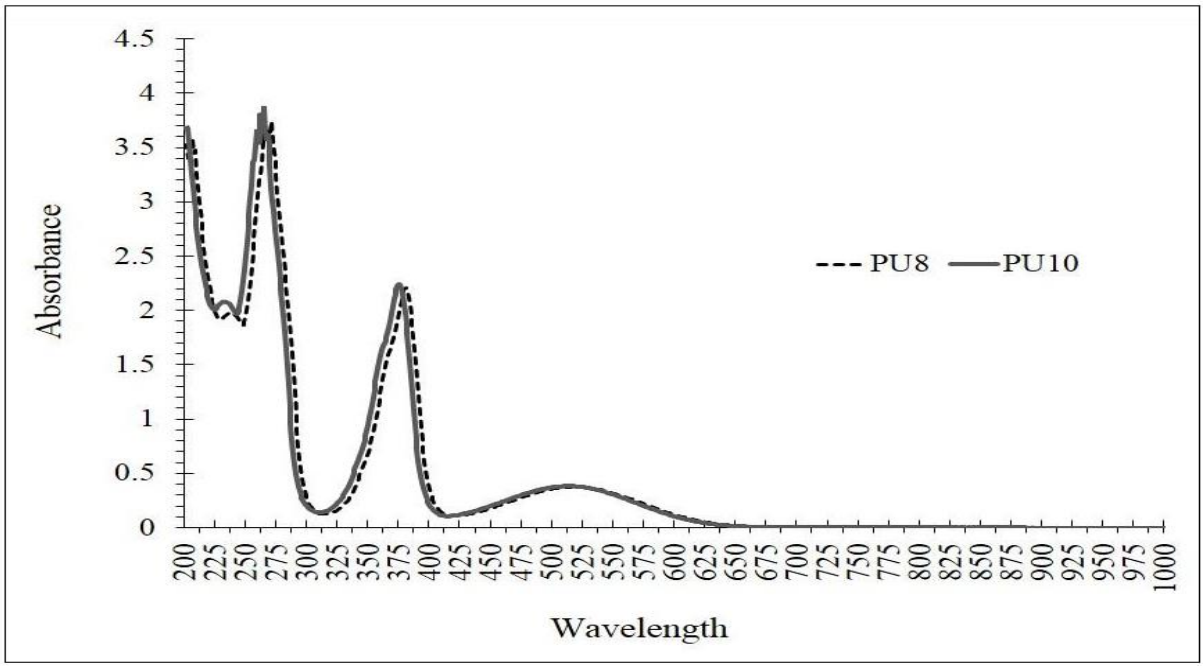

Fig. 2. UV absorption spectra of PU_{10} and PU_8.

They can produce different antimicrobial phenazine pigments which have been recognized for their antibiotic properties for over 150 years (Mavrodi *et al.,* 2006). Hence, in this study, we isolated *P. aeruginosa* from different clinical sources and recorded potent antifungal activity to inhibit the growth of *A. ochraceus*. As no previous data was available for inhibition of *A. ochraceus* by *P. aeruginosa*, the cross streak method was initially used to determine whether it has inhibiting activity.

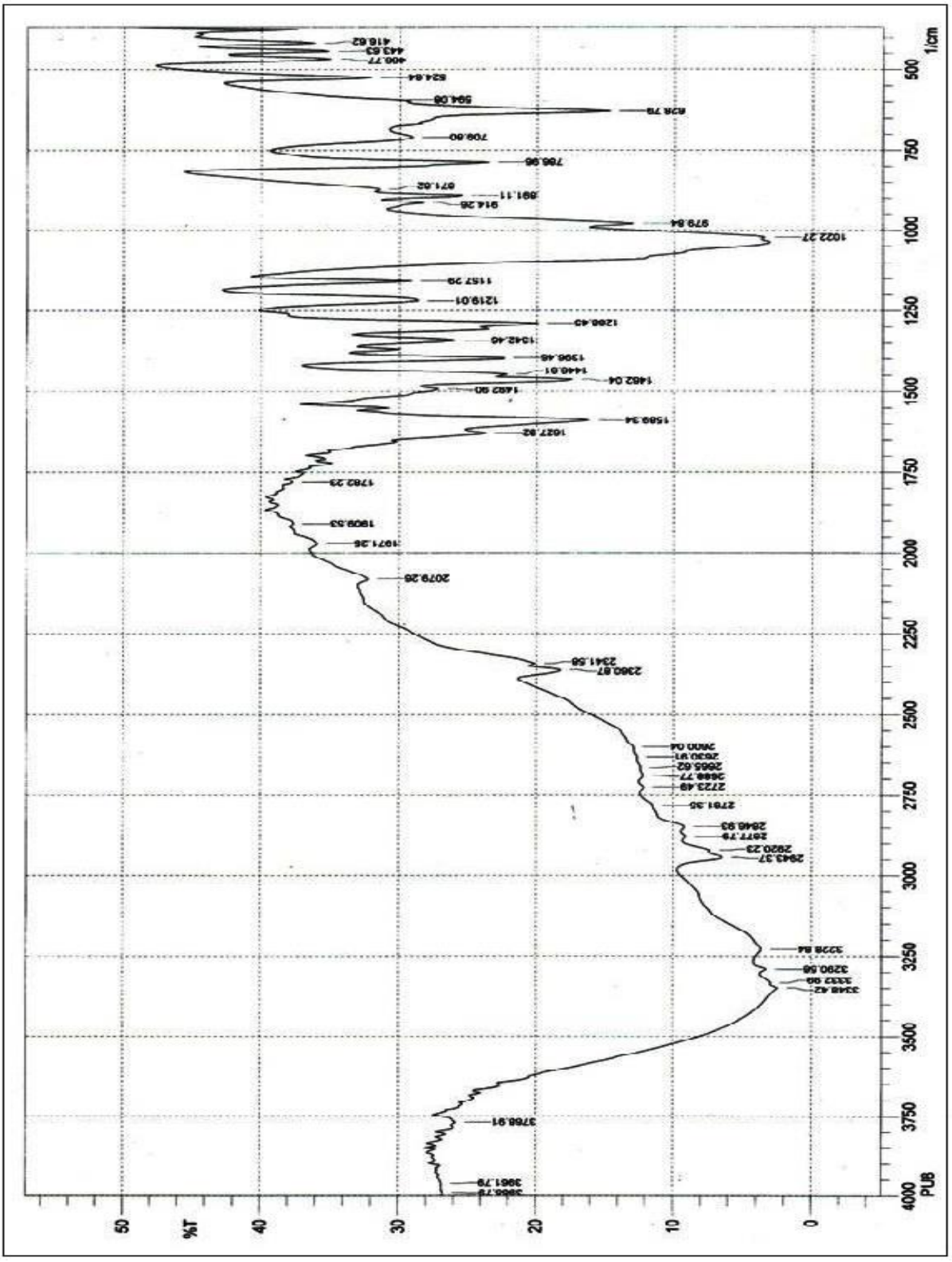

Fig. 3. FT-IR spectra of the pyocyanin pigment extracted from the isolate *P. aeruginosa* PU_8.

Pyocyanin production was high in glycerol alanine medium rather than commonly used PDA medium, so we used this medium for our initial purpose. We used it for fungal culture at the primary inhibition process and found suitable conditions for fungal growth. Primary antimicrobial activity against *A. ochraceus* by *P. aeruginosa* in the cross streak method showed hopeful outcome as an antagonistic compound secretion. Later on, an antagonistic compound from the culture supernatant of *P. aeruginosa* was also proved to be an efficient antifungal agent. Using the crude extract of pyocyanin producer can inhibit 59% to 72% of the pathogenic fungal growth by isolate PU_{10} and PU_8 respectively.

Several studies also reported that purified pyocyanin can inhibit mold such as *A. niger, A. fumigatus,* and yeast *Cryptococcus neoformans*, *C. tropicalis* and *C. albicans* (Özyürek *et al.,* 2016; Marrez and Mohamad, 2020).

Sometimes some of the other pigment may remain mixed with the chloroform extract of pyocyanin, *P. aeruginosa* can produce different pigments including pyocyanin (blue green), pyoverdin (yellow, green and fluorescent), pyomelanin (light-brown) and pyorubrin (red-brown) (Jayaseelan *et al.,* 2014). Thus, the pigment was later on extracted and purified through various downstream processes such as column chromatography and crystallization. Later on, confirmation of pyocyanin was done, where it was confirmed that the crude extract of the isolates surely has pyocyanin pigment. TLC, UV-Vis spectra suggest the characteristics of the pyocyanin compound, which similarly matched with previous data (Ohfuji *et al.,* 2004, Sudhakar and Karpagam 2011; Karpagam *et al.,* 2013).

We have further confirmed the structural characteristics of the pyocyanin pigment by FT-IR. Pyocyanin is a hetero-cyclic nitrogenous compound, the FT-IR spectra indicate the presence of –OH group (3400-3300 cm-1), C=N bonds (1590-1600 cm-1) and C-N (1280- 1250 cm-1) bonds, the presence of $–CH_3$ group is confirmed with the –C-H stretches of the alkyl (methyl) group in the 1380-1400 cm-1 range (Stuart 2005). These results mostly agreed with those of characteristics of pyocyanin pigment.

Limitations of our study that we did not apply purified pyocyanin compound to see its antifungal effect and couldn't confirm ochratoxin production by our *A. ochraceus* isolate.

Conclusion

This study concluded that *Pseudomonas aeruginosa* could effectively inhibit the growth of *Aspergillus ochraceus in vitro*. Continuation of this study with purified product may further evaluate its antifungal potentiality. Although the use of *Pseudomonas* as a fungal growth inhibitor was effective in a laboratory, its application in agriculture requires further studies and adequate implementations.

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