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Disruption of hyphal development in *Candida albicans* ATCC 10231 by bioactive compound from *Pseudomonas azotoformans* UICC B-91

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ABSTRACT

Candidiasis and other fungal infections are becoming more frequent. The search for appropriate antibiotics for fungal infections is still ongoing. *Pseudomonas azotoformans* University of Indonesia Culture Collection (UICC) B-91 has potential as an anticandidal. The purpose of this study was to determine the mechanism of inhibition of the metabolite of *P. azotoformans* UICC B-91 from the medium against morphologically pathogenic fungi. The results showed metabolite compound *P. azotoformans* UICC B-91 at a concentration of 100 and 80 mg/ml had strong inhibitory power, and 60 mg/ml had moderate inhibition. Determination of the minimum inhibitory concentration value of the medium solution of *P. azotoformans* UICC B-91 against *Candida albicans* American Type Culture Collection (ATCC) 10231 was at a concentration of 7.50 mg/ml. Microscopic observation of *C. albicans* ATCC 10231 after the addition *of P. azotoformans* UICC B-91 medium under the light microscope showed inhibition of the transition from yeast to hyphae. Microscopic observation of *C. albicans* ATCC 10231 after the addition of *P. azotoformans* UICC B-91 medium under scanning electron microscope showed changes in the yeast cell surface become uneven. The mechanism inhibition of metabolites from *P. azotoformans* UICC B-91 medium against *C. albicans* ATCC 10231 is through diffusion that disrupts cell membrane function or inhibits transition from yeast to hyphal form.

INTRODUCTION

The discovery of endophytic microbial that associated with *Neesia altissima*, we reported that three endophytic bacterial strains, namely *Pseudomonas aeruginosa* strains University of Indonesia Culture Collection (UICC) B-40 and UICC B-93, and *Pseudomonas azotoformans* strain UICC B-91, were discovered [1]. Previous research had identified and isolated endophytic *P. azotoformans* from stem bark tissue of the *N. altissima* plant in Halimun forest [1]. Research has been carried out on *P. azotoformans* UICC B-91 which is proven to be able to inhibit several pathogenic bacteria, such as *Escherichia coli* American Type Culture Collection (ATCC) 8739, *Salmonella typhimurium* ATCC 25241, *Staphylococcus aureus* ATCC 6583, *S. aureus* ATCC 25923, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 19659, *P. aeruginosa* ATCC 15442, and *Candida albicans* ATCC 10231, with a notable result on anticandidal activity [2]. In addition, other studies have reported that the supernatant from the fermentation medium of *P. azotoformans* UICC B-91 has anticandidal activity with the largest average value of the inhibition zone produced is 8.00 ± 0.00 mm at a concentration of 50 mg/ml [2].

Candida albicans is a fungal pathogen that can be found in the healthy human microbiome but can also cause mucosal infections or invasive candidiasis. Invasive candidiasis is a deadly disease with a high mortality rate. It has a mortality rate ranging from 20% to 50%. *Candida albicans* were included in the critical group of three priority groups based on numerical scores, as well as consensus discussions among the World Health Organization Advisory Group on the Fungal Priority Pathogens List [3]. Recent studies indicating higher rates of azole resistance, particularly in low-and

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middle-income countries, raise concerns that resistance is on the rise. Resistance to antifungals is uncommon. Despite this, resistance rates, particularly in nonsterile site isolates, appear to be increasing, highlighting the need for more effective and efficient therapy. This research was conducted to determine the mechanism of inhibition of the metabolite of *P. azotoformans* UICC B-91 from the medium on the morphological activity of *C. albicans* ATCC 10231.

MATERIALS AND METHODS

Microorganisms

The bacterium used is a collection of the UICC, is *P. azotoformans* UICC B-91, and the yeast used is a collection of the ATCC, is *C. albicans* ATCC 10231.

Isolation and extraction of anticandidal active compounds

The starter was made by inoculating one loop of *P. azotoformans* UICC B-91 colonies from the slant nutrient agar into 20 ml of rehydration-fluid bacteria (RB) medium. Then incubated in an incubator shaker at 30ºC for 24 hours at 100 rpm. The 1% inoculum was then transferred into 200 ml RB medium. Fermentation was carried out for 96 hours at a temperature of 30ºC in an incubator shaker at a speed of 100 rpm. The calculation of the number of total plate count (TPC) is carried out to determine the number of colony forming units per milliliter (CFU/ml) with an interval of 24 hours. The fermented culture of *P. azotoformans* UICC B-91 was then centrifuged at 2,000 g for 20 minutes. The supernatant was separated from the pellet and then macerated with ethyl acetate with a volume of 1:1 (*v*/*v*) overnight. The medium layer was then macerated again with ethyl acetate and the same steps were carried out up to two times. The ethyl acetate layer was evaporated using a rotary evaporator [2].

Anticandidal activity test

Suspension of *C. albicans* ATCC 10231 was prepared and inoculated using cotton and then smeared over the entire surface of sterile saboraud dextrose agar (SDA) medium with side-to-side movements. Smearing is done by rotating the petri dish at an angle of 60° twice. The anticandidal activity test was carried out in two batches. Each testing batch was done in triplicate. Each SDA medium was made into five quadrants in a petri dish. Each quadrant was placed on a disc that had been dripped with 50 µl *P. azotoformans* UICC B-91 with a concentration of 100, 80, 60, 0.1 mg/ml ketoconazole as a positive control, and 0.5% dimethyl sulfoxide (DMSO) as a negative control. SDA medium was incubated at 30ºC for 24–48 hours. The clear zone formed was measured with a calliper [4].

Determination of minimum inhibitory concentration (MIC) of *P. azotoformans* UICC B-91

A total of three sets of sterile test tubes with each set containing eight tubes were prepared. Tubes one through six are prepared for treatment. Tubes seven were prepared as a growth control, and tubeseight as a control medium without growth. A total of 3 ml of Potato Dextrose Broth was poured into tubes two to eight using a micropipette in each set. 4.5 ml of 60 mg/ml concentration medium was poured into tube one using a sterile micropipette in each set. Serial dilutions were performed. A total of 1.5 ml from tube one was transferred to tube two using a sterile micropipette and then tubes two was vortexed. The same procedure was repeated from tubes two to three to six in each set. The remaining 1.5 ml in tube six was discarded. After that, 0.15 ml of yeast suspension was poured into tubes two to seven in each set. Tubes eight as a control medium without growth were not poured with yeast suspension. The entire set of tubes was incubated for 12 to 18 hours at 30ºC [5].

Microscopic observation of *C. albicans* ATCC 10231 after addition of *P. azotoformans* UICC B-91 with light microscope

The MIC test tubes with a concentration of 60 mg/ml and the growth control which had been incubated were vortexed again for 30 seconds. A total of two slides were prepared and one drop of 10% potassium hydroxide (KOH) was added. The entire suspension was dripped onto a 10% KOH solution on each slide of 20 µl and covered with a cover slip. The preparations were fixed and observed under a microscope up to 1,000× magnification [6].

Microscopic observation of *C. albicans* ATCC 10231 after addition of *P. azotoformans* UICC B-91 with scanning electron microscope (SEM)

Suspension of MIC at a concentration of 60 mg/ml and growth control was transferred into 1.5 ml Eppendorf tubes of 200 µl each. Each suspension was immersed in 200 µl of 2.5% glutaraldehyde overnight. Then the suspension was centrifuged at 1,000 rpm for 10 minutes and the supernatant was discarded. The pellets were soaked in 200 µl of 2% tannin acid for 2 hours, then centrifuged and the supernatant was discarded. Pellets were immersed in caccodylate buffer for 10 minutes two times, then centrifuged and the supernatant was discarded. The pellets were immersed in 200 µl of 1% osmium tetraoxide solution for 1 hour. Then it was centrifuged again and the supernatant was discarded. The pellets were washed with 50% ethanol, left for 10 minutes, centrifuged, and then the supernatant was discarded. The pellets were washed again with 50%, 70%, 80%, and 95% ethanol for 10 minutes, respectively. Then washed with absolute ethanol for 10 minutes two times, after that, it was centrifuged and the supernatant was discarded. Pellets were added with tert-butanol for 10 minutes and performed two times. A small amount of tert-butanol was added to the cell precipitate and the cell smear was smeared on a cover slip. The washed cover slips were then smeared with cells, coated with gold under vacuum, and observed under SEM at Zoology Characterization Laboratories-BRIN at 5,000×, 10,000×, and 15,000× magnification [7].

RESULTS

Isolation and extraction of anticandidal active compounds

The production of anticandidal compounds was produced through a fermentation process with 200 ml RB medium. The total volume of RB medium used for the production of anticandidal compounds was 1,600 ml. The

starter was made by inoculating one loop of *P. azotoformans* UICC B-91 colonies into 20 ml RB medium. The medium color changed from transparent yellow to cloudy greenish–yellow after incubated in an incubator shaker at 100 rpm at 30ºC for 24 hours. As much as 1% (*v*/*v*) inoculum was transferred into 200 ml RB medium and fermented in an incubator shaker at 100 rpm at 30ºC.

Based on observations every 24 hours, the number of TPC *P. azotoformans* UICC B-91 at 0 hours had an average of 2.34×10^5 CFU/ml. After fermentation for 24 hours, the amount of TPC increased by an average of 1.80×10^6 CFU/ml. The number of TPC at the 48th hour still increased with an average of 1.62×10^8 CFU/ml. The highest number of TPC occurred at the 72th hour with an average of 1.11×10^{10} CFU/ml. The amount of TPC at 96th hour was 5.10×10^9 CFU/ml and the amount of TPC at the 120th hour decreased to 1.80×10^8 CFU/ml. The results of the TPC calculation were visualized by making a growth curve of *P. azotoformans* UICC B-91 (Fig . 1).

Fermentation of *P. azotoformans* UICC B-91 for 96 hours showed a change in the color of the medium from transparent brownish yellow to cloudy greenish-yellow. The fermented medium aged 96 hours was centrifuged and then macerated with ethyl acetate as solvent. The wet weight of the medium obtained from evaporation is 1.229 g. The liquid medium was re-evaporated using an oven at 40ºC until the medium thickened. The constant weight of the medium obtained was 0.86 g. A total of 0.86 g of thick medium *P. azotoformans* UICC B-91 was dissolved in 8.6 ml of 0.5% DMSO to make a medium solution with a concentration of 100 mg/ml. Serial dilutions were carried out to obtain a medium solution with a concentration of 80 and 60 mg/ml.

Anticandidal activity test

The results of observations after incubation for 24 hours were the zone of inhibition of several treatments and controls. The inhibition zone observed after the addition of the medium concentration of 100 mg/ml averaged 18.82 ± 0.54 mm, the inhibition zone at a concentration of 80 mg/ml averaged 13.75 ± 0.53 mm, the inhibition zone at a concentration of 60 mg/ml mean 9.49 ± 0.43 mm. The zone of inhibition observed in the positive control was 0.1 mg/ml with an average of 7.17 ± 0.26 mm. The zone of inhibition observed in the negative control at 0.5% concentration was not detected at all (Fig. 2) (Table 1).

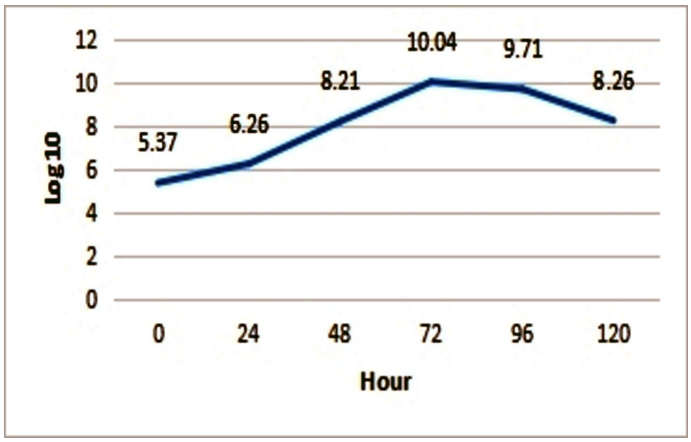

Figure 1. Growth curve of *P. azotoformans* UICC B-91.

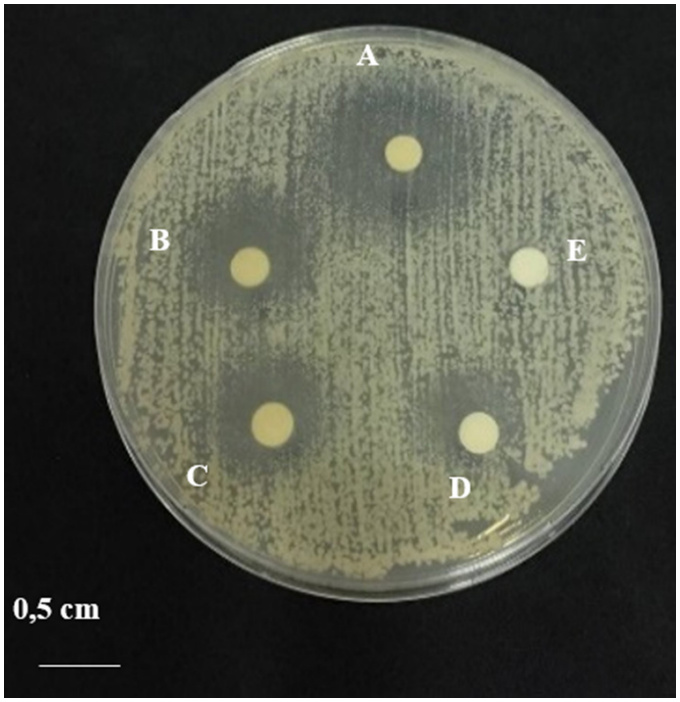

Figure 2. Anticandidal activity test of *C. albicans* ATCC 10231: (A) addition of 100 mg/ml concentration medium solution; (B) concentration 80 mg/ml; (C) concentration 60 mg/ml; (D) addition of 0.1 mg/ml ketoconazole; and (E) addition of 0.5% dimethyl sulfoxide (DMSO).

Table 1. Calculation results of the anticandidal activity of *P. azotoformans* UICC B-91.

BATCH	MEDIUM CONCENTRATION			CONTROL	
	100 mg/ml	80 mg/ml	60 mg/ml	POSITIVE	NEGATIVE
	(mm)	(mm)	(mm)	(mm)	(mm)
			BATCH I		
First repetition	17.73	14.30	10.00	7.00	0
Second repetition	18.96	13.38	9.50	7.50	0
Third repetition	19.00	14.00	10.00	7.00	0
BATCH II					
First repetition	19.20	13.95	9.21	7.00	0
Second repetition	19.00	12.86	9.20	7.00	0
Third repetition	19.00	14.00	9.00	7.50	0
Average ± SD	18.82 ± 0.54	13.75 ± 0.53	9.49 ± 0.43	7.17 ± 0.26	0

Determination of MIC medium solution of *P. azotoformans* UICC B-91 against *C. albicans* ATCC 10231

The results of the optical density (OD) measurement can be seen in Appendix 1. Based on the results obtained, the medium concentrations of 60.00, 30.00, 15.00, and 7.50 mg/ml had decreased OD values after incubation, while the concentrations of 3.75 and 1.87 mg/ml increased in OD values after incubation. The growth control OD value after incubation increased. The OD value of the control medium after incubation did not increase or decrease (fixed). The measurement results showed that the medium solution of *P. azotoformans* UICC B-91 with a concentration of 7.50 mg/ml was determined as the MIC value for *C. albicans* ATCC 10231 because it did not provide additional OD values at low concentrations (Fig. 3.).

Microscopic observation of *C. albicans* ATCC 10231 after addition of *P. azotoformans* UICC B-91 medium with light microscope

Microscopic changes of *C. albicans* ATCC 10231 after the addition of *P. azotoformans* UICC B-91 medium solution were compared with the growth control observed under a light microscope. The results of the growth control observations showed that yeast cells were oval in shape, pseudohyphae were found, and the distribution of cells tended to be clustered. The results of observations after the addition of a solution of *P. azotoformans* UICC B-91 at a concentration of 60 mg/ml were dominated by yeast cells with a less uniform shape because some cells were lysed, pseudohyphae were not found, and the distribution of cells tended to be scattered (Fig. 4).

Microscopic observation of *C. albicans* ATCC 10231 after addition of *P. azotoformans* UICC B-91 medium with SEM

Microscopic changes in *C. albicans* ATCC 10231 after the addition of *P. azotoformans* UICC B-91 medium compared to growth controls were observed under SEM. The results of the growth control observations showed that yeast cells were oval in shape with a length of 2.54–3.71 µm, width 2.05–3.40 µm, flat cell surface, normal yeast cell shape, and pseudohyphae were found. The addition of *P. azotoformans* UICC B-91 with a concentration of 60 mg/ml showed that yeast cells were oval in shape with a length of 2.48–3.66 µm, a width of 2.47–3.20 µm, the yeast cell surface was uneven and many cells was broken (ruptured) (Fig. 5).

DISCUSSION

Isolation and extraction of anticandidal active compounds

RB medium was used as the fermentation medium for *P. azotoformans*. There were 2 g of peptone, 0.4 g of yeast extract, and 0.2 g of $MgSO_4.7H_2O$ in 200 ml of RB medium. Peptone and yeast extract in the fermentation medium serve as nitrogen and carbon sources for the formation of secondary metabolites, respectively, while $MgSO_4.7H_2O$ aids in the formation of pigments [8]. The starter is designed to adapt to the substrate that will be used during the fermentation process [9].

The growth curve consists of four phases: the lag phase, the exponential phase, the stationary phase, and the death phase. The lag phase is the phase when bacteria adapt to new conditions, that is a fresh medium. Based on the observations, the lag phase is not observed. According to Madigan *et al*. [10], the lag phase was not observed because the inoculum inoculated into the new medium came from the same type of medium and conditions. The exponential phase is the phase when the cells enter a period of rapid growth. The exponential phase starts to appear at 0 to 72 hours. The cells in the population divide into two cells in these phases, the stationary phase is the phase when bacteria stop replicating due to depleted nutrients or product accumulation. The stationary phase in the observations occurred after 72 hours. The balance between the number of living and dead cells occurs in this phase. Finally, the death phase occurs when the number of cells that experience death exceeds the number of living cells. The death phase has begun to be seen at the 96th hour. Based on the results of TPC and growth curves obtained, the isolation of bioactive compounds was carried out when the *P. azotoformans* UICC B-91 culture was 96 hours old. Here, in the final stationary phase, there are limited nutrients remaining in microorganisms and sufficient accumulation of metabolites [11].

Anticandidal activity test

The inhibitory power can be said to be weak if the average value of the inhibition zone produced is <5 mm, moderate if the average value of the inhibition zone is 5–10 mm, strong if the average value of the inhibition zone is 5–10 mm [12]. The average inhibition zone produced is 10–20 mm, very strong if the average

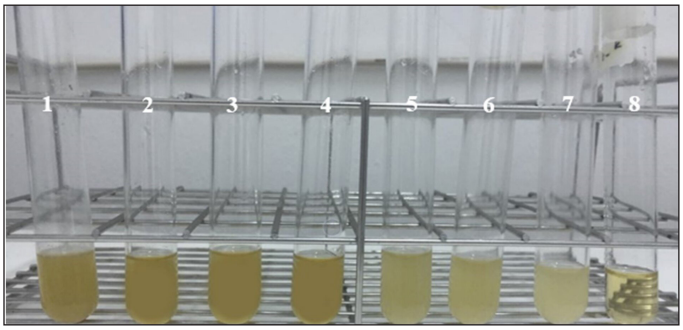

Figure 3. MIC test of *P. azotoformans* UICC B-91 medium solution against *C. albicans* ATCC 10231 (from left to right): (1) medium concentration 60.00 mg/ml; (2) 30.00 mg/ml; (3) 15.00 mg/ml; (4) 7.50 mg/ml; (5) 3.75 mg/ml; (6) 1.88 mg/ml; (7) growth control; and (8) control medium.

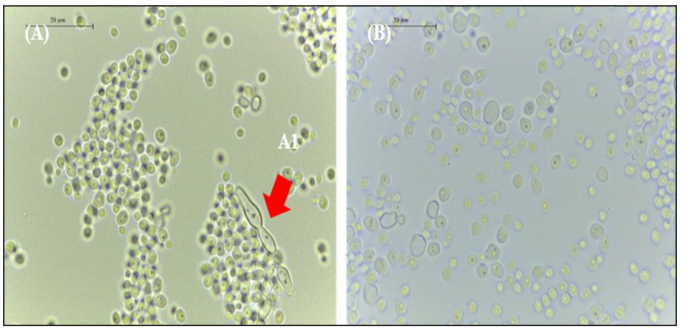

Figure 4. Morphology of *C. albicans* ATCC 10231 under a light microscope at 1,000× magnification: (A) in growth control; (A1) pseudohyphae; and (B) with the addition of 150 µl medium concentration 60 mg/ml.

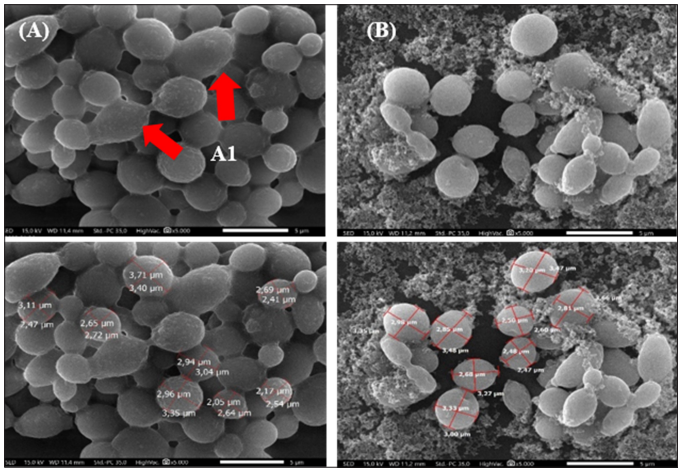

Figure 5. Yeast cell morphology of *C. albicans* ATCC 10231 under 5,000× magnification SEM: (A) in growth control; (A1) pseudohyphae; and (B) with the addition of 150 µl of medium concentration of 60 mg/ml.

value of the inhibition zone is >20 mm. Based on observations, medium *P. azotoformans* UICC B-91 concentration of 100 and 80 mg/ml had a strong inhibitory effect on *C. albicans* ATCC 10231 and a concentration of 60 mg/ml had moderate inhibition.

The anticandidal activity test has also been carried out by Oktarina *et al*. [2]. Testing of *P. azotoformans* UICC B-91 medium against *C. albicans* ATCC 10231 was carried out using the disc diffusion method. Medium with concentrations of 0.1, 1, and 5 mg/ml had no zone of inhibition against *C. albicans* ATCC 10231. Increasing concentrations at 10 and 50 mg/ml had an average zone of inhibition of 4.25 ± 0.25 and 8.00 ± 0.00 mm, respectively.

Determination of MIC medium solution of *P. azotoformans* UICC B-91 against *C. albicans* ATCC 10231

Cell suspensions can scatter light and the amount of light scattered is proportional to the number of cells present. The more cells, the more light is scattered, and the cloudier the suspension will look. The decrease in OD value of *C. albicans* ATCC 10231 at concentrations of 60.00, 30.00, 15.00, and 7.50 mg/ml was due to the higher the concentration of the medium, the greater the amount of bioactive compounds contained so that more and more antifungal compounds were absorbed by *C. albicans* ATCC 10231 and caused the growth of *C. albicans* ATCC 10231 to be inhibited and the measured OD value decreased [10].

The OD values in the medium of *C. albicans* ATCC 10231 with a concentration of 3.75 and 1.87 mg/ml after incubation were increased. The addition of OD values occurred due to an increase in the number of cells after incubation [13]. Furthermore, the addition of the OD value to the growth control occurred because no additional medium was added. The control medium did not increase or decrease the OD value because suspension and medium were not added.

Changes in yeast morphology confirmed with microscopy suggests *P. azotoformans* disrupts the cell membrane

According to Kabir *et al*. [14], *C. albicans* ATCC 10231 has dimorphism ability by forming pseudohyphae. The accumulation of yeast and pseudohyphae forms a biofilm. *Candida albicans* biofilm structure can cause antimicrobial resistance [15]. Changes in yeast cell morphology after being given the medium occurred due to the bioactive compounds contained in it. According to Pratiwi *et al*. [16] reported that the medium solution of *P. azotoformans* UICC B-91 contained alkaloids. According to Priya *et al*. [17], alkaloid compounds are able to downregulate several adhesion and hyphal-specific genes, such as the *Unscheduled Meiotic gene Expression 6* (*UME6*), *Agglutinin-Like Sequence* 1 and 3 (*ALS1* and *ALS3*) genes, and *Hyphal Wall Protei*n 1 genes (*HWP1*) in various strains of *C. albicans*. The *UME6* gene regulates the transition of the yeast form to the hyphal form. The *ALS1* and *ALS3* genes are involved in the process of adhesion to host cells and biofilm formation in both *in vitro* and *in vivo* conditions. The *HWP1* gene is a hyphal wall regulator and plays a role in hyphae development, mating, and attachment to host cells. The *hwp1* mutant *C. albicans* was shown to inhibit hyphal germination and pathogenicity to host cells *in vivo* [18].

Changes in yeast cell morphology after being given the medium occurred due to the bioactive compounds contained in it. Pratiwi *et al*. [16] reported that *P. azotoformans* UICC B-91 medium contained alkaloids. According to Tsuchimori *et al*. [18], the mechanism of action of the alkaloids is thought to be by disrupting cell membranes or the transition of yeast to hyphae. Martins De Andrade *et al*. [19] reported that alkaloids were able to disrupt cell membranes as evidenced by irregular yeast cell surface changes, and the release of cell components was also known. In addition, the mechanism of action also occurs through the process of inhibiting the transition of yeast to hyphae as reported by Ma *et al.* [20] that alkaloid compounds significantly inhibit the morphological transition of yeast to hyphae. Inhibition of morphological transition from yeast to hyphae contributes to the inhibition of virulence and pathogenicity that can penetrate host cells. This process makes the alkaloids potential to inhibit the pathogenicity of *C. albicans* ATCC 10231 [18].

CONCLUSION

The zone of inhibition produced by the medium solution of *Pseudomonas azotoformans* UICC B-91 against *C. albicans* ATCC 10231 at a concentration of 80 and 100 mg/ml had a strong inhibitory effect. The mechanism of inhibition of metabolites from *P. azotoformans* UICC B-91 medium against *C. albicans* ATCC 10231 is occur through diffusion that disrupts cell membrane function or inhibits the transition from the yeast form to the hyphal form. Endophytic *P. azotoformans* from *N. altissima* plant stem bark tissue has pharmaceutical potential because it produces anticandidal compounds, and the medicinal properties of this plant may be due to the ability of its endophytic microorganisms to produce biologically active secondary metabolites. More research is needed to evaluate the spectral composition produced so that new anticandidal drugs can be developed.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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Appendix 1. MIC test results from *P. azotoformans* UICC B-91 medium solution towards *C. albicans* ATCC 10231.

Concentration (mg/ml)	First repetition		Second repetition		Third repetition		Average		ΔOD*	Annotation
	Before	After	Before	After	Before	After	Before	After		
60.00	0.622	0.567	0.604	0.543	0.529	0.470	0.585	0.527	−0.058	Decrease
30.00	0.349	0.324	0.325	0.296	0.291	0.265	0.322	0.295	−0.027	Decrease
15.00	0.155	0.141	0.163	0.146	0.135	0.122	0.151	0.136	−0.015	Decrease
7.50	**0.094**	**0.085**	**0.081**	**0.074**	**0.068**	**0.059**	**0.081**	**0.073**	**−0.008**	**Decrease**
3.75	0.047	0.891	0.044	0.769	0.035	0.769	0.042	0.810	0.768	Increase
1.87	0.029	1.061	0.02	1.024	0.015	1.024	0.021	1.036	1.015	Increase
Growth control	0.011	1.109	0.006	1.083	0.008	1.083	0.008	1.092	1.083	Increase
Medium control (blank)	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.000	Fixed

*ΔOD = difference from the average OD value before and before incubation.
The bold values show that the medium solution of *P. azotoformans* UICC B-91 with a concentration of 7.50 mg/ml was determined as the MIC value for *C. albicans* ATCC 10231 because it did not provide additional OD values at low concentrations.