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ENHANCEMENT OF ANTIBACTERIAL COMPOUNDS PRODUCTION BY *ASPERGILLUS FLAVUS* AND *PENICILLIUM CITRINUM* ISOLATED FROM LOCALS FOODS IN BOBO DIOULASSO AND OUAHIGOUYA, BURKINA FASO

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ABSTRACT

This current study was carried out to optimize media components and culture conditions for maximum antibacterial compound production and extraction by *Aspergillus flavus* and *Penicillium citrinum*. A total of five (5) isolates of *Aspergillus* and six (6) isolates of *Penicillium* were collected from groundnut and rice. Out of which one of each genera found to be promising. These isolates were identified as *Aspergillus flavus* and *Penicillium citrinum* according to respectively Raper and Fennell[1] and Pitt[2] key. The influence of culture conditions including incubation duration, incubation temperature, initial pH, agitation rate and different carbon and nitrogen sources on growth and bioactive compound production was investigated. This production was done by fermentation in synthetic broth: 5 g/l yeast extract, 1 g/l KH_2PO4, 0.5 g/l $MgSO_4.7$ H2O, 0.01 g/l $FeSO_4.7$ H_2O, 15 g/l NaCl, 30 g/l Glucose during one week in shaker. The disc diffusion method was used to study the antibacterial activities against two (2) Gram negative and two (2) Gram positives bacteria. The antibacterial activity was estimated on the basis of diameter of inhibition zone. Fungi showed high rate of antibacterial compound with yeast as nitrogen sources on the 7-9 days in fermentation at temperature of 30ºC and 150 rpm of the rotary shaker. The best carbon source was starch and glucose for *Aspergillus flavus* at pH 7 and starch and sucrose at pH 6 for *Penicillium citrinum.*

KEYWORDS: antibacterial compound, *Aspergillus flavus*, *Penicillium citrinum*, Culture media, culture condition, Enhancement.

1. INTRODUCTION

Emerging infectious diseases and the significant clinical implication of drug resistance has led to heightened interest in the study of antimicrobial resistance from different angles. One solution of this problem is the research for news antimicrobials agents. The search on antibiotic from fungi started with the discovery of penicillin, produced by *Penicillium notatum.*[3] Fungi are ubiquitous eukaryotic and heterotrophs which provide an abundant reservoir of bioactive metabolites for medicinal exploitation. Fungi from genera *Aspergillus* and *Penicillium* represented the best source of biologically active metabolites.[4] Antibiotics and other secondary metabolites are synthesized in response to physiological stress due to the nutrients limitation e.g. in response to limitation of phosphate or easily assimilable carbon and nitrogen sources.[5,6]

Aspergillus flavus is the most producer of aflatoxin, but it could produce many types of bioactive metabolites. For example aspergillic acid is secondary metabolite derivate from hydroxypyrazine which has bactericide property.[7] *Aspergillus flavus* could also produce kojic acid which has antibacterial and insecticide properties.[8][9]

Penicillium citrinum is capable to produce citrinin which is reported to have a broad spectrum antibiotic especially against Gram positive bacteria and PcPAF, protein with antifungal activity.[10]

In this study an effort has been made to enhance the process parameters and culture conditions of isolates fungi and facilitate improved production of antibacterial compound.

2. MATERIALS AND METHODS

2.1 *Sampling*

Samples were collected from center market of Bobo Dioulasso and Ouahigouya from March 2014 to august 2015. Eight (8) samples of groundnuts and four (4) samples of rice were taken in sterile containers. Samples are stored at 4ºC until analysis.

Figure 1: Sites of sample collection

2.2 *Isolation and identification of fungi*

Fungi were isolated according to Ulster or direct method.[11] It involved depositing directly food samples on PDA (Potato Dextrose Agar). Mycelia of each isolate were cultured in this medium and incubated at 30ºC. Purification of the isolates was performed by successive subcultures to obtain pure isolates.[12] In our research project on bioactive compound from fungi five (5) isolates of *Aspergillus* and six (6) isolates of *Penicillium* were collected from groundnut and rice and characterized. One isolate for both genera (S_2 and S_3) was found to be potent and was selected for further studies. The macroscopic and microscopic characterization strongly suggests that isolate S_2 was *Aspergillus flavus* and isolate S_3 *Penicillium citrinum*.

The identification was based on the conventional keys of Raper and Fennell[1] for *Aspergillus* and of Pitt[2] for *Penicillium.* Thus the main criteria used are growth time, colonies colors during incubation time, diameter and texture of colonies. This identification was completed with microscopic characteristics for example length of conidiophores, the hyphae texture and the number of divergent metulae in a whorl. The number of phialides which bearing conidia was also described.

2.3 *Indicators organisms*

Pure cultures of bacterial used as indicator strains were obtained from Centre Hospitalier Universitaire- Yalgado Ouédraogo (CHU-YO). It included two (2) Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 19249, *Salmonella* Typhymirium ATCC 13311) and two (2) Gram positive bacteria (*Listeria monocytogenes* CRBIB 13134, *Staphylococcus aureus* ATCC 9144).

2.4 *Process optimization of culture conditions for antimicrobial compounds production*

The optimum growth conditions of fungi and its antimicrobial activity was examined. The focus parameter were the incubation duration, pH of the broth, temperature of incubation, agitation rate and carbon and nitrogen source for the higher production of antimicrobial compound by potent *Aspergillus flavus* and *Penicillium citrinum*. The antimicrobial activity evaluation was carried out in 25 ml of synthetic broth in Erlenmeyers (100 ml). Spores were suspended in 5 ml of sterile water containing 0.1% of Tween 80. The broth was aseptically inoculated with spore suspensions of isolates S_2 and S_3. All the assays were carried out in triplicate.

2.4.1 Fermentation time

For the optimum incubation time for antimicrobial activity, fourteen (14) sterile Erlenmeyers (100 ml) containing 25 ml of synthetic broth whose the pH is adjusted at (7 for S_2 and 6 for S_3) were aseptically inoculated with spores suspensions of fungi. The Erlenmeyers were incubated for two weeks at 30°C in an orbital shaker at 150 rpm. At a every 24 hours, one Erlenmeyer was removed from the shaker and the antimicrobial activity was evaluated using discs diffusion method.[13] The culture filtrate was separated from the mycelium by centrifugation at 10000 rpm for 20 minutes in 4°C.

2.4.2 Initial pH

The effect of pH on antimicrobial activity was evaluated during a week of incubation. The initial pH of the synthetic broth was then adjusted with NaOH (0.1N) and HCl (0.1N) at pH6, pH6.5, pH7, pH7.5, pH8. Each isolate of S_2 and S_3 was inoculated in five (5) Erlenmeyers corresponding to a specific pH and then incubated at 30°C in the orbital shaker at 150 rpm.[14]

2.4.3 The temperature of incubation

The influence of the incubation temperature on the antimicrobial activity was evaluated using five gradients. Five (5) Erlenmeyers containing the synthetic broth whose the pH is adjusted at (7 for S_2 and 6 for S_3) was used for each isolate. The incubation temperature were respectively 20°C, 30°C, 35°C, 37°C and 44°C. The incubation was done on rotary shaker at 150 rpm for one week.[15]

2.4.4 The agitation frequency

The agitation frequency regulates the oxygenation rate in the Erlenmeyer. The agitation effect on antimicrobial activity was tested using six different frequencies. Then, for each isolate, Six (6) Erlenmeyers containing the synthetic broth whose the pH is adjusted at (7 for S_2 and 6 for S_3) was used. Each suspension spores of both isolates were inoculated. All Erlenmeyers were incubated

at 30°C a week for each specific frequency. The incubation was then respectively performed on the rotary shaker at 0 rpm, 50 rpm, 100 rpm, 150 rpm, 200 rpm and 250 rpm.[14]

2.4.5 The medium composition

To evaluate the effect of the sources of carbon and nitrogen on the antimicrobial activity, the synthetic broth was employed as an original medium. Five (5) Erlenmeyers were used for each isolate, whose the pH is adjusted at (7 for S_2 and 6 for S_3). Glucose, starch, fructose, sucrose and lactose with 30g/l as concentration are used as carbon source in the Erlenmeyers contained synthetic broth without Glucose.

Concerning the nitrogen sources effect evaluation, four (4) other Erlenmeyers for each isolate, whose the pH is adjusted at (7 for S_2 and 6 for S_3) were used. The first one was prepared with yeast extract as nitrogen source and the other one without yeast extract, and supplement respectively with 5 g/l of NH_4Cl, $(NH_4)_2SO_4$ or $NaNO_3$. All the Erlenmeyers were incubated at 30°C for one week on rotary shaker at 150 rpm.

2.5 Production of antimicrobial compounds

2.5.1 The fungal medium preparation

From the five (5) *Aspergillus* and the six (6) *Penicillium* initial isolates, one of both genera were identified as *Aspergillus flavus* and *Penicillium citrinum*. These isolates are then tested for their potentiality to produce antimicrobial compound. Fungi were inoculated in Potato Dextrose Agar (PDA) for three days. Fungal growth in synthetic broth is needed for antibacterial compounds production and extraction. Erlenmeyers of 100 ml volume containing 25 ml of synthetic broth with pH adjusted at (7 for S_2 and 6 for S_3) were sterilized at 121°C for 21 min. The inoculum was prepared by suspending spores harvested with 0.1% Tween 80 from a three (3) day old PDA cultures. Sterilized Erlenmeyer were aseptically inoculated with spore suspensions. Erlenmeyers were incubated at 30°C for a week.

2.5.2 Antimicrobial compound extraction

For the nature of the antimicrobials compound is initially unknown, four (4) solvents were used in order to optimize their extraction. It was acetonitrile, chloroform, ethyl acetate and methanol. The fungi metabolites extraction from synthetic broth is performed three times.[14] After incubation, each solvent at equal ratio (1/1) were added to the Erlenmeyers and the crude solvents were collected. This substrate solvent mixture was allowed to become thoroughly wet at refrigerator for 2 hours, followed by 2 hours of mechanical agitation, and then it was filtered using filter paper (Whatman n° 4). The filtrated extract (5 ml) was dried under a flow with rotavapeour at 45°C with a slight rotation of 150 rpm.[16] The extract was taken with 1 ml of each four solvents and conserved at 4°C before using for antimicrobial activity test.[17]

2.5.3 Test for activity against indicator bacteria

The conserved extract filtrates were screened for antimicrobial activity using disc diffusion method on Mueller- Hinton agar plates seeded with the target indicator bacteria as described by Rodrigues and Tilvi.[17] Briefly, sterile and virgin discs of 5 millimeters in diameter were impregnated with 250 µg. $disc^{-1}$ of extracted filtrate. Each indicator bacteria was grown in 10 ml of nutrient broth and its turbidity was adjusted to be equal to or greater than 0.5 McFarland turbidity standard.[18] They were homogeneously spread with flame-sterilized handle on the surface of freshly prepared Mueller-Hinton agar.[19, 20] The impregnated discs were placed on the agar. The Petri dishes was pre incubated at 4°C for 2 hours to allow uniform diffusion into the agar. After pre-incubation, the plates were incubated for 24 hours at 37°C. The diameters of the clear zones around each disc were measured and compared with control agar plates containing disc with solvent only (negative control).[21] Triplicates tests were done for each of the fungal extract.

2.5.4 Statistical analysis

The collected data were subjected to analysis of variance (ANOVA) considering the inhibition diameter, the growing factor and indicator bacteria were done using XLSTAT-Pro 7.5.2 software. The internal variation was evaluate using Newman-Keuls test at probability level p = 5% to compare the average inhibition zone of the extracts. The results were expressed as mean ± SD and the measures were repeated three times (n=3).

3. RESULTS

3.1 Macroscopic morphology of the colonies

The isolates S_2 and S_3 grew well on rich medium like PDA plates. They easily produce superficial and submerged hyphae with fruiting bodies. Colonies of isolates S_2 attained 40 to 50 mm as diameter in 7 days of incubation at 30°C. They were firstly white, yellow-green thereafter dark green above all in the center where texture was velvety and more dense, with yellow reverse. As for the isolate S_3, its colonies with a regular margin attained 15 to 20 mm as diameter in 7 days at 30°C. Colonies were white in the center and green dark frame, with yellow reverse, they produce exudate (Fig 1).

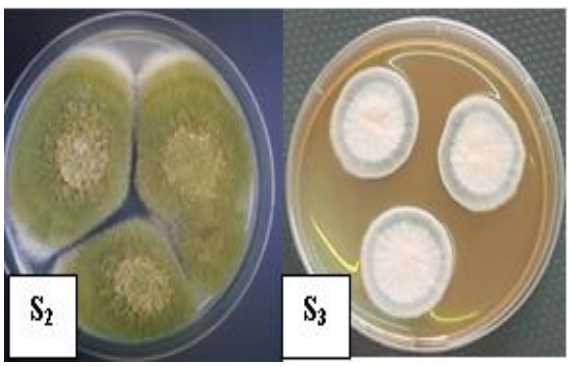


Figure 1: Macroscopic aspects of isolates S_2 and S_3 in PDA media at 7 days of incubation at 30°C

3.2 Microscopic morphology of the cells

In optical microscopy, isolate S_2 showed long and dense felt of yellow conidiophores, which were non partitioned hyalines up to 0.85 mm in lenght. Aspergillaires heads were biseriates and radiaires, they have globose to subglobose vesicles, 30-40 µm in diameter. Phialides borne directly on the metulae which bear numerous round and refractive conidia. Isolate S_3 showed uncommon conidia, conidiophores were biverticillate and partitioned with 3-5 divergent metulae in a whorl. Each metulae bear 4-6 phialides. According to the caracteristics described respectively by Raper and Fennell[1] and by Pitt[2] key, isolate S_2 was *Aspergillus flavus* and S_3 was *Penicillium citrinum* (Fig 2).

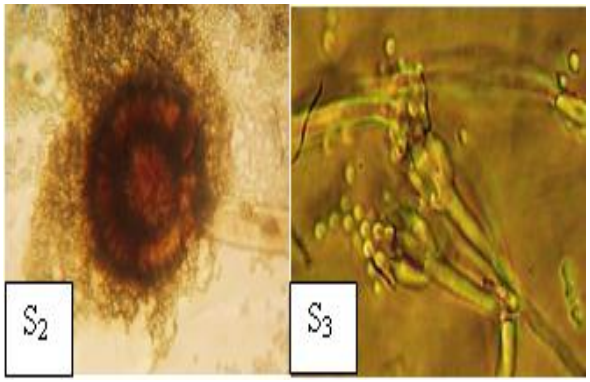


Figure 2: Microscopic aspects of isolate S_2 and S_3 (x100)

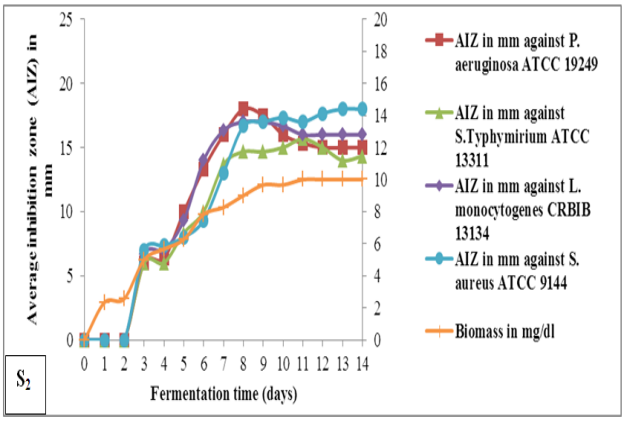
3.3 Isolation and Screening for antimicrobial activity

About eleven (11) fungi were isolated from groundnut and rice samples. They were then used in the antibiosis trial against indicators bacteria. It show that isolates S_2 and S_3 were capable to produce antimicrobial substances against both Gram negative and Gram positive bacteria used as indicators strains. These isolates were selected for optimization.

3.4 Optimization of culture conditions for higher production of antibacterial compounds

a. Effect of the incubation duration

The antibacterial compound production by isolated fungi was monitored over two weeks in synthetic broth. The course of antibacterial compound production and growth by isolates (S_2, S_3) are shown in Fig 3. The production started only after 48 hours for both isolates. The highest rate of antimicrobial activity by the isolates was observed from days 7 up to 9 after its subculture into fermentation broth. After the 9th day, no increase in antibacterial activity was observed. Antimicrobial compound biosynthesized is correlate with fungal growth and development. It was highest in the stationary phase where diameter of inhibition zone reached 18 mm average with 10 mg/dl as biomass value for isolate S_2 and 7 mg/dl for isolate S_3.



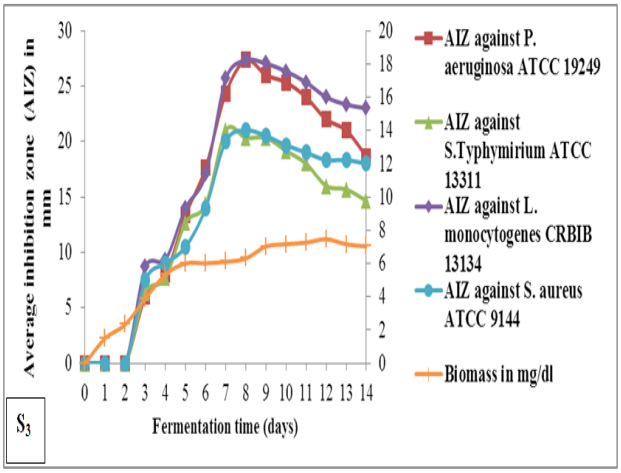


Figure 3: Time course of biomass and antibacterial activity of isolates S_2 and S_3 during the fermentation.

b. Effect of pH of the broth

The effect of initial pH of the medium on antibacterial compounds production was performed starting from 6 to 8 with 0.5 differences. The production was maximum for isolate S_2 and S_3 respectively at pH 7 and pH 6. Both isolates preferred neutral pH range rather than acidic or alkaline for the antibacterial activity. The average inhibition zone reached 22 mm at pH 7 for isolate S_2 and 19 mm at pH 6 for isolate S_3. There is a relationship between the broth pH and zone of inhibition, for the increase in pH correlate with an increase in zone diameter (Table 1).

Table 1: Effect of the pH on the size of inhibition zone (mm) of the antimicrobial activity of isolates S_2 and S_3. Data expressed as mean ± SD (n=3)

Test bacterial	pH	Isolate S_2	Isolate S_3
P. aeruginosa	6	14.33 ± 0.58^{d}	21 ± 1^{a}
	6.5	18 ± 1^{c}	16 ± 1^{b}
	7	22.67 ± 0.58^{a}	11 ± 1^{d}
	7.5	20.33 ± 0.58^{b}	13.33 ± 0.58^{c}

	8	11.67±1[e]	11±1[d]
significance level		< 0.0001	< 0.0001
S. Typhymirium	6	11±1[d]	19±0.58[a]
	6.5	15.33±0.58[c]	15.33±1[b]
	7	19.67±0.58[a]	9.67±0.58[d]
	7.5	14.67±0.58[c]	9±1[d]
	8	17.67±0.58[b]	12.33±0.58[c]
significance level		< 0.0001	< 0.0001
L. monocytogenes	6	11±1[d]	23±0.58[a]
	6.5	13±1[c]	11±0[c]
	7	22±1[a]	13.33±1[b]
	7.5	17±1[b]	13±1[a]
	8	14.67±0.58[c]	11±0[c]
significance level		< 0.0001	< 0.0001
S. aureus	6	8.33±0.58[d]	13±0.58[a]
	6.5	8.33±0.57[d]	8.33±1[c]
	7	19±1[a]	10.33±0.58[b]
	7.5	13.33±0.58[b]	6.67±0.58[d]
	8	11±1[c]	9.33±0.58[bc]
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

c. Effect of temperature of incubation

Among the five (5) tested temperature, 30˚C was observed to be optimum temperature for antimicrobial activity. Table 2 depicts the effect of incubation temperature on antibacterial production by the fungi isolates. The mesophylic range of temperature was determined as an optimum temperature for antimicrobial activity. Thus the average inhibition zone reached 18 mm and 19 mm diameter respectively for isolate S_2 and S_3 at 30°C.

Table 2: Effect of the temperature on the size of inhibition zone (mm) of the antimicrobial activity of isolates S_2 and S_3. Data expressed as mean ± SD (n=3)

Test Bacterial	Temperature (°C)	Isolate S_2	Isolate S_3
P. aeruginosa	20	9±1[d]	7±1[d]
	30	23±1[a]	16.33±0.58[a]
	35	17±1[b]	17±1[a]
	37	14.33±0.58[c]	13.67±0.57[b]
	44	8.67±0.58[d]	10.33±0.58[c]
significance level		< 0.0001	< 0.0001
S. Typhymirium	20	11.67±0.58[c]	11±1[c]
	30	17.67±0.58[a]	15±0[a]
	35	14.67±0.58[b]	12.67±0.58[b]
	37	12.67±0.58[c]	10.33±0.58[c]
	44	7.33±0.58[d]	7±1[d]
significance level		< 0.0001	< 0.0001
L. monocytogenes	20	13.67±1[b]	10.33±0.58[e]
	30	19±1[a]	23.33±0.58[a]
	35	12.67±0.58[bc]	18±1[b]
	37	18±0.58[a]	14.67±0.58[c]
	44	11±0.58[c]	12.33±0.58[d]
significance level		< 0.0001	< 0.0001
S. aureus	20	12.33±1[b]	8.33±0.58[d]
	30	13.33±1[b]	23±0[a]
	35	18.33±0.58[a]	19±1[b]
	37	10±0.58[c]	18.33±0.58[b]
	44	8.67±0.58[c]	15.67±0.58[c]
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

d. Effect of the agitation rate

The optimal amount of shaker movement was noticed at 150 rpm. The higher average inhibition zone was 22 mm at 150 rpm and the little was 10 mm at 50 rpm. For both isolate any antibacterial activities were observed without agitation. The rate of agitation and the amount of yield were linked (Table 3).

Table 3: Effect of the aeration on the size of inhibition zone (mm) of the antimicrobial activity of isolates S_2 and S_3. Data expressed as mean ± SD (n=3)

Tests bacterial	Aeration levels (rpm)	Isolate S_2	Isolate S_3
P. aeruginosa	0	0^{d}	0^{d}
	50	14 ± 1^{c}	10.67 ± 0.58^{c}
	100	18 ± 1^{b}	15.33 ± 0.58^{b}
	150	22.33 ± 0.58^{a}	23^{a}
	200	15 ± 1^{c}	11 ± 1^{c}
significance level		< 0.0001	< 0.0001
S. Typhymirium	0	0^{d}	0^{d}
	50	10.33 ± 0.58^{c}	9.67 ± 0.58^{c}
	100	15 ± 1^{b}	13 ± 1^{b}
	150	19.33 ± 0.58^{a}	15.67 ± 0.58^{a}
	200	10.33 ± 0.58^{c}	13^{b}
significance level		< 0.0001	< 0.0001
L. monocytogenes	0	0^{d}	0^{e}
	50	11 ± 1^{c}	13.33 ± 0.58^{d}
	100	17.33 ± 0.58^{b}	17.33 ± 0.58^{b}
	150	22.67 ± 0.58^{a}	19.67 ± 0.58^{a}
	200	11.67 ± 0.58^{c}	15.67 ± 0.58^{c}
significance level		< 0.0001	< 0.0001
S. aureus	0	0^{e}	0^{e}
	50	7.67 ± 0.58^{d}	7.67 ± 0.58^{d}
	100	19 ± 1^{b}	14.33 ± 0.58^{b}
	150	23.67 ± 0.58^{a}	22.33 ± 0.58^{a}
	200	12.67 ± 0.58^{c}	8.67 ± 0.58^{c}
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

e. Effect of the medium composition

The investigation on the enhanced production of antimicrobial compounds usually involves an exploration of appropriate fermentation medium. The effect of carbon source on antibacterial compound production by isolated fungi is presented in table 4. In our study, carbon sources used in the five reconstructed media evaluated included simple sugar (glucose, fructose) and complex sugar (starch, sucrose, lactose). Among of them starch and glucose for isolate S_2 and starch and lactose for isolate S_3 produced the high rate of inhibition. The diameter of the average inhibition zone reached 20 mm and 21 mm respectively for isolate S_2 and S_3.

Table 4: Effect of different carbon sources supplemented in modified synthetic broth on the size of the inhibition zone (mm) the antimicrobial activity of isolates S_2 and S_3. Data expressed as mean ± SD (n=3)

Tests bacterial	Carbon source	Isolate S_2	Isolate S_3
P. aeruginosa	Glucose	21 ± 1^{b}	9 ± 1^{e}
	Fructose	8.67 ± 0.58^{e}	11 ± 1^{d}
	Lactose	18 ± 1^{c}	21 ± 1^{b}
	Starch	23.33 ± 0.58^{a}	23.67 ± 0.58^{a}
	Sucrose	13 ± 1^{d}	18.67 ± 0.58^{c}
significance level		< 0.0001	< 0.0001
S. Typhymirium	Glucose	17.33 ± 0.58^{b}	9.33 ± 0.58^{d}
	Fructose	7.33 ± 0.58^{e}	8 ± 0^{d}
	Lactose	14 ± 1^{c}	14 ± 1^{b}
	Starch	19.67 ± 0.58^{a}	19 ± 1^{a}
	Sucrose	10 ± 1^{d}	11 ± 1^{c}
significance level		< 0.0001	< 0.0001

L. monocytogenes	Glucose	20.67±0.58b	12.33±0.58^{e}
	Fructose	7.67±0.58^{e}	13.33±0.58^{d}
	Lactose	16±1^{c}	20±0^{b}
	Starch	23±1^{a}	22.33±0.58^{a}
	Sucrose	13.33±0.58^{d}	15.33±0.58^{c}
significance level		< 0.0001	< 0.0001
S. aureus	Glucose	19.33±0.58^{a}	11±1^{d}
	Fructose	11±1^{b}	14.33±1.15^{c}
	Lactose	12.33±0.58^{b}	17.67±0.58^{b}
	Starch	20±1^{a}	19.33±0.58^{a}
	Sucrose	8.67±0.58^{c}	16.67±0.58^{b}
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

The nitrogen source used in this study include yeast extract, NH_4Cl, $(NH_4)_2SO_4$ and $NaNO_3$. Thus yeast extract proved itself to be the best nitrogen supply for both isolates. The fungi showed the higher diameter of inhibition zone of 20 mm average against the indicator bacteria when the fermentation broth contained yeast extract for nitrogen source. The diameter zone was decrease when yeast extract was replaced by NH_4Cl, $NaNO_3$ and $(NH_4)_2SO_4$ (Table 5).

Table 5: Effect of different nitrogen sources supplemented in modified synthetic broth on the size of the inhibition zone (mm) of the antimicrobial activity of isolates S_2 and S_3. Data expressed as mean ± SD (n=3)

Test Bacterial	Nitrogen source	Isolate S_2	Isolate S_3
P. aeruginosa	Yeast extract	23.67±0.58^{a}	23±1^{a}
	NH_4Cl	19±1^{b}	19.33±0.58^{b}
	$NaNO_3$	14.33±0.58^{c}	15.33±0.58^{c}
	$(NH_4)_2SO_4$	10.67±0.58^{d}	10.33±0.58^{d}
significance level		< 0.0001	< 0.0001
S. Typhymirium	Yeast extract	17.33±0.58^{a}	18.67±0.58^{a}
	NH_4Cl	13±0^{b}	11.67±1.52^{b}
	$NaNO_3$	11.67±0.58^{c}	8.67±0.58^{c}
	$(NH_4)_2SO_4$	11±1^{c}	11±0^{b}
significance level		< 0.0001	< 0.0001
L. monocytogenes	Yeast extract	22.67±0.58^{a}	21±1^{a}
	NH_4Cl	17.67±0.58^{b}	18.67±0.58^{b}
	$NaNO_3$	15.33±0.58^{c}	16±0^{c}
	$(NH_4)_2SO_4$	12.33±0.58^{d}	12.67±0.58^{d}
significance level		< 0.0001	< 0.0001
S. aureus	Yeast extract	16.67±0.58^{a}	19.33±0.58^{a}
	NH_4Cl	11±1^{b}	11.67±0.58^{c}
	$NaNO_3$	8.67±0.58^{c}	14.33±0.58^{b}
	$(NH_4)_2SO_4$	7.67±0.58^{c}	9.33±0.58^{d}
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

f. Solvent extraction method for the recovery of antimicrobial compounds

Different organic solvents in decreasing polarity like acetonitrile, chloroform, ethyl acetate and methanol were used to extract the antimicrobial compounds from the filtrates of fermented broth by solvent extraction method. Ethyl acetate and chloroform reveal themselves to be suitable solvents. Nevertheless a lower antimicrobial activity was obtained with methanol and acetonitrile (Table 6).

Table 6: Efficiency of various solvents for extraction of bioactive compound produced by isolates S_2 and S_3. Data expressed as mean ± SD (n=3).

Test Bacterial	Solvents	Isolate S_2	Isolate S_2
P. aeruginosa	Acetonitrile	10.67 ± 1^{d}	15.33 ± 0.58^{c}
	Chloroform	18 ± 0.58^{b}	23.33 ± 0.58^{a}
	Methanol	14.33 ± 1^{c}	13 ± 1^{d}
	Ethyl acetate	23.67 ± 0.58^{a}	18.33 ± 0.58^{b}
significance level		< 0.0001	< 0.0001
S. Typhymirium	Acetonitrile	8.67 ± 0.58^{d}	10.67 ± 0.58^{c}
	Chloroform	14.67 ± 1^{b}	16.33 ± 0.58^{a}
	Methanol	11.33 ± 0.58^{c}	9.67 ± 0.58^{c}
	Ethyl acetate	17 ± 1^{a}	13.67 ± 0.58^{b}
significance level		< 0.0001	< 0.0001
L. monocytogenes	Acetonitrile	12.33 ± 1^{b}	13.33 ± 0.58^{c}
	Chloroform	20.67 ± 0.58^{a}	19.67 ± 0.58^{a}
	Methanol	13.33 ± 0.58^{b}	13.67 ± 0.58^{c}
	Ethyl acetate	19.67 ± 0.58^{a}	16.67 ± 0.58^{b}
significance level		< 0.0001	< 0.0001
S. aureus	Acetonitrile	7 ± 1^{d}	8.67 ± 0.58^{c}
	Chloroform	13.33 ± 0.58^{b}	12 ± 1^{b}
	Methanol	10 ± 1^{c}	15 ± 1^{a}
	Ethyl acetate	15 ± 1^{a}	9.33 ± 0.58^{c}
significance level		< 0.0001	< 0.0001

The values which are in common the same letter are not significantly different according to the Newman-Keuls test at probability level of 5%.

4. DISCUSSION

In recent years, great attention has been paid to study the secondary metabolites of fungi due to their potential pharmacological utilization.[22] Development of an efficient fermentation process for the production of secondary metabolites by fungi isolate requires examination of a diverse array of species-specific features, including physical and chemical factors. Carbohydrates and nitrogen play key roles as structural and energy compounds in cell. Also several cultivation parameters like pH, incubation period, rotary shaker and temperature play a major role in the production of bioactive metabolites. The antibacterial compound production started the 3rd day. Yield of antibacterial compound was high between 7-9 days during the stationary phase, showing that the metabolite production was directly proportional to the growth rate (Fig 3). The obtained results are in agreement with those of Asnaashari and Ghanbary[15] who obtained penicillin G production on the 8th day from *Penicillium chrysogenum*. Furthermore, Gallagher and Richard[23] also observe a production of cyclopiazonid by *Aspergillus flavus* one week on rice media.

The highest antibacterial activity by *Aspergillus flavus* and *Penicillium citrinum* was achieved respectively at pH 7 and pH 6, but a sensible variation of the pH reduces significantly the production of metabolite compounds (Table 1). Generally, in most published literature, optimum pH for antibiotic production by imperfect fungi cultures has been reported to be near neutral.[24,25] This is in adequation with result obtained by Devi and D'Souza[10] who produced citrinin by *Penicillium chrysogenum* at pH 6.5. According to Guimaraes and Furlan[26], the pH of the medium culture is one of the most important environmental factors, because it exerts a marked effect on the activity of several enzymes that catalyze metabolic reactions, as well as exerting significant influence on complex physiological phenomena such as membrane permeability and cell morphology.[26]

The incubation temperature was also found to have an effect on growth as well as bioactive metabolite production. The temperature of 30˚C was observed to be the optimum temperature for the antibacterial activity by both fungi in this study (Table 2). Similar results have been reported by Devi and D'Souza[10] in their work on citrinin production by *Penicillium chrysogenum* where, the optimum temperature was slightly different (27±2˚C). In addition Rosfarizan and Ariff[27] also incubated Erlenmeyer flasks at 30˚C to produce kojic acid by *Aspergillus flavus*.

Agitation affects aeration and mixing of the nutrients in the fermentation medium, it led to increased secondary metabolite synthetize. Although agitation is usually considered only from the viewpoint of oxygen supply. A high agitation may damage cells and fragment hyphae. The consequence is a reduction in antibiotic production associate with an absence of aerial mycelium. Agitation speed of 150 rpm was found as an optimum speed for both isolates (Table 3). This result also corroborated with those of Atalla and Zeinab[22] who incubated Erlenmeyers flasks of fungus *Varicosporina ramulosa* on a rotary shaker at 150 rpm for biologically active

compound production. However, our results is incoherent with those of Rosfarizan and Ariff[27] who obtained kojic acid production by *Aspergillus flavus* with 250 rpm as rotary shaker. Devi and D'Souza[10] also incubated Erlenmeyer flask at 200 rpm to produce citrinin by *Penicillium chrysogenum*.

The carbon sources used in the synthetic broth evaluated in this study included simple and complex sugar. Starch and glucose for isolate S_2 and starch and lactose for isolate S_3 proved themselves to be the most appropriate carbon source for eliciting higher production of secondary metabolites by the selected isolates evaluated (Table 4). Similar results for isolate S_2 were reported by Rosfarizan and Ariff[27] who found the best carbon sources for kojic acid production by *Aspergillus flavus* to be glucose and Starch. For strain S_3 this is incoherent with methodologies utilizing glucose to increase the antibiotic productivity. It has also reported that lactose was the best carbon for penicillin production by *Penicillium chrysogenum* probably due to the slow hydrolysis of the disaccharide as the result of the very low β-galactosidase activity.[6]

In this study, it was clear from the results that the growth of the isolates fungi was greatly influenced by the nature and type of nitrogen source supplemented in the medium. In comparison with the inorganic nitrogen sources, organic nitrogen sources induced relatively bioactive metabolite production. In this study, yeast extract proved to be the most appropriate nitrogen source for eliciting higher production of secondary metabolites by both isolates (Table 5). According to Emelda and Vijayalakshmi[28] slowly utilizable organic nitrogen sources such as yeast extract have been shown to benefit for antibiotic production. It is due to the avoidance of immediate interference of ammonium on synthases of secondary metabolism, which can be generated by rapidly utilizable nitrogen sources such as inorganic ammonium salts (NH_4Cl, $NaNO_3$ and $(NH_4)_2SO_4$). This was corroborated by Adinarayana and Prabhakar[29] who showed that the highest cephalosporin C production by *Acremonium chrysogenum* were obtained with starch, sucrose and yeast extract as respectively carbon and nitrogen source.

Antibacterial compound was extracted by four solvents used in this study. Chloroform and ethyl acetate were the best solvents for both isolates (Table 6). The previous study done by Rosfarizan and Ariff[27] and those of Devi and D'Souza[10] has also revealed a similar statement. The extraction of the cell free supernatant of *Aspergillus flavus* and *Penicillium citrinum* with chloroform and ethyl acetate demonstrated bioactive crude extract that displayed activity against a panel of pathogens tested.

5. CONCLUSION

The present study showed that pH, temperature, incubation duration, rotary shaker, carbon and nitrogen source directly influenced the production of these bioactive metabolites. The two strains tested here behaved differently, each one requiring specific conditions for maximum growth as well as bioactive metabolite production. As indicated by the results, the antibacterial compound produced by isolate S_2 could be kojic acid and that from isolate S_3 may be a citrinin. Confirmations need further investigations to determine the functional group, elucidate the structure and physicochemical nature of the compounds. Further studies are under progress for molecular characterization of the isolates to confirm morphological identification. From the above results it can be concluded that fungi have proven even today are rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity.

Conflict of Interests

The authors have not declared any conflict of interests.

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