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Application of GC–MS Technique for the Detection of Secondary Metabolites of *Penicillium Citrinum*

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ABSTRACT

Objective: *The effect of culture conditions on the production of secondary metabolites by Penicillium citrinum was studied via Gas Chromatography-Mass Spectrometry (GC-MS). The culture filtrate of the fungus was obtained from two different media, Potato Dextrose Broth (PDB) and Yeast Extract, grown at two temperatures (25°C and 28°C), to assess the effect of culture conditions on the production of metabolites.* ***Methods:*** *The culture filtrate of the fungus was obtained from two different media, Potato Dextrose Broth (PDB) and Yeast Extract, grown at two temperatures (25°C and 28°C), to assess the effect of culture conditions on the production of metabolites.* ***Results:*** *The GC–MS results showed differences in the qualitative and quantitative composition of metabolites produced under various conditions. Cultivation in PDB at 28°C led to a wide variety of compounds, with prominent metabolites being derivatives of 1,2-benzenedicarboxylic acid and dimethyl phthalate. However, growth at 25°C resulted in the dominance of a smaller number of compounds, such as cyclohexasiloxane derivatives. Cultivation in yeast extract medium at 25°C improved the metabolic diversity, while higher temperatures (28°C) decreased diversity but increased the amount of dominant metabolites.* ***Novelty:*** *These findings suggest that P. citrinum's metabolic pathways are significantly affected by external conditions like temperature and nutritional conditions. Our results align with the OSMAC (One Strain - Many Compounds) principle, which shows that a single fungus can yield many different metabolites under different conditions. The detection of phenolic and phthalate compounds indicates potential antimicrobial activity, suggesting P. citrinum has significant potential as a source of bioactive metabolites for drug discovery.*

INTRODUCTION

Penicillium citrinum is a saprophytic, filamentous fungus of the genus *Penicillium*, a very widespread type of mold that is typically found in soil, fallen vegetation, food products kept in storage, as well as indoor environments. It belongs to the phylum Ascomycota, and it is distinguished by its quick growth, septate hyphae, and asexual spores in the form of conidia. *P. citrinum* colonies are generally yellow to green and have a powdery texture because they have a high rate of sporulation in the favorable environmental circumstances [1].

Penicillium citrinum has gained an increasing scientific interest because of its capacity to synthesize a broad spectrum of biologically active secondary metabolites with antimicrobial effects. Despite its well-established reputation in producing mycotoxin citrinin, recent research has shown the possibility of using it as a source of new antimicrobial agents against many pathogenic microorganisms [2].

Various strains *of P. citrinum* have been reported to produce compounds with antibacterial activity against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Escherichia coli,* which are clinically relevant. These bioactive

metabolites are polyketides, alkaloids, and peptides, whose action is through interference with the cell walls of microorganisms by disrupting protein synthesis, blocking essential metabolic pathways, or inhibiting protein synthesis [3].

Besides its antibacterial effect, *P. citrinum* has also been shown to have an antifungal effect against other competing fungal species. This is an ecological benefit that enables it to survive in competitive ecological conditions, such as soil and rotting organic materials. The antifungal products formed can cause inhibition of the spore germination or the growth of the hyphae in other fungi, and are of special interest in agricultural and pharmaceutical use [4].

Penicillium citrinum has a high antibacterial activity because of its synthesis of bioactive secondary metabolites, including polyketides, alkaloids, and peptides that interfere with vital bacterial activities. Its extracts have been reported to prevent Gram-positive, as well as Gram-negative, bacteria, such as *Staphylococcus aureus* and *Escherichia coli* [5].

The inhibitory mechanism is mainly linked to impairment of the cell wall and cell membrane integrity of the bacteria, resulting in the leakage of intracellular components and subsequent death of the cell. Moreover, certain metabolites of *P. citrinum* are able to prevent the synthesis of proteins and the multiplication of nucleic acids, which further increases their antimicrobial effect [6].

Gas Chromatography-Mass Spectrometry (GC-MS) is a very advanced method of analysis, a combination of the separating ability of gas chromatography and the identification ability of a mass spectrometric method. It is also believed to be one of the surest ways of analyzing volatile and semi-volatile organic compounds. The high sensitivity and accuracy of GCMS make it widely applicable in the pharmaceuticals, environmental analysis, forensic science, and food safety [7].

In gas chromatography, the separation of the compounds is done according to their volatility and their interaction with the stationary phase of the column. The sample is vaporized in this process, and the inert carrier gas, say helium, transfers the product through a capillary column. The various compounds have varied retention times based on their boiling points and polarity. Nonetheless, the use of GC is insufficient to allow unambiguous identification of compounds, and it is combined with mass spectrometry [8].

Mass spectrometry gives a positive identification through ionizing the chemical compound and measuring the mass-to-charge ratio (m/z). Electron impact ionization is usually used in GC-MS systems, which results in compound-specific fragmentation patterns. These patterns are molecular fingerprints that are capable of easy comparison with spectral libraries to identify them accurately. It renders GCMS very helpful even with complicated mixtures [9].

Among the benefits of GC-MS are that it is very sensitive and specific, as it can detect compounds at very low levels, including parts per million or billion. It also gives both qualitative and quantitative results with a single run, which saves a lot of time in

terms of efficiency and time to analyze. Separation and detection provide a significant enhancement in the accuracy of the analysis [10].

The recent developments in GC-MS technology, such as tandem mass spectrometry (GC-MS/MS) and high-resolution systems, have significantly increased performance in sensitivity, resolution, and speed. The technique also has more convenient software with automated analysis and interpretation of data to further make it more accessible and dependable in daily laboratory work [11].

Conclusively, GC -MS is a vital analytical instrument because it can separate, identify, and quantify chemical substances accurately. Its further evolution guarantees its further relevance to various fields of science [12].

RESEARCH METHOD

1. Preparing Fungal Filtrate

Potato Dextrose Broth (PDB) was prepared in four 250 mL Volumetric flasks under sterile conditions, and 4 extra flasks with Yeast Extract medium [13] were prepared using 250 mL Volumetric flasks. Each of the flasks was aseptically inoculated with the fungal isolate of *Penicillium citrinum*, and all the cultures were then incubated in a shaking incubator at 28 °C with an agitation rate of 100-150 rpm to allow the cultures to grow without being disturbed and produce all the possible metabolites.

With the aim of optimizing production efficiency, identical amounts of the same media were inoculated under the same conditions but incubated in a static incubator at 25 °C [14]. The media were filtered using sterile gauze, fungal growth was removed, and only the filtrate was collected. The filtrate was placed in tubes.

2. Determination of the active compounds in the fungal filtrate using GC–MS

A 50 µL sample of the fungal filtrate was injected into the GC-MS system. A Shimadzu 15A gas chromatograph with a split/splitless injector (250 °C) and a flame ionization detector (250 °C) was used for GC/MS analysis. The carrier gas used was nitrogen (1 ml/min), and the capillary column was DB-5 (50 m × 0.2 mm, film thickness 0.32 µm.

The column temperature program started from 60 to 220 °C at a temperature gradient of 5°C per minute, and then held at 220°C for 5 min**.**

The relative percentage of the components was calculated by the peak area using a Shimadzu C-R4A Chromatopac without correction factors.

RESULTS AND DISCUSSION

To study the secondary metabolite profile of the fungal filtrate of *Penicillium citrinum* under various cultivation conditions, we used Gas Chromatography-Mass Spectrometry (GC-MS) with different growth media and temperatures. This analysis showed variations in the qualitative and quantitative profiles of metabolites under these different conditions.

Under the culture conditions of potato dextrose broth (PDB) at 28°C, the chromatographic profile revealed a rich and diverse profile with several peaks observed

across a broad range of retention times, suggesting the presence of a variety of compounds. The most abundant compound was identified by a major peak at 23.731 min. This was corroborated by the GC–MS data table, which listed 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester as the most abundant compound at 35.55%. Other major compounds included dimethyl phthalate (29.59%) at 16.233 min and propane, 1-bromo-3-chloro- (15.21%) at 32.235 min. Furthermore, a number of minor compounds were present, such as phenolic compounds like phenol, 2-methyl-5-(1-methylethyl)-, which could have biological effects. The differences in peak intensities indicate varying concentrations of compounds present and suggest the capability of the fungus to produce diverse secondary metabolites under the given conditions.

After culturing the fungus in PDB medium at 25°C, there were fewer major compounds with higher intensities. Cyclohexasiloxane, dodecamethyl- was the dominant compound, comprising 54.59% of the total peak area with a retention time of 13.66 min. This was followed by dimethyl phthalate (16.01%) and tetracosamethyl-cyclododecasiloxane (14.34%). This suggests that the decrease in temperature resulted in the production of a single major compound rather than a complex spectrum, which was reflected in the metabolic changes of the fungus.

On the other hand, growth in yeast extract medium at 25°C led to a greater metabolic diversity. The chromatogram showed several peaks with a wide range of retention times, with no dominant metabolites. The predominant compounds identified were dodecamethylcyclohexasiloxane (10.71%), eicosane (10.09%), and gentisic acid (7.24%), with other compounds present in lesser amounts (2-5%). This profile suggests a more varied metabolite production and that yeast extract medium stimulates metabolic diversity at a middle temperature.

But, when the incubation temperature was increased to 28°C with the same yeast extract medium, a decrease in the number of compounds detected but an increase in the proportion of some metabolites was observed. This chromatogram had fewer but stronger peaks, suggesting the prominence of certain compounds. The predominant compounds included cyclohexasiloxane, dodecamethyl- (47.23%), methoxy-6-aza-9-beta-estra-1,3,5(10),6,8(9)-pentaen-17-ol (26.60%), N-benzylideneisopropylamine-N-oxide (15.55%), and 1,9b-dihydro-4-methyl-1H-phenanthro [9,10-bazirine (10.62%). This result implies that the higher temperature may inhibit metabolic diversity but improve the production of dominant compounds.

The findings clearly show that the choice of growth media and temperature of incubation have a significant impact on the metabolism of *Penicillium citrinum*. The PDB medium incubated at 28°C enabled the production of a variety of metabolites with dominant compounds while the yeast extract medium at 25°C enabled a more diverse range of metabolites without a dominant compound. On the other hand, at higher temperatures in yeast extract media, the metabolic diversity decreased and the production of a few major compounds increased, showing a temperature-induced metabolic shift.

Table 1. GC–MS analysis of secondary metabolites of *Penicillium citrinum* grown in PDB medium at 28°C.

No.	Name	RT (min)	Area%	Quality	CAS Number
1	1,1-Diisobutoxy-butane	9.919	0.60	32	0-00-000000
2	ethyl E-2-methyl-2-butenoate	11.865	1.29	15	0-00-000000
3	Phenol, 2-methyl-5-(1-methylethyl)-	13.349	4.79	93	2-75-000499
4	Phenol, 2-(1,1-dimethylethyl)-	13.639	1.23	38	6-18-000088
5	Dimethyl phthalate	16.233	29.59	97	3-11-000131
6	Imidazole, 2-bromo-4-methyl-5-nitro-	18.636	2.33	49	8-46-105983
7	Ledene	18.776	1.40	53	6-46-021747
8	2',4'-DIMETHYLOXANILIC ACID N'-VERATRYLIDENEHYDRAZIDE	20.498	1.14	64	0-00-000000
9	o-Cresol, .alpha.,.alpha.'-(propylenedinitrilo)di-	21.541	0.81	41	7-91-000094
10	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	23.731	35.55	83	5-69-000084
11	Tetradecane	24.364	0.51	53	4-59-000629
12	Propane, 1-bromo-3-chloro-	32.235	15.21	35	6-70-000109
13	Bis(2-ethylhexyl) phthalate	33.543	4.36	59	7-81-000117

Table 2. GC–MS analysis of bioactive secondary metabolites produced by *Penicillium citrinum* cultured in PDB medium at 25°C.

No.	Name	RT (min)	Area%	Quality	CAS Number
1	Cyclohexasiloxane, dodecamethyl-	13.66	54.59	35	6-97-000540
2	Dimethyl phthalate	16.851	16.01	41	3-11-000131
3	Tetracosamethyl-cyclododecasiloxane	20.499	14.34	38	3-94-018919

Table 3. GC–MS analysis of fungal metabolites from *Penicillium citrinum* cultured in yeast extract medium at 28°C.

No.	Name	RT (min)	Area%	Quality	CAS Number
1	Cyclohexasiloxane, dodecamethyl-	13.66	47.23	58	6-97-000540
2	N-Benzylideneisopropylamine-N-oxide	16.799	15.55	46	0-00-000000
3	3-METHOXY-6-AZA-9.BETA.-ESTRA-1,3,5(10),6,8(9)-PENTAEN-17-ONE	17.245	26.60	46	0-00-000000
4	1a,9b-dihydro-4-methyl-1H-phenanthro[9,10-b]azirine	24.369	10.62	43	1-47-111005

Table 4. GC–MS analysis of fungal metabolites from *Penicillium citrinum* cultured in yeast extract medium at 25°C.

No.	Name	RT (min)	Area%	Quality	CAS Number
1	Dodecamethylcyclohexasiloxane	13.67	10.71	90	6-97-000540
2	GENTISIC ACID	20.499	7.24	64	0-20-003618
3	Eicosane	24.374	10.09	94	8-95-000112
4	3,6-Bis(N,N-dimethylamino)-9-methylcarbazole	24.764	4.30	42	8-55-119046
5	6-Aza-5,7,12,14-tetrathiapentacene	28.167	3.51	50	0-00-000000
6	4-Hydroxyphenyllactic acid, ethyl ester, di-TMS	30.902	4.68	50	0-00-000000
7	3-Quinolinecarboxylic acid, 6,7-difluoro-1,4-dihydro-4-oxo-, ethylester	31.991	2.61	49	0-00-000000
8	Gibberellin A3	32.24	3.80	43	5-06-000077
9	3-METHYL-5-DIPHENYLDIHYDRAFURAN	34.082	3.72	86	0-00-000000
10	Tetrasiloxane, decamethyl-	35.81	4.42	50	8-62-000141
11	Cyclotrisiloxane, hexamethyl-	37.258	2.92	58	9-05-000541
12	BENZENE, 1,4-BIS(TRIMETHYLSILYL)-	37.424	2.93	58	0-00-000000
13	1,1,1,3,5,5,5-Heptamethyltrisiloxane	38.929	3.02	64	7-88-001873

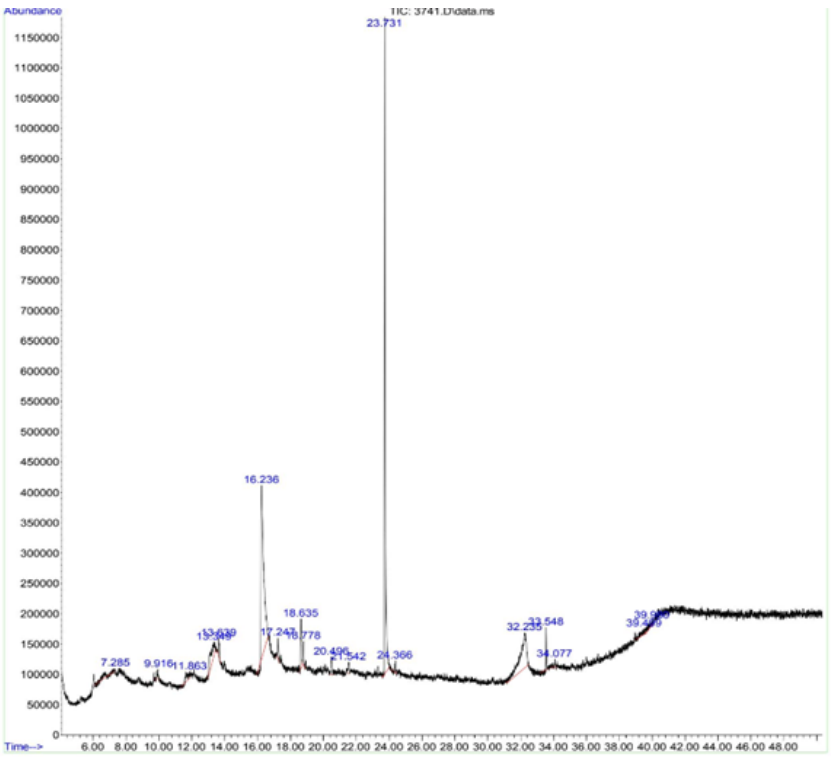

Figure 1. GC–MS chromatogram of fungal filtrate of *Penicillium citrinum* cultured in PDB medium at 28°C.

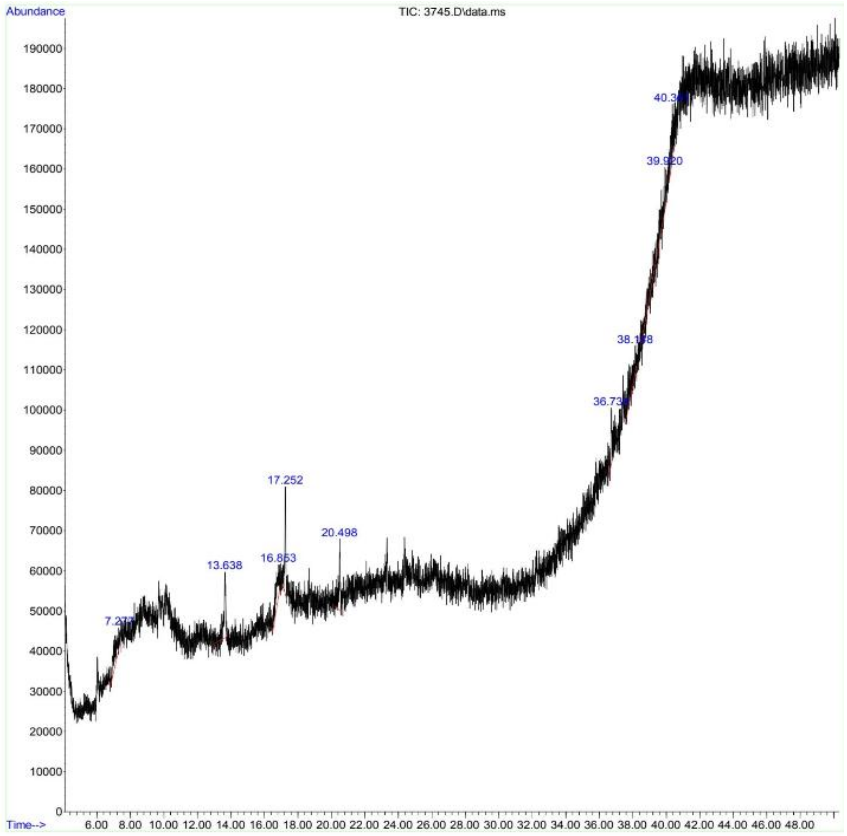

Figure 2. GC–MS chromatogram of fungal filtrate of *Penicillium citrinum* cultured in PDB medium at 25°C.

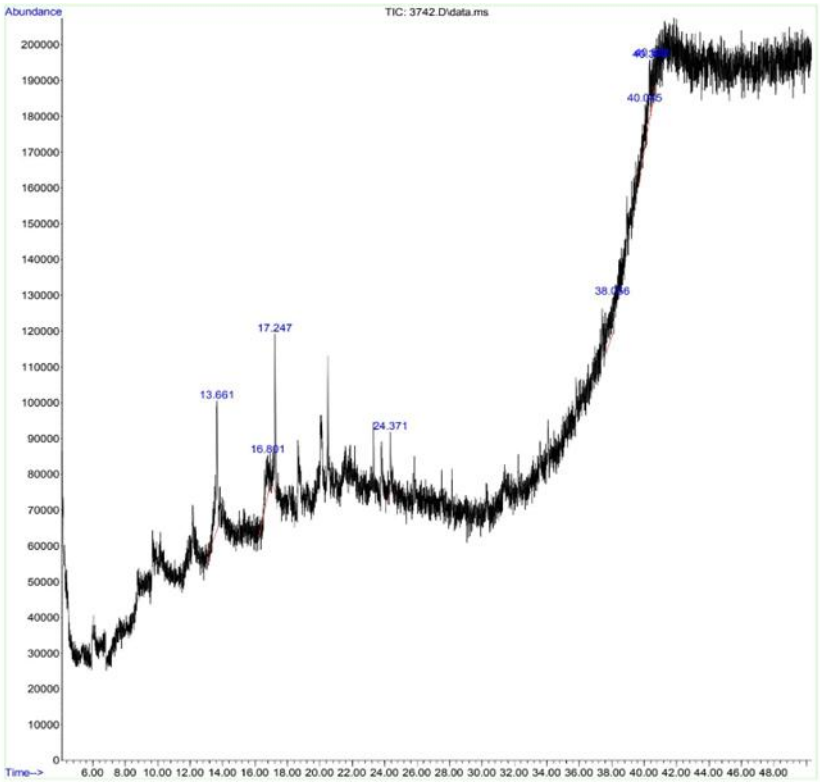

Figure 3. GC–MS chromatogram of fungal filtrate of *Penicillium citrinum* cultured in yeast extract medium at 28°C.

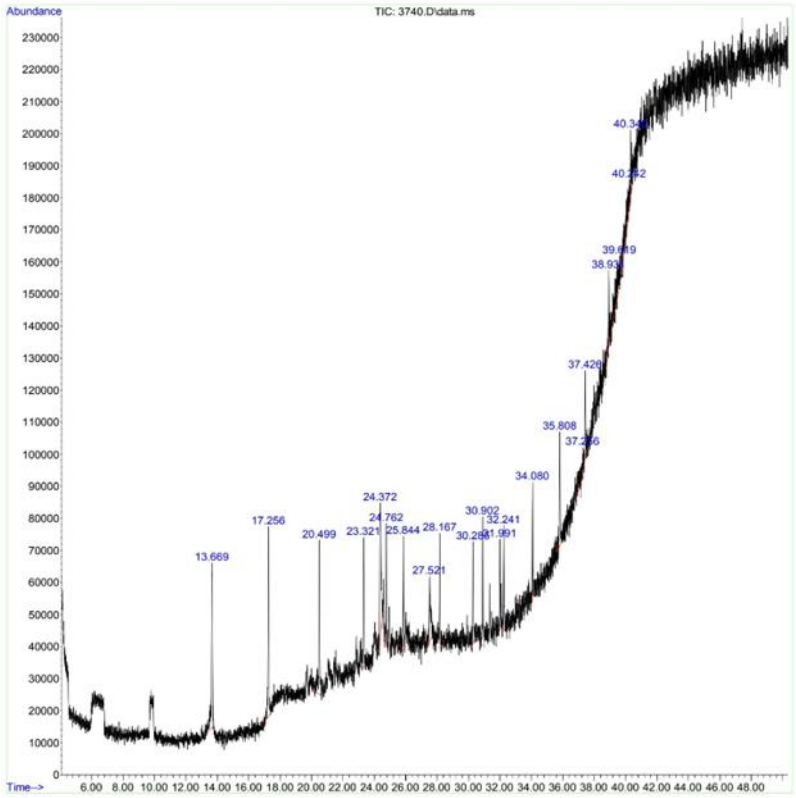

Figure 4. GC–MS chromatogram of fungal filtrate of *Penicillium citrinum* cultured in yeast extract medium at 25°C.

This study shows that the culture medium and temperature used for incubation play a crucial role in the production and diversity of secondary metabolites *of Penicillium citrinum*, as analysed by GC-MS. The differences in GC profiles in response to varying environmental conditions suggest metabolic adaptation strategies by the fungus. Our results are in agreement with other studies that show the significant influence of the environment on fungal secondary metabolism [15], [16]. When cultivated in PDB medium at 28°C, the fungal filtrate contained a diverse set of compounds, with some of them occurring at a high relative abundance. The presence of 1,2-benzenedicarboxylic acid derivatives and dimethyl phthalate indicates the production of ester compounds, which are known to be frequently produced by fungi and may have antimicrobial properties [17]. This finding is supported by Kumar et al., where phthalate derivatives were found to be major components in antifungal extracts. Further, the phenolic compounds observed in the current study, including substituted phenols, have been reported to alter the microbial cell membrane, which may lead to antimicrobial activity, as reported by Raut et al.. These results are consistent with reports that fungal metabolites frequently show potent biological activities, including antibacterial activity [18].

At a lower temperature (25°C) in PDB medium, the metabolite composition changed to be dominated by one compound, cyclohexasiloxane, dodecamethyl-, which implies that lower temperatures may limit the variety of enzymes and lead to the synthesis of specific metabolites. The findings with yeast extract medium at 25°C showed enhanced metabolic diversity with no one compound dominant. This suggests that activating

multiple biosynthetic gene clusters can be promoted in a nutrient-rich medium such as yeast extract. This is reflected in the presence of compounds like gentisic acid, which is an antioxidant and antifungal compound. This is in line with observations made by Rateb and Ebel describing the key role of nutrition in the activation of cryptic fungal secondary metabolic pathways. This finding is consistent with those that highlight the influence of nutrients on metabolite diversity [19].

On the other hand, with a higher temperature (28°C) using yeast extract medium, the metabolite diversity was reduced and a few compounds dominated. This could be due to the fact that higher temperatures create stress for the cell and the production of some pathways may be preferred over others. These temperature-induced metabolic changes were also observed by Bode et al., who noted that the environmental conditions play a major role in the qualitative and quantitative production of fungal secondary metabolites. These findings are also consistent with reports on the role of environmental stress on metabolite production [20].

In general, the results of this study support the "One Strain - Many Compounds" (OSMAC) theory, which suggests that different metabolites can be produced by a single microbial strain grown under different conditions. The diversity observed in this study confirms that the variations in growth conditions (medium and temperature) are a good approach to increase the diversity of metabolites and their bioactivities.

In addition, the detection of phenolic compounds and phthalate derivatives in the fungal filtrate may account for the observed antibacterial activity towards selected bacteria, as these compounds are known to have an inhibitory effect on bacterial growth. This suggests the potential use of *Penicillium citrinum* as a source of active compounds for drug development and as an antimicrobial agent.

CONCLUSION

Fundamental Finding : The study explored the influence of culture conditions, specifically temperature and growth medium, on the production of secondary metabolites by Penicillium citrinum, analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The findings revealed that temperature and medium significantly affect the metabolic diversity and concentration of compounds produced by the fungus. Cultivation in Potato Dextrose Broth (PDB) at 28°C resulted in a broad variety of metabolites, with prominent compounds like 1,2-benzenedicarboxylic acid derivatives and dimethyl phthalate. On the other hand, growth at 25°C reduced metabolic diversity, leading to the dominance of specific compounds, such as cyclohexasiloxane derivatives. Yeast extract medium, especially at 25°C, enhanced the diversity of metabolites, while higher temperatures (28°C) diminished this diversity but increased the production of some dominant metabolites. The results support the "One Strain - Many Compounds" (OSMAC) theory, indicating that environmental factors can trigger different biosynthetic pathways, resulting in varied metabolites with potential biological activities. **Implication :** The results imply that manipulating environmental conditions such as temperature and nutrient availability can be a powerful tool in optimizing the production

of specific bioactive metabolites in fungi like Penicillium citrinum. The study suggests that P. citrinum can be a valuable source of antimicrobial compounds, as evidenced by the detection of phthalate and phenolic compounds known for their antimicrobial properties. These findings are significant for drug discovery, especially in the search for new antimicrobial agents. The ability to control and optimize the production of metabolites through environmental conditions can enhance the development of P. citrinum as a sustainable source of bioactive compounds for pharmaceutical applications. **Limitation :** Despite the valuable insights provided, the study is limited by the small scale of the sample and the use of a cross-sectional design, which limits the generalizability of the findings. The small sample size and lack of longitudinal data mean that the observed effects of temperature and medium on metabolite production may not be representative of broader or long-term trends. Additionally, the study only examined a limited set of metabolites, and future research could consider a broader range of compounds. The metabolic shifts observed under different conditions also suggest that other environmental factors, such as pH or light exposure, could further influence metabolite production, an aspect that was not explored in this study. **Future Research :** Future research should focus on expanding the sample size and utilizing a longitudinal study design to better understand the long-term effects of environmental conditions on metabolite production in Penicillium citrinum. Additionally, exploring other environmental variables, such as pH levels, light exposure, or varying nutrient sources, could provide a more comprehensive understanding of how these factors affect fungal metabolism. Further investigations into the pharmacological activities of the identified metabolites are essential, particularly their efficacy against a broader range of pathogens. Finally, research should aim to optimize culture conditions to maximize the yield of bioactive compounds, with an emphasis on scaling up production for potential industrial or therapeutic applications.

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