

Production of Secondary Metabolites from Two *Penicillium* Strains Adapted to Different Temperature Conditions: A Study on Differential Response of Fungal Strains to Temperature Stress

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Abstract

In the present investigation, temperature dependent production of secondary metabolites of two *Penicillium* strains i.e., cold tolerant *Penicillium oxalicum* originally isolated from a low temperature environment of Leh (Ladakh), India and the other one is mesophilic *Penicillium citrinum* (KR150257) isolated from Lucknow (Uttar Pradesh), India. The psychrotolerant *P. oxalicum* can grow at low temperature (4°C) and shows optimum growth at 15°C, while the mesophilic *P. citrinum* exhibits optimum growth temperature at 35°C. The study of secondary metabolites produced by both *Penicillium* strains, studied by UV-Visible Spectroscopy, GC-MS, confirmed the presence of alkaloids, mycotoxins, antibiotics, hydrocarbons and fatty acids. The maximum production of alkaloids by cold tolerant *Penicillium oxalicum* is detected under temperature stress (35°C). On the other hand, mesophilic *Penicillium citrinum* produced maximum alkaloids with different absorption characteristics at 35°C. The GC-MS analysis of secondary metabolites revealed the presence of number of unique biochemical compounds in both the *P. oxalicum* and *P. citrinum* strains grown under temperature stress conditions (35°C and 4°C, respectively). The common biochemical in the secondary metabolites produced by both the *Penicillium* strains grown under temperature stress condition are 3-dodecene, 2-dodecanol and 1-hexadecanol, eicosane, dibutyl, phthalate, 9-hexacosene, propanoic acid, 2-(aminoxy). The three-unique biochemical produced by *P. oxalicum* grown at low temperature (4°C) are 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent [f] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The five-unique biochemical produced by *P. oxalicum* at high temperature (35°C) are 2-Methyl-2-propylmethylphospho nofluoridate, Pyridine, 2[(1,1dimethylethyl) thio], 4(1H) Pyrimidinone,6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di(2-propylpentyl). The seven unique biochemical produced by *P. citrinum* at low temperature (4°C) are Cyclohexanone, 4-ethyl-4-methyl-3-(1-methylethyl)-,trans-, 3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl, Glycyl-L-proline, Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, 11,14-Eicosadienoic acid, methyl ester. The unique derivative of β -lactam antibiotic produced by the *P. citrinum* at 35°C is 2,4-Azetidinedione,3,3-diethyl-1-methyl.

Keywords: Penicillium; Secondary metabolites; GC-MS; Temperature stress; Alkaloids

Introduction

Psychrotolerant microorganisms are mostly present in the extremely cold environment [1,2] but exhibit slower growth rates, as they automatically encounter number of growth limiting conditions such as reduced efficiency of nutrient uptake, membrane disorders and decrease in the enzyme activity [3]. The psychrotolerants survive at low temperature due to their better nutritional adaptability [4] and have unique cold shock and cold acclimation proteins and enzymes [5]. Fungi often provide plentiful and diverse bioactive metabolites which are medicinally important such as Penicillin, Lovastatin [6], fingolimod [7] and caspofungin [8]. The search for new and bioactive secondary metabolites is still going on, particularly from the extremophilic microorganisms. The psychrotolerant fungi can be a good tool to explore the new bioactive metabolites of pharmaceutical importance due to uniqueness of their habitat and changes in the metabolic systems, amenable for their adaptation to extreme cold environmental conditions.

Penicillium generally, is a genus of ascomycetous fungi, known for its growth and survival as mesophiles or thermophiles, but rarely in a very low temperature conditions. *Penicillium* have great major economic importance in the field of agriculture and pharmaceuticals and many of its species known to produce a highly diversified spectrum of bioactive secondary metabolites, including antibacterial [9,10], antifungal substances [11], immune suppressants, cholesterol-lowering agents [12], and potent mycotoxins [13,14]. Worldwide,

Penicillium is also known to produce secondary metabolites such as ergot alkaloids, diketopiperazines, quinolines, quinazolines, polyketides [15], camazulene and azetidine [16]. *Penicillium* is also known for production of essential fatty acids and hydrocarbons and their therapeutical applications [17] by combating a number of human diseases [18]. Production of these biomolecules from *Penicillium* strains is intensely being examined, particularly from the strains of unexplored habitats. Earlier studies have revealed that the temperature is one of the most important factors which affects the growth and survival of these microorganisms [19]. In the present study, there is successfully isolation of cold tolerant *Penicillium oxalicum* from the cold deserts Leh, Ladakh (J&K), India has been done and efforts have been made to screen its secondary metabolite production under temperature stress and that was compared with mesophilic strain of *Penicillium citrinum* isolated from sub-tropical region i.e., Lucknow (Uttar Pradesh), India. Efforts were

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made to see that whether the isolated strains of *Penicillium* respond to extreme temperature conditions by producing different metabolites. Further the metabolites were identified using spectroscopic techniques including GC-MS.

Materials and Methods

Growth condition

Isolated strains of *Penicillium* were grown aseptically at different temperature ranges from 0°C, 4°C, 15°C, 25°C, 35 to 45°C for 21 days' incubation time at a static condition in basal aqueous medium (50 ml) containing yeast extract, 2.5 g; KH_2PO_4 , 0.05 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; FeSO_4 , 0.01 g; KNO_3 , 1.55 g and 1000 cm^3 of distilled water [20]. The basal aqueous medium was supplemented separately with 1% of glucose as source of carbon.

Extraction of secondary metabolites produced by *Penicillium* strains

The basal aqueous medium of both the *Penicillium* strains was subjected to a liquid-liquid extraction with ethyl acetate (EtOAc) thrice at different time of interval i.e., 3rd, 7th, 15th, and 21st day and the crude extracts of secondary metabolites were quantified by taking absorption spectra (250–400 nm) of the extract by using UV-Visible Spectrophotometer. After the completion of incubation time of 21 days, the whole *Penicillium* biomass was harvested by filtration of liquid medium through filter paper whatman-42. After washing with distilled water, the biomass was weighed before and after drying. Solvent ethyl acetate (99.5% purity) of LC grade were used and purchased from Qualigens (Thermofisher Pvt Ltd, India).

TLC analysis

Thin layer Chromatography (TLC) was used to separate the compounds present in the crude ethyl acetate extract of the secondary metabolites. The extracts of secondary metabolites were applied on thin-layer silica gel plates (Silica gel F254, Merck, Germany). The compounds were separated on TLC plates, run by using solvent systems CAP (chloroform/acetone/2-propanol 85:15:20 v/v/v) and chloroform/methanol/25% NH_4OH (90: 10: 0.1 v/v/v), the plates were sprayed with 6 N sulfuric acid /methanol (1:1 v/v) for the detection of mycotoxins [21], the Dragendorff reagent used for detection of nitrogen containing metabolites and Ehrlich reagent for detection of the indole alkaloids [22]. The R_f values were calculated. The chromatogram was also visualized under both normal and short and long-UV light system.

R_f value = Distance travelled by the solute/Distance travelled by the solvent

GC-MS analysis

Gas chromatography and mass spectroscopy analysis of secondary metabolites was carried out in ethyl acetate crude extract of *Penicillium* strains using GC-MS/MS Triple Quadrupole Mass analyzer ThermoFisher Scientific-Model Name GC1310/TSQ8000-Evo system fitted with liquid auto sampler-Triplus RSH, by Thermofischer Scientific Pvt Ltd. Mumbai (India). The Column selected was -TG-5MS (30 m × 0.25 mm × 0.25 μm) with column conditions- 40°C/5 min, Ramp rate 10°C/min - 260°C/10 min, Injector -260°C, Ion source-200°C, Interface-260°C, Mass range: 35-550. The injection volume was 1.0 μL . The mass spectrum of the unknown component was compared with the spectrum of the known components using the database of National Institute Standard and Technology (NIST) library.

Results

Growth and production of secondary metabolites

Growth of both the *Penicillium* strains was first monitored in terms of fresh weight (grams) of fungal biomass. The *P. oxalicum*, an isolate from temperate regions of Leh, Ladakh, was grown in basal medium at different temperatures (0°C to 45°C). The results showed minimum growth at 4°C and 35°C, but optimum growth at 15°C (data not shown) after 21 days of incubation time. On the other hand, *Penicillium citrinum*, an isolate from sub-tropical regions of Uttar Pradesh (Lucknow, India) exhibited optimum growth at 30°C to 35°C, but exhibited poor growth at 4°C and 15°C. Hence, it is proved that *P. oxalicum* is a psychrotolerant and *P. citrinum* is a mesophilic strain (Figure 1). The broth of fungal culture was withdrawn at different time interval during the incubation and was used for extraction of secondary metabolites by using ethyl acetate (EtOAc). The absorption spectra (250–400 nm) of EtOAc extracts from *P. oxalicum*, grown at two different temperatures (4°C and 35°C), showed distinct absorption peak (275 nm) at 35°C, but the absorption spectra of secondary metabolites (Figures 2A and 2B) obtained from the culture broth incubated at 4°C showed reduction in the overall absorbance along with spectral shift in the absorption peak (260 nm). On the opposite side, extracted secondary metabolites of *P. citrinum* at 35°C showed absorption peak (328 nm), while the metabolite extract obtained at 4°C showed absorption maxima (258 nm) (Figures 2C and 2D). In both the fungal strains, the production of secondary metabolites was higher at 35°C than that 4°C. The results suggested that production of secondary metabolites in the cold tolerant *P. oxalicum* strain was enhanced under high temperature stress, while imposition of low temperature stress on mesophilic *P. citrinum* could not elicit the similar response and exhibited maximum production of metabolites only under optimum growth temperature conditions (Figures 3A and 3D).

TLC analysis

The ethyl acetate extract of secondary metabolites from both the *Penicillium* strains were concentrated by evaporation and drying. The solid residues were re-dissolved in the small amount of methanol and applied on the TLC plates. The chromatogram prepared for each strain was sprayed with 6 N sulfuric acid /methanol (1:1 v/v) [21]. A single spot was observed in case of *P. oxalicum* with R_f value of 0.5 was carefully removed and eluted with EtOAc. The absorption spectrum (250–400 nm) of the separated spot showed absorption peak at 274.8 nm. On the contrary, there was no spot found on TLC plate prepared for *P. oxalicum*

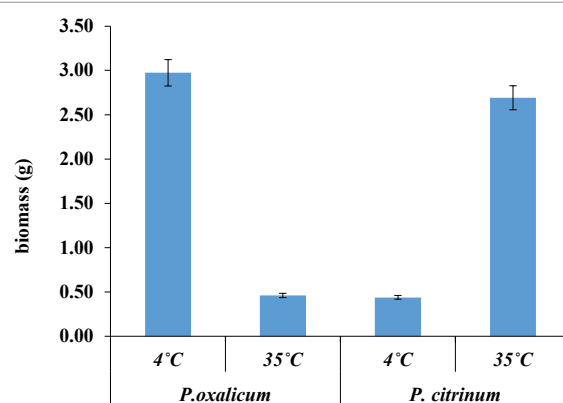


Figure 1: Growth of psychrotolerant *P. oxalicum* and mesophilic *P. citrinum* at low (4°C) and high temperatures (35°C).

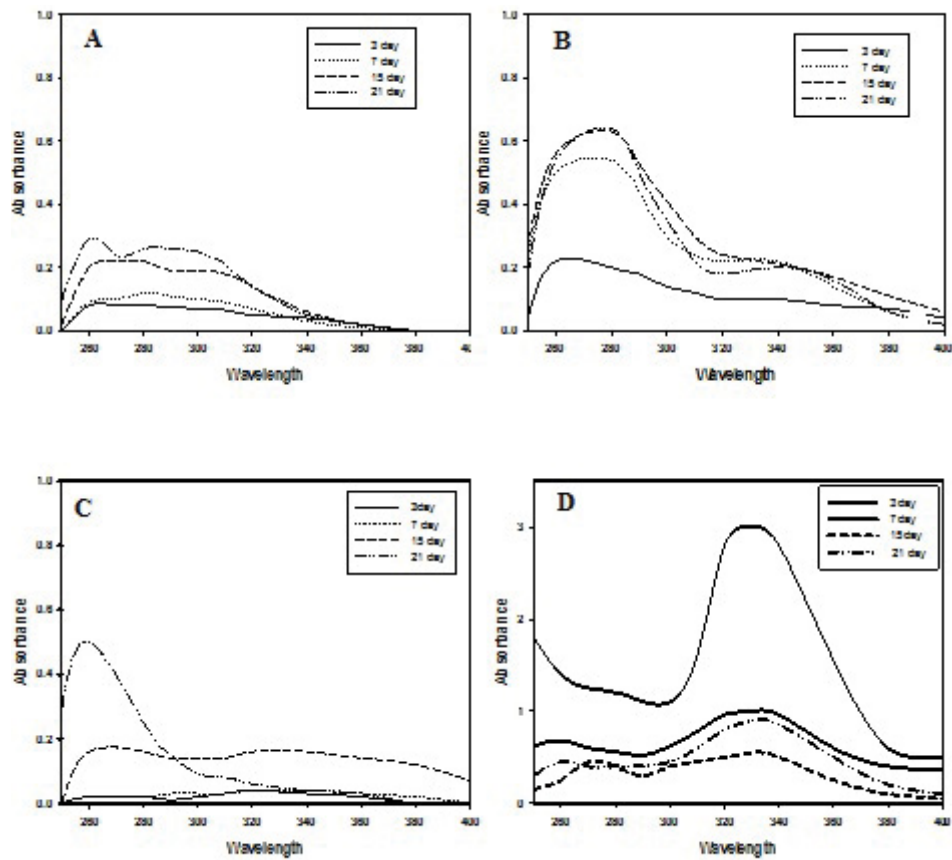


Figure 2: A and B: Absorbance spectra (250–400 nm) of ethyl acetate extract of secondary metabolites produced by *P. oxalicum* grown at temperatures 4°C and 35°C; C and D: Absorbance spectra (250–400 nm) of ethyl acetate extract of secondary metabolites produced by *P. citrinum* grown at temperatures 4°C and 35°C.

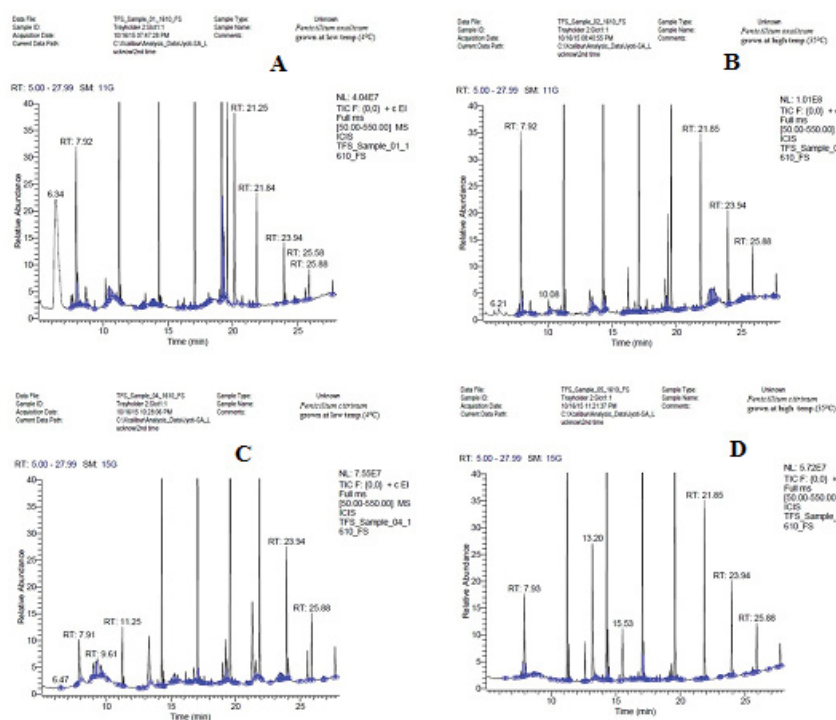


Figure 3: A-D: Chromatogram of GC-MS/MS analysis of ethyl acetate extract of secondary metabolites produced by *P. oxalicum* and *P. citrinum* grown at temperatures 4°C and 35°C respectively.

<i>P. oxalicum</i> (4°C)				
S.no.	Compounds	R _p min	Chemical Formula	Abundance (%)
1	1-Dodecene	7.92	C ₁₂ H ₂₄	6.87
2	2-Dodecanol	11.25	C ₁₄ H ₂₈	9.44
3	2-Propyn-1-ol,acetate	14.02	C ₅ H ₆ O ₂	0.13
4	4(1H) Quinazolinone	16.24	C ₈ H ₆ N ₂ O	0.45
5	2-Hexadecanol	17.06	C ₁₆ H ₃₄ O	8.45
6	1,4,8-Metheno-1H-cyclopent[<i>f</i>] azulene,3a,4,4a,7,7a,8,9,9a-octahydro	19.13	C ₁₄ H ₁₆	19.07
7	Propanoic acid,2-(aminoxy)-	19.2	C ₃ H ₇ NO ₃	4.79
8	Dibutyl phthalate	19.32	C ₁₆ H ₂₂ O ₄	2.02
9	10-Heneicosene	19.56	C ₂₁ H ₄₂	5.95
10	6-Quinazolinol	20.13	C ₈ H ₆ N ₂ O	7.77
11	9-Hexacosene	21.84	C ₂₆ H ₅₂	3.34
<i>P. oxalicum</i> (35°C)				
1	3-Dodecene	7.92	C ₁₂ H ₂₄	8.15
2	6,7-Dodecanedione	10.08	C ₁₂ H ₂₂ O ₂	0.61
3	2-Dodecanol	11.25	C ₁₂ H ₂₆ O	12.47
4	2-Methyl-2-propyl methyl phosphonofluoridate	13.25	C ₅ H ₁₂ FO ₂ P	1.96
5	Pyridine,2[(1,1dimethylethyl)thio	13.81	C ₇ H ₁₃ NS	0.21
6	1-Hexadecanol	14.3	C ₁₆ H ₃₄ O	10.27
7	4(1H)Quinazolinone	16.24	C ₈ H ₆ N ₂ O	1.7
8	2-Hexadecanol	17.06	C ₁₆ H ₃₄ O	12.69
9	Eicosane	17.1	C ₂₀ H ₄₂	0.19
10	4(1H)Pyrimidinone,6-amino-2-methyl-5-nitroso	19.11	C ₅ H ₆ N ₄ O ₂	1.51
11	Propanoic acid,2-(aminoxy)-	19.2	C ₃ H ₇ NO ₃	0.58
12	Dibutyl phthalate	19.32	C ₁₆ H ₂₂ O ₄	2.82
13	10-Heneicosene	19.57	C ₂₁ H ₄₂	9.37
14	9-Hexacosene	21.85	C ₂₆ H ₅₂	5.55
15	4(3H) Quinolinone	25.27	C ₉ H ₇ NO ₂	0.15
16	Phthalic acid, di-(hex-3-yl)ester	25.5	C ₂₀ H ₃₀ O ₄	0.18

Table 1: Tentative identification of the compounds resulting after GC-MS/MS analysis of ethyl acetate extracted secondary metabolites produced by *P. oxalicum* grown at temperatures 4°C and 35°C.

<i>P. citrinum</i> (4°C)				
S.no.	Compounds	R _p min	Chemical Formula	Abundance (%)
1	Cyclobutanone, 2,2-dimethyl	7.91	C ₆ H ₁₀ O	3.3
2	2-Dodecanol	11.25	C ₁₂ H ₂₆ O	1.95
3	Cyclohexanone,4-ethyl-4-methyl-3-(1-methylethyl)-,trans-	13.3	C ₁₂ H ₂₂ O	4.45
4	1-Hexadecanol	14.3	C ₁₆ H ₃₄ O	11.5
5	3-Methyl-1,4diazabicyclo[4.3.0]nonan-2,5-dione , N-acetyl	16.17	C ₁₀ H ₁₄ N ₂ O ₃	0.41
6	Glycyl-L-proline	16.77	C ₇ H ₁₂ N ₂ O ₃	0.74
7	Hexadecane	17.14	C ₁₆ H ₃₄	0.39
8	Pyrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	19.01	C ₁₁ H ₁₈ N ₂ O ₂	1.07
9	2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone	19.23	C ₁₀ H ₁₈ O ₂ S ₂	1.85
10	Dibutyl phthalate	19.32	C ₁₆ H ₂₂ O ₄	0.92
11	9-Hexacosene	19.57	C ₂₆ H ₅₂	14.25
12	11,14-Eicosadienoic acid, methyl ester	21.29	C ₂₁ H ₃₈ O ₂	5.96
13	Propanoic acid, 2-(aminoxy)-	21.55	C ₃ H ₇ NO ₃	1.1
14	Phthalic acid, di(2-propylpentyl)	25.6	C ₂₄ H ₃₈ O ₄	1.37
<i>P. citrinum</i> (35°C)				
1	3-dodecene	7.93	C ₁₂ H ₂₄	4.99
2	2-Dodecanol	11.25	C ₁₂ H ₂₆ O	10.42
3	Methenamine	12.6	C ₆ H ₁₂ N ₄	1.37
4	Phenol,2,4-bis(1,1-dimethylethyl)-	13.2	C ₁₄ H ₂₂ O	6.36
5	2-Hexadecanol	14.3	C ₁₆ H ₃₄ O	17.32
6	Hexadecane	14.4	C ₁₆ H ₃₄	1.64
7	2,3-Dimethylhydroquinone	15.53	C ₈ H ₁₀ O ₂	2.24
8	2,4-Azetidinedione,3,3-diethyl1-methyl	16.14	C ₈ H ₁₃ NO ₂	0.29
9	Eicosane	17.1	C ₂₀ H ₄₂	1.19
10	Phthalic acid, hex-3-yl isobutyl ester	18.15	C ₁₈ H ₂₆ O ₄	0.2
11	Propanoic acid,2-(aminoxy)-	19.19	C ₃ H ₇ NO ₃	0.36
12	Dibutyl phthalate	19.32	C ₁₆ H ₂₂ O ₄	0.68
13	9-Hexacosene	21.8	C ₂₆ H ₅₂	6.82

Table 2: Tentative identification of the compounds resulting after GC-MS/MS analysis of ethyl acetate extracted secondary metabolites produced by *P. citrinum* grown at temperatures 4°C and 35°C.

by spraying with Ehrlich reagent. However, black/brown spot observed on TLC plate in case of metabolite extract of *P. citrinum* under short wavelength UV-light. The same plate stained Ehrlich reagent showed no spot. But spraying the same TLC plate with Dragendorff reagent showed an orange spot with Rf value around 0.3. The same spot eluted with EtOAc showed absorbance maxima at 255.2 and 326.4 nm. The results suggested that secondary metabolites of *P. oxalicum* include some mycotoxins, whereas extracts of *P. citrinum* exhibited presence of alkaloids based on absorbance and colour of the chromatogram.

GC-MS analysis of the secondary metabolites of *Penicillium* strains

Gas chromatography and mass spectroscopy analysis of compounds was carried out in ethyl acetate crude extract of *Penicillium* strains as shown in Tables 1 and 2. Chromatogram GC-MS analysis of the ethyl acetate extract of *Penicillium oxalicum* grown at two different temperatures (4°C and 35°C) showed the presence of 11 and 16 major peaks at respective temperature. On the other hand, EtOAc extract of *Penicillium citrinum* grown at 4°C and 35°C exhibited 14 and 13 major peaks at respective temperature. The common metabolites produced by both the *Penicillium* strains grown under different temperature regimes (4°C and 35°C) included 3-dodecene, 2-dodecanol, 1-hexadecanol at 4°C and Eicosane, Dibutyl phthalate, 9-Hexacosene, Propanoic acid, 2-(aminooxy) at high temperature (35°C). The three unique metabolites produced by *P. oxalicum* only when grown at low temperature (4°C) included 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent[*f*] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The five-inimitable biochemical present in high temperature (35°C) stress grown *P. oxalicum* were 2-Methyl-2-propyl methyl phosphonofluoridate, Pyridine, 2[(1,1dimethylethyl) thio], 4(1H) Quinazolinone, 4(1H) Pyrimidinone, 6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di(2-propylpentyl). Similarly seven unique biochemical produced by *P. citrinum* grown at low temperature stress (4°C) were Cyclohexanone, 4-ethyl-4-methyl-3-(1-methylethyl)-, trans-, 3-Methyl-1,4-diazabicyclo [4.3.0] nonan-2,5-dione, N-acetyl, Glycyl-L-proline, Pyrrolo [1,2-*a*] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, 11,14-Eicosadienoic acid or its methyl esters. Further results on high temperature (35°C) grown *P. citrinum* showed the presence of unique derivative of beta-lactam antibiotic produced i.e., 2,4-Azetidinedione, 3,3-diethyl-1-methyl. The other metabolites produced by this strain at 35°C include Methylamine, Phenol, 2,4-bis(1,1-dimethylethyl)-, 2,3-Dimethylhydroquinone.

Discussion

In the present investigation, the psychrotolerant *P. oxalicum* has potential to grow at extremely low temperature (4°C) with growth optima at 15°C. The mesophilic *P. citrinum* showed zero tolerance to low temperature and showed optimum growth between 30°C to 35°C. Two extreme temperature conditions (4°C and 35°C) were selected to study effect of temperature on the production of secondary metabolites from the isolated *Penicillium* strains. The results on the production of secondary metabolites in both *Penicillium* strains showed temperature dependent increase in the quantity of secondary metabolites with rising temperature. The results revealed both quantitative as well as qualitative changes in production of secondary metabolites with changing temperature regimes for growth in both the strains of *Penicillium*. The results revealed the presence of three unique metabolites produced by *P. oxalicum* only when grown at extremely low temperature (4°C), which included 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent

[*f*] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The other five inimitable biochemicals produced by *P. oxalicum* at high temperature (35°C) were 2-Methyl-2-propyl methyl phosphonofluoridate, Pyridine, 2[(1,1dimethylethyl) thio], 4(1H) Quinazolinone, 4(1H) Pyrimidinone, 6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di(2-propylpentyl). These unique compounds reported above were produced by the psychrotolerant *P. oxalicum* only, they were found to be absent in case of mesophilic strain *P. citrinum*. However, few common metabolites produced by *Penicillium oxalicum* respond to changing growth temperatures in terms of percent increase or decrease in the production of compounds such as percent abundance of Dodecanol at 4°C (9.44%) declined to the level of 1.95% at 35°C. Similarly, percent abundance of 4(1H) Quinazolinone at 4°C (0.44%) inclined to 1.7% at 35°C and abundance of 10-Heneicosene at 4°C (5.95%) inclined to 9.37% at 35°C. Another view of secondary metabolites production proposes that the genes involved in secondary metabolism provide a “genetic playing field” that allows mutation and natural selection to fix new positive characters via evolution and secondary metabolism to make them an integral part of cellular metabolism [23,24]. For e.g. the secondary metabolite quinoline produced by *P. oxalicum* has been reported to have an antiprotozoal activity [25], antimalarial, anti-bacterial, antifungal, antihelmintic, cardiotoxic, anticonvulsant, anti-inflammatory, anticancer and analgesic activity [26,27] and it is also known as an alkaloid mycotoxin [28]. The other bioactive compound Quinazolinone produced by this fungus constitutes a class of drugs that works as sedatives and contains a 4-quinazolinone core. The 2,4-Azetidinedione, 3,3-diethyl-1-methyl produced by *P. citrinum* is derivative of 2-azetidinone (β -lactam) ring system is precursor for number of broad spectrum β -lactam antibiotics [23,29-34]. Other common bioactive compound Dodecanol produced by both the *Penicillium* strains is a saturated 12-carbon fatty alcohol, has a floral odor and is used in detergents, lubricating oils, and pharmaceuticals. Several unique metabolites produced by *P. oxalicum* under extreme temperature conditions are the biochemicals which need to be explored for structural details and as well as for application part. Hence, the foregoing results revealed that the unique characteristics of the psychrotolerant *P. oxalicum* and its temperature stress dependent production of number of new secondary metabolites with potential industrial application, particularly in pharmaceutical and therapeutical fields.

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