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, 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-propylmethylphosphono nfluoridate, Pyridine, 2(1,1dimethyl)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-methylthetyl)-trans-, 3-Methyl-1,4diazabicyclo[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-Dimethylpropyl, 2,2-dimethyl-propanesulfonfluor 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 revealed the presence of number of unique biochemical compounds in both the *P. oxalicum* and *P. citrinum* strains isolated from a low temperature environment of Leh (Ladakh), India and the other one is mesophilic *Penicillium citrinum* originally isolated from 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; KH₂PO₄, 0.05 g; MgSO₄·7H₂O, 0.05 g; FeSO₄·0.01 g; KNO₃, 1.55 g and 1000 cm³ 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% NH₄OH (90: 10: 0.1 v/v/v), the plates were sprayed with 6 N sulfuric acid/methanol (1:1 v/v) [21]. A single spot was observed in case of *P. oxalicum* with Rf value of 0.5 was carefully removed and eluted with EtOAc. The absorption spectrum (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 from 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 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 Rf 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*.

**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 from both the *Penicillium* strains (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).
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.
### 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.

<table>
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<th>S.no.</th>
<th>Compounds</th>
<th>R&lt;sub&gt;t&lt;/sub&gt;, min</th>
<th>Chemical Formula</th>
<th>Abundance (%)</th>
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<tr>
<td>1</td>
<td>1-Dodecene</td>
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<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;</td>
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### 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.

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<tr>
<th>S.no.</th>
<th>Compounds</th>
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<th>Chemical Formula</th>
<th>Abundance (%)</th>
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<td>Cyclobutanone, 2,2-dimethyl</td>
<td>7.91</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O</td>
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<td>2-Dodecanol</td>
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<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;</td>
<td>1.95</td>
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<td>3</td>
<td>Cyclohexanone, 4-ethyl-4-methyl-3-(1-methylthethyl)-trans-</td>
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<td>4.45</td>
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<td>11.5</td>
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<td>8</td>
<td>Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-</td>
<td>19.01</td>
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<td>Dibutyl phthalate</td>
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### Table 3: Tentative identification of 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 Dragendorf 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 on ethyl acetate crude extracts 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- dodecane, 2-dodecanol, 1-hexadecanol at 4°C and Eicosane, Dibutyl phthalate, 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-cyclopet[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(1H-dimethyl-ethyl) 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-1,4diazabicyclo [4.3.0] nonan-2,5-dione, N-acetyl, Glycyl-L-proline, Pyrrole [1,2-a] pyrazine-1,4-dione, hexahydro-3(2-methylpropyl)-, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfate, 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-cyclopet

**References**


