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Morphophysiological variations in two *Penicillium* strains isolated from different climatic zones

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ABSTRACT

The present investigation is a comparative study of the morphological and physiological characteristics of two *Penicillium* strains isolated from different climatic regions. A psychrophilic strain *Penicillium oxalicum* isolated from Leh (Ladakh) - a cold desert in J & K (India) was able to grow upto 4°C and other one was a mesophilic *Penicillium citrinum*, isolated from Lucknow (U.P.), India, was able to grow upto 35°C. The Fungal Taxonomical classification of both the strains was primarily based on the morphology of hyphae, spores, and spore-bearing (conidial) structures of isolates. The **ITS region of 18s rDNA** was successfully amplified using universal primers ITS4 & ITS5 for molecular identification fungal isolates. The Psychrophilic strain was identified as *Penicillium oxalicum* (accession no. KR150256) and mesophilic strain as *Penicillium citrinum* (accession no. KR150257). Physiological studies pertaining to preference of growth temperature and nutritional (C, N) conditions on the growth of both the *Penicillium* strains was studied to understand their physiology response. The study revealed interesting results regarding the growth and reproductive behaviour of both *Penicillium* strains adapted to different climatic zones. The temperature range of 4-25°C was found to be optimum range for growth of Psychrophilic *Penicillium oxalicum.* However, maximum growth of the psychrophilic strain was achieved at 15°C at acidic pH 4.0. The mycelial growth of mesophilic *P. citrinum* occurred between 15-35°C at acidic pH 5.0; but its optimum growth was obtained between 25-30°C. The best carbon source for the growth of *P. oxalicum* was glucose, followed by sucrose. On the other hand, the best carbon source for the growth of *P. citrinum* was found to be sucrose, followed by glucose. The best nitrogen source for growth of *P. oxalicum* was found to be sodium nitrate, followed by organic nitrogen glycine, and L-tryptophan. On the contrary, *P. citrinum* could grow well in the presence of both glycine and L-tryptophan. Thus, an opposite morphophysiological characteristics of both the fungal strains could be associated with their adaptation to their respective climatic conditions and might be helpful in their taxonomic classification.

1) INTRODUCTION

Microorganisms that live in the extreme environment usually modify their physiology to ensure their survival and growth under harsh conditions. Psychrophilic fungi are known to survive at extremely low temperature and are commonly found in polar and non-polar habitats [1]. Therefore, optimal temperature for the growth of psychrophiles is ≤ 15°C, maximum temperature is ≤ 20°C [2]. The Leh, Ladakh in J & K has all the characteristics of a cold desert and is accredited with very low temperature, high Ultra-Violet-B radiation, low water & nutrient availability, and repeated freeze and thaw cycles. Most of the psychrophilic fungi are acclimated to such harsh conditions by adapting various morphological and physiological strategies. Sometimes low temperature condition influence fungal cells by increasing their water viscosity, denaturation of proteins, slowing down of their enzymatic reactions, and membrane stability [3, 4]. Psychrophilic metabolic changes in psychrotrophic fungi make them more valuable in biotechnological and pharmaceutical fields due to diverse metabolic and morphological characteristics evolved during adaptation to such extreme environments. Such extremophiles are exploited for production of cold-active enzymes, bioactive metabolites and exo-polysaccharides, potential application for biofertilizer and bioremediation [1].

Microfungi of the genus *Penicillium* are one of the most promising sources of physiologically active compounds such as alkaloids, antibiotics, hormones, mycotoxins etc. *Penicillium* (a mold) is a very large and omnipresent genus which currently contains approx. 354 accepted species [5]. *Penicillium* spp. are typically fast growing, mostly in green coloured shades, often white, tailing of a dense felt of conidiophores and are recognized by their dense brush-like spore bearing structures. Spore bearing systems in most *Penicillium* spp. are biverticillate, sparsely monoverticillate or terverticillate [6]. Some fungi are tolerant to the external extreme environmental factors such as dryness and low or high temperature [7]. *Penicillium* spp. is one of the fungi that has the ability to survive in the extreme environmental conditions [8, 9]. According to Brandt and Warnock [10], the taxonomical classification of fungi is mainly centred on morphology of hyphae, spores, and spore-bearing (conidial)

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structures of isolate. The sexual spores and their mode of reproduction have historically formed the main basis for the classification of fungi into the Zygomycota, Ascomycota, and Basidiomycota. In recent years, introduction of rapid DNA sequencing has revolutionized fungal taxonomy based on phylogenetic approach to species recognition (PSR concept). More or less in this fungi, the asexual stage has demonstrated successful means of fast dispersal to new habitats where sexual stage has become extinct [11]. In spite of such a large number of commercial attributes of the *Penicillium* fungus; there is need to explore new *Penicillium* strains, particularly from extreme habitat and to understand more about commercial application of fungi in combination with morphological and physiological variations.

2) MATERIALS AND METHODS

2.1. Reagents and solvents used

For culture preparation, Potato Dextrose broth/agar and the basal medium containing Yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, KNO_3, Glucose was purchased from Qualigens and Himedia (India). Morphological study of both *Penicillium* strains was carried out by using a Light microscope and Scanning Electron Microscopy (SEM JSM-6490LV, JEOL Japan).

2.2. Isolation and morphological identification of both *Penicillium* strains

Isolation of psychrophilic *Penicillium oxalicum* [soil samples of Leh Ladakh (J& K, India)] and mesophilic *Penicillium citrinum* (soil sample of B.B.A.U. Campus, Lko, India) was performed on Potato dextrose agar (PDA) containing petri plates by following serial dilution method. After isolation of axenic culture of both *Penicillium* strains on PDA plates using standard microbiological techniques, morphological study of 3-7 day old culture was carried out. Macro-morphological changes was observed by measuring radial growth on PDA containing petriplates, changes in sporulation, colour and texture of mycelium and pigment production on both sides of the petriplates. Microscopic examination such as size of the mycelium, spore shape and size, arrangements of conidia and conidiophores, division of hyphae was done by using a lightmicroscope, later confirmed by using Scanning Electron Microscopy. The PDA plates were inoculated with a 4-mm mycelial disc from fresh fungal cultures, the cultures were incubated at their respective optimum temperature, and after 7 days, morphological examination of *Penicillium* strains was done by using SEM. For SEM study, fixation of specimen was done in 2 % (v/v) glutaraldehyde in 0.1 mM$NaPO_4$ buffer and washed in buffered 1 % OsO_4 for 2 h. Dehydration of specimen was done using an ethanol series (10, 25, 40, 60, 75, 85, 95 and 100 %) for 15 min/conc. The specimen was dried in a critical point drying apparatus, sputter-coated with gold and observed with a field emission SEM. For successfully completion of all experiments, the fungus was sub-cultured on the PDA medium and the routine cultures were kept at ±4°C in the refrigerator.

2.3. Molecular identification of isolated fungal strains-

The **ITS region of 18s rDNA** was successfully amplified using universal primers ITS4 & ITS5 by National Fungal Culture Collection of India (NFCCI-ARI) Pune, India for Molecular identification of both the fungal cultures commenced with the isolation of genomic DNA from pure culture. The **ITS region of rDNA** was successfully amplified using universal primers ITS4 & ITS5. The sequencing PCR was set up with ABI-Big Dye® Terminatorv3.1 Cycle Sequencing Kit. The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences & analysed for identification.

2.4. Physiological studies

2.4.1. Temperature-dependent growth of both *Penicillium* strains

Isolated fungal strains were grown aseptically under varying temperatures regimes ranging from 0-45°C for 28 days, in basal medium (50ml) containing yeast extract, 2.5g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.01 g;KNO_3, 1.55 g and 1000 cm^3 of distilled water [12]. The broth medium was supplemented separately with 1% glucose as carbon source.

2.4.2. pH dependent growth of both *Penicillium* strains

Isolated *Penicillium* strains were aseptically inoculated in autoclaved potato dextrose broth (50 ml) with varying pH (pH4-10) and were incubated for 21 days at their respective moderate temperatures, pH was adjusted by adding NaOH or HCl to growth media. Growth was measured in terms of fresh weight (g) after incubation time period of 21 days. Harvesting of fungal biomass was done through filtration using whatman filter paper and fresh weight was taken.

2.4.3. Effect of carbon and nitrogen on the growth of *Penicillium* strains

2.4.3.1. Carbon source

Effect of the different concentrations of carbon sources (glucose, cellulose, and sucrose) on mycelial growth of both *Penicillium* Isolated fungal strains were inoculated in basal medium containing agar plates with varying concentrations carbon and nitrogen sources and incubated for 15 days at their respective optimum temperature and pH for 21 days of incubation. The glucose of the basal medium was replaced by each of the carbon compounds so as to offer 1% of carbon - a substituent of glucose (10g/l) in the basal medium.

2.4.3.2. Inorganic and organic nitrogen source

Effect of different concentrations of inorganic and organic nitrogen sources (sodium nitrate, ammonium sulphate, yeast extract, glycine, and L-tryptophan) on the mycelial growth of *Penicillium* strains was evaluated, replacing inorganic nitrogen compound in glucose supplemented basal medium. A control set without addition of nitrogen source was run in parallel. 100 ml Erlenmeyer flasks containing 50 ml of growth medium, were inoculated with a 10 mm surface agar plug from 7 day old culture grown on PDA petriplates for all the experiments. Three replicates were continued for each nitrogen source for 21 days. Cultures were filtered through Whatman filter paper No. 1 and moisture content was dry using tissue paper before taking fresh weight of biomass.

3) RESULTS

3.1. Macroscopic and Microscopic Characters

Morphology has been used to define the fungal diversity as primary criterion for identifying any fungus. Macroscopic features of psychrophilic *Penicillium* strain on petridish containing PDA media showed moderate growth, a green

color colony with white periphery, velvet appearance. The back side of the colony was found to be in creamish yellow in color (Fig.1A).

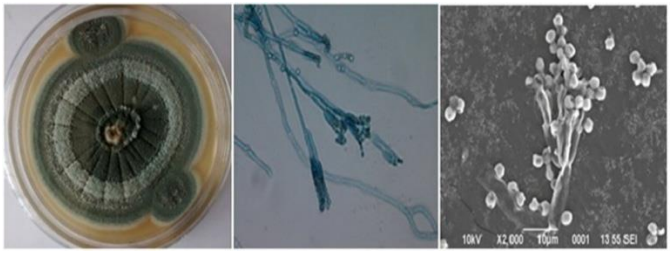

Fig. 1 A. Morphological studies of *Penicillium oxalicum* using Light microscope and SEM.

While on Czapek Dox media, this species exhibited slow growth, white matte like appearance with back side white in colour. The pigment produced by Psychrophilic *Penicillium* strain on PDA plates was yellow in color. The mesophilic strain on PDA plates showed rapid growth, dark green colour granular appearance and the back side of the colony was reddish in colour. On the other hand, this strain on the Czapek Dox media showed moderate growth, green colour and smooth appearance (Fig.1B).

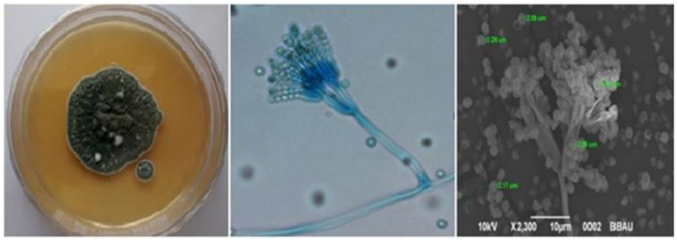

Fig. 1 B. Morphological studies of *Penicillium citrinum* using Light microscope and SEM.

The pigment produced by the Mesophilic strain of *Penicillium* on the PDA plates was orange in color. Similar macroscopic results have been reported by [13] for both the *Penicillium* strains. Microscopic study using light microscope and Scanning Electron Microscope showed that the hyphae of *P. oxalicum* are septate, spore size approx. 2.70 µm and conidiophores are terverticillate. On the other hand, hyphae of *P. citrinum* were smooth, spore size approx. 2.35µm and conidiophores are terverticillate. Morphological characters such as elliptical conidia and smooth-walled conidiophores were used for the identification of the fungal strains. The *P. citrinum* is reported to have globose conidia and conspicuously rough-walled conidiophores [14].

3.2. Molecular identification of isolated fungal strains-

Phylogenetic tree in relation with their identification is shown in Fig. 2 & 3. ITS and β-tubulin loci are recommended for identification of *Penicillium* species [5]. On the basis of sequencing of the ITS regions and BLASTn comparisons with GenBank, the size of the amplified ITS region of psychrophilic *P. oxalicum* (KR150256) with universal ITS4 & ITS5 is similar to that described for other *Penicillium* species with 99% similarity *P. oxalicum* and nearest similar strain was KX090323.1 *Penicillium oxalicum* strain S1111 and HG798733.1 *Penicillium oxalicum*, genomic DNA containing ITS1 5.8S rRNA gene and ITS2 strain TUEF25. Mesophilic *P. citrinum* (KR150257) was found similar with KP329672.1 *Penicillium citrinum* strain DTO: 133-B6. The phylogenetic relationship of both the *Penicillium* sp. among other different *Penicillium* species mentioned in NCBI database in the form of phylogenetic tree is shown in Fig. (2 & 3).

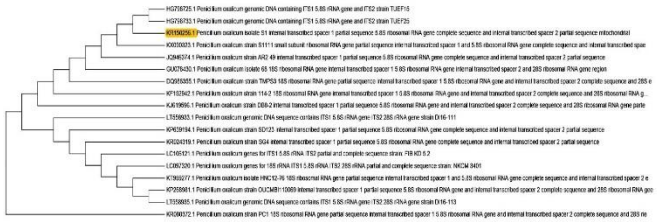

Fig. 2. Phylogenetic analysis of psychrophilic *Penicillium oxalicum* and reference (R) sequences of its nearest relatives based on the ITS rRNA gene.

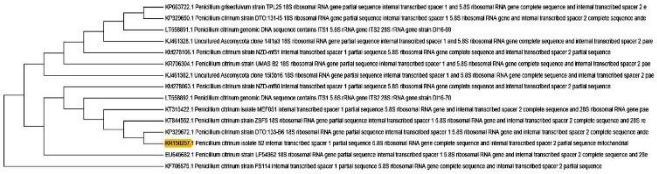

Fig. 3. Phylogenetic analysis of psychrophilic *Penicillium citrinum* and reference (R) sequences of its nearest relatives based on the ITS rRNA gene.

Gonc¸alves et al.[15] equated the ITS sequence of the Antarctic marine *Penicillium* with sequences from the deposited in the Gen Bank database, and found that the Antarctic *Penicillium* was 100% identical to the species *P. solitum* (AF000934), and it was 98.85, 98 and 97.4 % similar to *P. discolor* (AY674349), *P. chinulatum* (AF003536) and *P. cavernicola* (AY674337), respectively.

3.3. Results of Physiological studies

3.3.1. Temperature-dependent growth of *Penicillium* strains

Biomass of psychrophilic *Penicillium oxalicum* harvested from the basal medium was incubated at different temperatures (0, 4, 15, 25, 35 and 45˚C) for 28 days. Results revealed that 15˚C is the optimum growth temperature for *P. oxalicum*. However, it could grow well at low temperature (at 4˚C) and showed sluggish growth at 25˚C, 35˚C, but it failed to

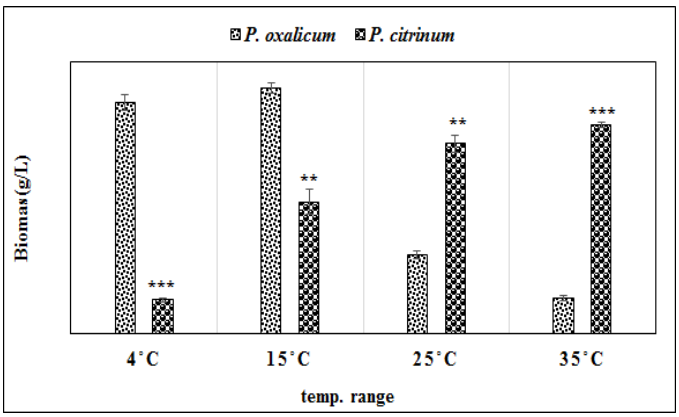

Fig. 4. Temperature dependent change in Biomass (g/L) of both *Penicillium* strains grown in basal medium. Student's paired sample't-test' showing significant difference between both *Penicillium* strains, *p<.0.05, **p<0.01, ***p<0.001. Error bar showing the mean±SD.

grow at 0˚C and 45˚C temperature. On the other side, the optimum temperature for *P. citrinum* was 35˚C, but it grew well at 25˚C. However, it failed to grow at 0˚C and 45˚C temperature. Hence, these results suggested that isolate from cold desert of Ladakh was psychrophilic (15˚C) fungal strain and *P. citrinum* required moderate to high temperature (25-30˚C) as mesophilic strain (Fig 4).

3.3.2. pH dependent growth of *Penicillium* strains

The filtered mycelia were dried and weight was taken for both the *Penicillium* strains grown at different range of pH 4.0 to 10. The mycelial growth of *P. oxalicum* was significantly higher at pH 4.0 when measured in terms of mycelial dried weight, while the *P. citrinum* showed the highest mycelia dried weight at pH 5.0 (Fig.5).

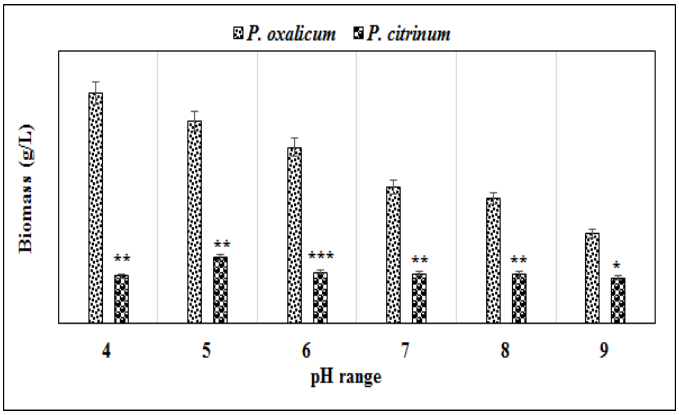

Fig. 5. pH dependent change in biomass (g/L) of both *Penicillium* fungal strains grown in basal medium (21 day of incubation time). Student's paired sample't-test' showing significant difference between both *Penicillium* strains, *p<.0.05, **p<0.01, ***p<0.001. Error bar showing the mean±SD.

These results indicated that both the *Penicillium* strains can grow well at acidic pH, where alkaline pH condition restricted their growth.

3.3.3. Effect of Carbon source on growth of *Penicillium* strains

The best carbon source for the growth of psychrophilic *P.oxalicum* was found to be 2 %glucose whereas the fungal growth declined with increase in concentration of sucrose and cellulose (Fig. 6. A, B).

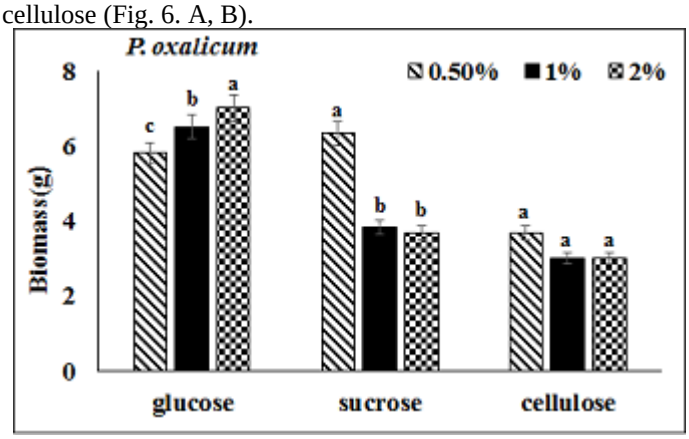

Fig. 6 A. Nutrition dependent (carbon) growth of *Penicillium oxalicum* grown in basal medium (15 day of incubation time). Nutritional dependent growth has been analysed by one way analysis of variance (ANOVA), Different letters within the same group indicate statistically significant difference ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$), Error bar showing the mean±SD.

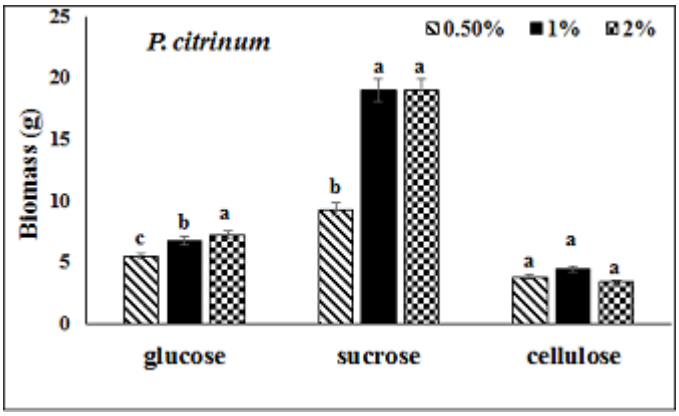

Fig. 6 B. Nutrition dependent (carbon) growth of *Penicillium citrinum* grown in basal medium (15 day of incubation time). Nutritional dependent growth has been analyzed by one way analysis of variance (ANOVA), Different letters within the same group indicate statistically significant difference ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$), Error bar showing the mean±SD.

The production of biomass (average mycelial dry weight) in relation to the preference for carbon source by *P. oxalicum*, was glucose> sucrose> cellulose, whereas on the other hand, mesophilic *P. citrinum* showed different order of preference for carbon source sucrose> glucose > cellulose.

3.3.4. Effect of Inorganic nitrogen source on growth of *Penicillium* strains

The best inorganic nitrogen source for the growth of psychrophilic *P. oxalicum* was found to be sodium nitrate. The least mycelial growth of the fungus was observed on ammonium sulphate as nitrogen source (Fig. 7. A & B).

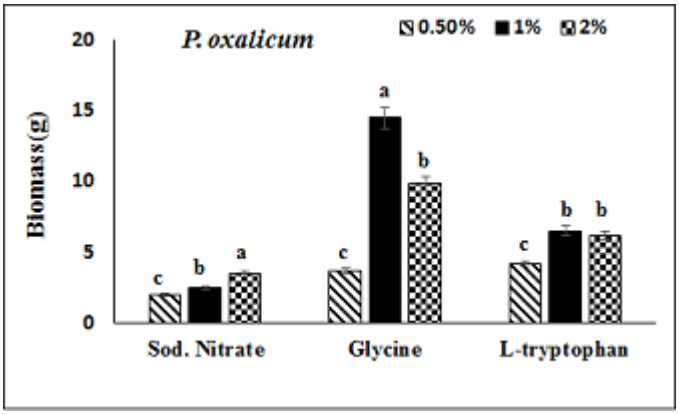

Fig. 7 A. Nutrition dependent (nitrogen) growth of *Penicillium oxalicum* grown in basal medium (15 day of incubation time).Nutritional dependent growth has been analysed by one way analysis of variance (ANOVA), Different letters within the same group indicate statistically significant difference ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$), Error bar showing the mean±SD.

The optimum level of biomass production (average mycelial dry weight) in *P. citrinum* relation to the inorganic nitrogen

consumed was in descending order as sodium nitrate> ammonium sulphate. The nutritional dependent optimum growth of *P. oxalicum* was recorded in the presence of 1% glycine when growth was measured in terms of mycelial fresh weight (g), whereas the *P. citrinum* showed optimum growth in the presence of 2 % glycine, followed by L-tryptophan.

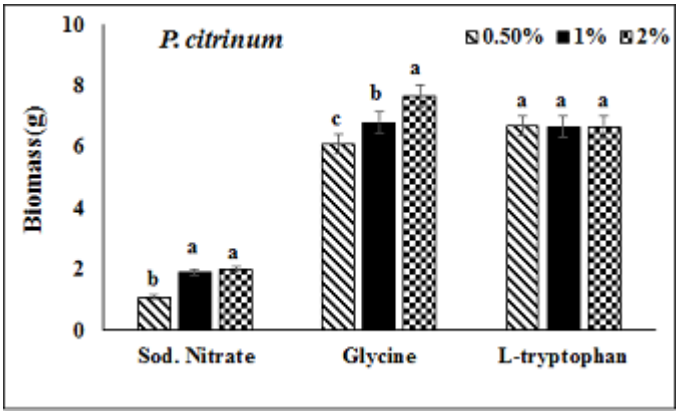

Fig. 7 B. Nutrition dependent (nitrogen) growth of *Penicillium citrinum* grown in basal medium (15 day of incubation time).Nutritional dependent has been analysed by one way analysis of variance (ANOVA), Different letters within the same group indicate statistically significant difference ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$), Error bar showing the mean±SD.

4) DISCUSSION

Morphological study of fungi has been used as a classical approach for identification of fungal strains. Isolation of psychrophilic fungi with optimum growth temperature between 20-30˚C have been reported from the cold desert of Ladakh (J&K), India, and Antarctic region [16]. The present isolate is identified as cold tolerant, *Penicillium oxalicum* (KR150256), which is found to be a true psychrophilic fungus capable of growing at 4°C. The isolated psychrophilic *Penicillium* strain exhibits optimum growth at 15°C, but fails to grow above 25°C. On the other hand, mesophilic *P. citrinum* was capable of within a broad range of temperature conditions (25°C to 35°C), but exhibited zero tolerance to low temperature condition (5-10°C). The present results also revealed interesting facts regarding growth and reproductive behaviour of the psychrophilic *P. oxalicum* and mesophilic *P. citrinum*. The growth of both the *Penicillium* strains was found to be better on PDA media, but moderate growth was observed on Czapek Dox media. The spore size, colour and texture of both fungal strains on different media plates were dissimilar. Similar results were also recorded by Tiwari et al. [13] for *Penicillium* strains. In a previous study, it has been reported that the fungal strain *P. oxalicum* can easily adapt to a wide range of pH conditions and also from cold dry to warm environmental conditions [17]. The results in the present study showed that mesophilic *P. citrinum* grew well at pH 5.0 only, whereas *P. oxalicum* could grow well at pH 4.0. However, alkaline pH (7-10) condition retarded the growth of both the fungal isolates. According to Chattopadhyay [18], the mechanism cold tolerance in fungal strains are not fully understood. They opined that the cellular response of Antarctic *Penicillium* strains to the low temperature regime was similar to that of temperate fungi and major effect of low temperature condition on fungus was production of oxidized proteins. It is also observed that once the fungal cells are exposed to low temperature condition, rate of enzymatic reactions is slowed down, leading to decrease in the demand for ATP and accumulation of reductants mainly derived from respiratory chain. Such metabolic condition stimulates an unexpected increase in the production of reactive oxygen species (ROS), which cause extensive damage to the cellular constituents like lipids, proteins, and DNA molecules. In a previous study, it was observed that *G. papaya* failed to sporulate on glycine, L-leucine, and glutamic acid, but these nitrogen sources supported fair degree of sporulation in *G. musarum* and *C. Papaye* [19]. Steinberg [20] observed good sporulation in *Aspergillus niger* in the presence of above said nitrogen sources. These results again suggested that better growth conditions in fungal strain prevent the sporulation. In our present investigation, glycine supported better growth and sporulation get retarded in case of both the *Penicillium* isolates. The psychrophilic *P. oxalicum* showed maximum biomass production in the presence of 2 % glucose, which is in accordance with the previous reports [21, 22, 23, 24, 25]. The preference fungal strains for glucose over the other carbon sources may be due to the ease with which glucose is metabolized to produce cellular energy [26, 27]. Microbial propagation at cold temperatures is built on the capacity to synthesize cold-adapted enzymes. There is need to explore the new extremophilic microorganisms, capable of producing specific enzymes, extremozymes, which help in the degradation of several polymeric substrates such as xylan, cellulose, pectin and chitin [28]. The present study on the psychrophilic *P. oxalicum* and mesophilic *P. citrinum* can provide useful markers for taxonomical studies of *Penicillium* spp. The present investigation paves the way for further study which can help in the exploitation of these extremophiles for their potential application in the field of industry, pharmaceuticals and environment.

5) STATISTICAL ANALYSIS

Mean and standard error was calculated. The difference between time and pH dependent growth of both *Penicillium* strains has been analysed by student's paired 'T-test' and significant variation in growth has been recorded. On the other hand, nutritional dependent (C, N) growth of both the *Penicillium* strains has been analysed by one way analysis of variance (ANOVA).

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