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ORIGINAL PAPER

High throughput de novo RNA sequencing elucidates novel responses in *Penicillium chrysogenum* under microgravity

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Abstract In this study, the transcriptional alterations in *Penicillium chrysogenum* under simulated microgravity conditions were analyzed for the first time using an RNA-Seq method. The increasing plethora of eukaryotic microbial flora inside the spaceship demands the basic understanding of fungal biology in the absence of gravity vector. *Penicillium* species are second most dominant fungal contaminant in International Space Station. *Penicillium chrysogenum* an industrially important organism also has the potential to emerge as an opportunistic pathogen for the astronauts during the long-term space missions. But till date, the cellular mechanisms underlying the survival and adaptation of *Penicillium chrysogenum* to microgravity conditions are not clearly elucidated. A reference genome for *Penicillium chrysogenum* is not yet available in the NCBI database. Hence, we performed comparative de novo transcriptome analysis of *Penicillium chrysogenum* grown under microgravity versus normal gravity. In addition, the changes due to microgravity are documented at the molecular level. Increased response to the environmental stimulus, changes in the cell wall component ABC transporter/MFS transporters are noteworthy. Interestingly, sustained increase in the expression of Acyl-coenzyme A: isopenicillin N acyltransferase (Acyltransferase) under microgravity revealed the significance of gravity in the penicillin production which could be exploited industrially.

Keywords *Penicillium chrysogenum* · RNA-Seq · Microgravity · ABC transporter · Acyl-coenzyme A: isopenicillin N acyltransferase

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Introduction

In the present study, the transcriptomic alterations in *Penicillium chrysogenum* under simulated microgravity conditions were analyzed for the first time using an RNA-Seq method. The majority of the previously published works were performed on prokaryotes [1, 2] but very limited studies focused on eukaryotes [3]. Fungi are usually overlooked and remain uncharacterized at the molecular level. Although other microorganisms like green algae [4], bacteria, cyanobacteria [5] and lichens [6] are able to survive under microgravity conditions [7]. Fungi are more important to be focused because of their harsh fungal enzymatic is capable of degrading spacecraft hardwares [8]. Our previous report showed that low shear microgravity did not pose any stress on *Penicillium chrysogenum*

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and *Aspergillus niger* [3], which could be due to the over-expression of certain stress-resistant proteins [9].

Microgravity modulates numerous features and functions on biological organisms through its effects on physical phenomena, such as hydrostatic pressure in the fluid-filled compartments, organelles sedimentation rate and convection processes of flow and heat [10]. These physical parameters can directly and indirectly influence signaling, metabolism, cell morphology and consequently, a wide range of cell functions [11].

Nowadays, microgravity conditions are achieved at the laboratory scale since the access to real space experiment is limited. Gravitational effects are investigated using a range of methods, including centrifugation (Hypergravity), clinorotation, magnetic levitation, random positioning machines, hind limb models, etc. In addition, few outdoor facilities like parabolic flight, drop towers are also available [12]. In the present study, we have used high aspect ratio vessel (HARV) designed by NASA to provide microgravity condition. HARV is a special variant of NASA bioreactor which is proved to provide microgravity conditions by various research groups [13]. *P chrysogenum* spores suspended in potato dextrose broth inside the HARV are constantly under the effect of earth's $\times 1$ g gravitational force. But, the orientation of gravity vector is constantly changed as the vessel rotates not allowing the cells to sense the gravity, spores thus experience the state of weightlessness [10]. The rotation of the HARV is adjusted such that the fungal biomass is suspended in the medium without creating any centrifugal effect on the cells [14]. A detailed protocol for achieving microgravity condition on fungal spores using HARV can be obtained from our previously published report [3].

Penicillium chrysogenum can cause fatal conditions in immune compromised humans [15]. *Penicillium chrysogenum* is also reported to cause eye infection [16], lung infection [17] and skin infection in normal individuals [18]. In vitro studies have shown that proteins from *Penicillium chrysogenum* can activate human Eosinophils through G-coupled receptors and cause allergic reactions [19]. Several allergens like beta-N-acetylglucosaminidase (68 kDa), vacuolar serine protease (32 kDa) and alkaline serine proteases (34 kDa) from *Penicillium chrysogenum* can initiate immunological [12] response in humans [20, 21]. Secondary metabolite production (Penicillin) is not hampered under microgravity [22]. Hence, studying the detailed biological changes in *Penicillium chrysogenum* under microgravity conditions has become a serious concern for the welfare of the astronauts.

The proteomic approach to fungal biology under extreme space conditions is not always feasible due to the low yield protein concentration and cannot not be visualized by silver staining [23]. The availability of the biomass for analysis is limited by the size of the HARV (50 ml). Also, there is lack of RNA-Seq reference genome for *Penicillium chrysogenum* even though whole-genome sequencing has already been performed. Hence, an attempt was made to perform de novo assembly and gene annotation. Fortunately, the advancement in the transcriptome sequencing provides an alternative to the whole-genome sequencing. Transcriptome sequences include only the introns, thus the data obtained contain a high content of molecular information [24] and reveal the functional gene sets more efficiently [25]. Recently, de novo assemblies using sequence reads from Illumina technology have been reported in many non-model organisms without a reference sequence [26].

Materials and methods

Fungal strain

The *Penicillium chrysogenum* (KACC 425892) strain was purchased from Korean Agriculture Culture Collection (Suwon, South Korea) and maintained on Potato dextrose agar (BD Difco, MD, USA) supplemented with streptomycin (100 mg L^{-1}). Approximately, 10^3 spores from 7-day-old culture were introduced into the HARV and the fungal mycelia were grown under the experimental condition.

High aspect ratio vessel

Autoclavable 50 ml capacity high aspect ratio vessel was purchased from Synthecon, Incorporated (Texas, USA). The system was operated in a temperature- and humidity-controlled chamber set at 25 °C and 90 % humidity was maintained to avoid air bubble formation inside the HARV. The principle of HARV and detail of the instrumental setup can be found elsewhere [22].

RNA isolation

Samples at 12 and 48 h under microgravity and normal gravity were harvested in tubes with snap freeze using Liquid nitrogen. RNA isolation was performed using the RNAeasy plant kit Qiagen) following manufacturer's protocol. Total RNA extracted was eluted in 20 µl of nuclease-free water and the final concentration was determined by NanoDrop (Epoch Microplate spectrophotometer). An aliquot of the samples was run on an Agilent RNA Bioanalyzer chip to check the RNA integrity. The quality of the RNA was excellent with intact 18S and 28S band (supplementary Fig. 1).

Library preparation

Library preparation was performed using SureSelect Strand-Specific RNA Library Prep Kit protocol outlined in "SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing mRNA Library Preparation Protocol" [10 5500-0116]. Details of the reagents used are SureSelect Strand-Specific RNA Library Prep Kit, ILM, Box 1 (Agilent, Cat # 5500-0116), SureSelect Strand-Specific RNA Library Prep Kit, ILM, Box 2 (Agilent, Cat # 5190-6410), HighPrep PCR (MAGBIO, Cat # AC-60050), Nuclease-Free Water (Ambion, Cat # AM9938), High Sensitivity Bioanalyzer Kit (Agilent, Cat # 5067-4626), Qubit DNA Assay (Invitrogen, Cat # Q-33120).

Briefly, the mRNA was fragmented for 4 min at elevated temperature (94 °C) in the presence of divalent cations and first-strand cDNA was synthesized. Strand specificity was maintained by the addition of Actinomycin D. The single-stranded cDNA was cleaned up using HighPrep PCR (MAGBIO, Cat # AC-60050). Second-strand cDNA was synthesized using a second-strand synthesis mix. The cDNA was cleaned up using HighPrep PCR (MAGBIO, Cat # AC-60050). Adapters were ligated to the cDNA molecules after end repair and the addition of "A"-base. SPRI cleanup was performed after ligation. The library was indexed and amplified using 12 cycles of PCR for the enrichment of adapter ligated fragments. The prepared library was quantified using Qubit and validated for quality by running an aliquot on a High Sensitivity Bioanalyzer Chip (Agilent).

Sequencing

Prior to cluster generation, library concentration and size were assayed using the Agilent DNA1000 kit (Agilent, USA) on a 2100 Bioanalyzer (Agilent, USA). The library Bioanalyzer showed expected peak between 200 and 600 bp as shown in Fig. 1. The effective sequencing insert size was 80–480 bp, with combined adapter size being 120 bp. Libraries were sequenced as 75 × 2 on an Illumina Nextseq platform equipped with the 75 bp paired-end module.

Illumina reads processing and assembly

The Illumina reads were quality checked using FASTQC [27]. The raw Illumina reads were processed by an in-house script for adapters and low-quality bases trimming towards 3′-end. The high-quality reads were assembled by Trinity [28] with default settings K-mers set at 25 to construct unique consensus sequences. The transcripts were annotated against protein sequences (from Uniprot Protein Database) and the transcripts are annotated with the Pfam database using ncbi-BLAST-2.2.29+ [29].

Pathway analysis and differential gene expression

Pathway analysis was done using KAAS [30]. Transcripts were combined and clustered using CD-hit [31]. Alignment of the reads was performed using Bowtie 2 [32]. A detailed schematic representation of work pipeline followed in this study is shown in Fig. 2. DESeq was used for DGE for 12 h normal gravity vs 12 h microgravity and 48 h normal gravity vs 48 h microgravity as required [33].

Results and discussion

The present study demonstrated the gene expression changes in *Penicillium chrysogenum* under microgravity conditions and the obtained results were compared with the normal gravity conditions. Based on the results, we concluded that

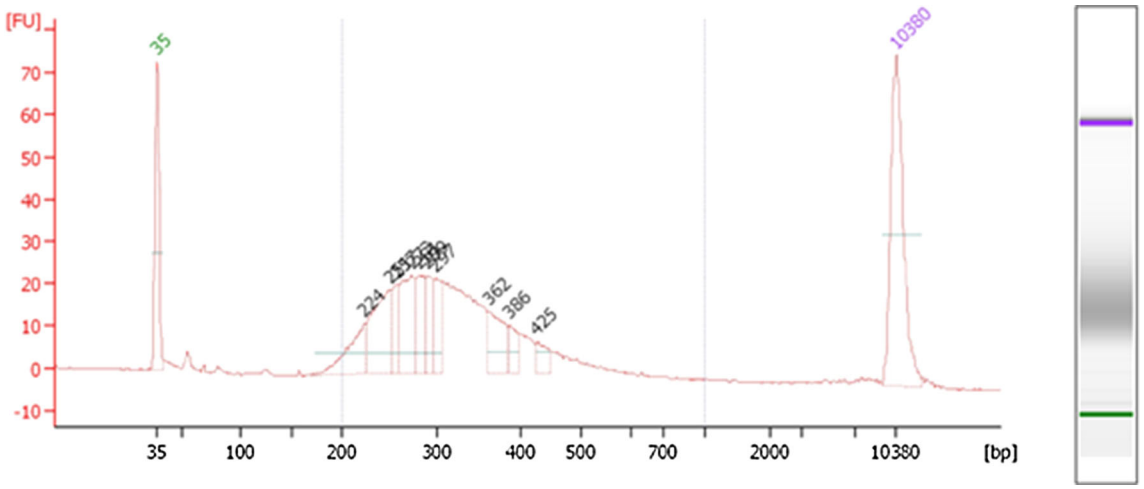


Fig. 1 Electrophoretogram of the prepared library showing expected peak from 200 to 600 bp. The effective insert size is 80–480 bp with combined adapter size being 120 bp

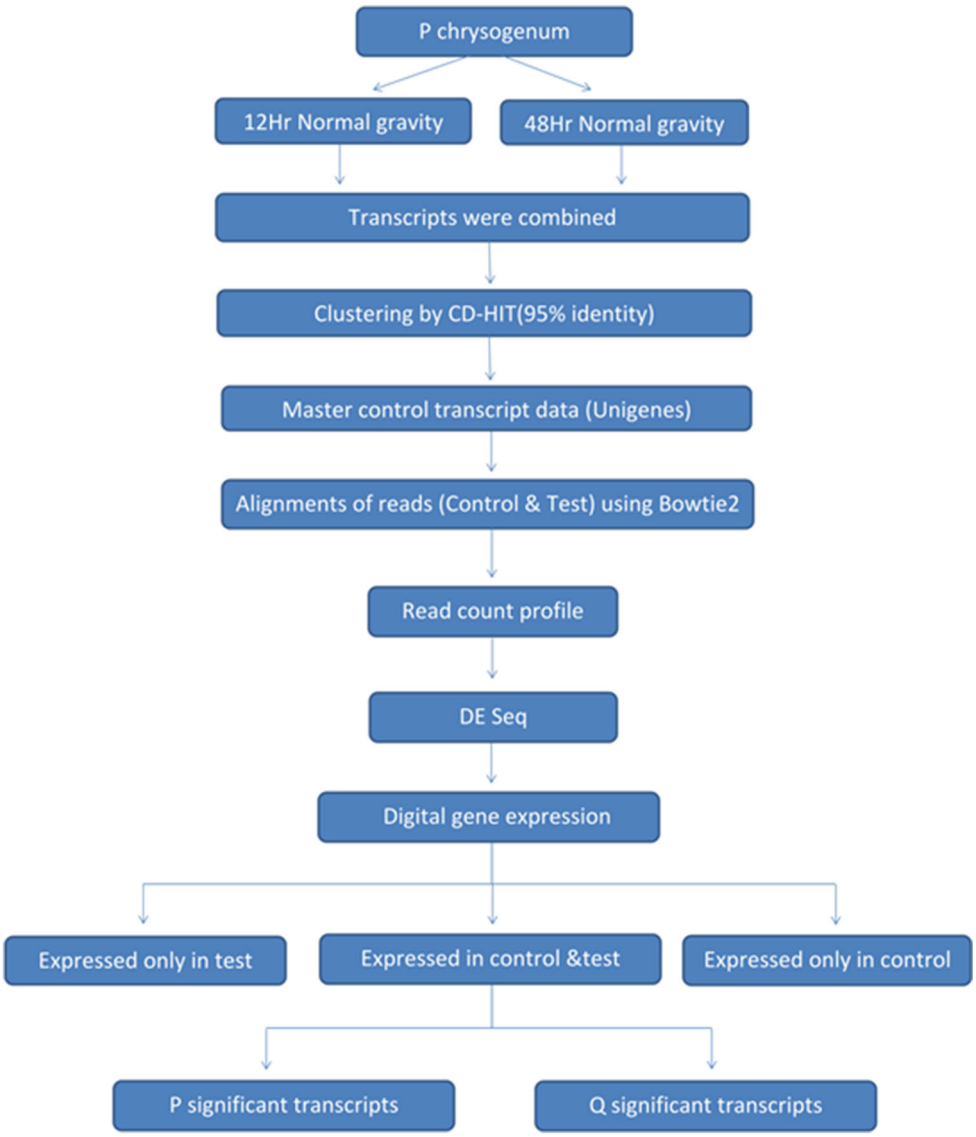


Fig. 2 Schematic representation of the workflow pipeline carried out in the de novo RNA-Seq experiment for *P. chrysogenum*

the fungi exposed under microgravity conditions resulted in number of biological changes at molecular level. Sudden environmental changes from normal to microgravity conditions lead to changes in fungi cell wall components (ABC transporter/MFS transporter). Moreover, a sustained increase in the expression of genes coding for Acyl-coenzyme A: isopenicillin N acyltransferase (Acyltransferase) under microgravity conditions revealed the significance of gravity in the penicillin production.

High aspect ratio vessel

The simulated microgravity condition was provided to the *Penicillium chrysogenum* for 12 and 48 h time period, care was taken to make sure that the biomass remains suspended as the fungal mass grows bigger during longer incubation. HARV was rotated in an axis perpendicular to the gravity vector and the air bubble formation was avoided by placing the humidity chamber set at 90 % in HARV. The rotation was adjusted to 25 rpm as the spore aggregates formed a fluid orbit within the vessel. Continual fall without contacting the vessel wall was attained according to the manufacturer's instruction (Operation Manual, The Rotary Cell Culture System, Synthecon, Inc., USA).

Read processing

Currently, illumina reads became the basis for a series of biological applications [34], both in diagnostic and in research fields [35, 36]. These include the de novo assembly of genomes and transcriptomes and the alignment of short reads over an existing reference [28, 31] chimeric

transcript detection [37], haplotype inference [38] and methylation detection [39]. Herein, we obtained enormous transcriptome dataset using relatively short reads generated by illumina paired-sequencing technology. This was the first attempt on *Penicillium chrysogenum*, an organism lacking reference genome. Two biological replicates were included at each time point to counterbalance the signal to noise ratio during data acquisition [40]. A total of 38,788 contigs was generated out of which 33,339 were successfully annotated. Contigs stat are provided in Table 1. The quality of the de novo assembly was validated by comparison with known databases and functional annotation. More biological replicates were avoided as some significant gene expressions could be masked during data transformation averaging and also minimizing errors introduced during sample preparation (Fig. 3).

Table 1 Contigs stat for *Penicillium chrysogenum*

Contigs generated	38,788
Maximum contig length	17,685
Minimum contig length	201
Average contig length	1,216.2 ± 1,398.3
Median contig length	1,718.00
Total contigs length	47,173,335
Total number of non-ATGC characters	0
Percentage of non-ATGC characters	0
Contigs ≥100 bp	38,788
Contigs ≥200 bp	38,788
Contigs ≥500 bp	22,291
Contigs ≥1 Kbp	15,041
Contigs ≥10 Kbp	71
Contigs ≥1 Mbp	0
N50 value	2200

Gene expression

The gene expression changes due to simulated microgravity havebeen reported earlier in various fungi like *Pleurotus ostreatus* [41] and *Saccharomyces cerevisiae* [42] to name a few. To find out differentially expressed genes, two- fourfold-change filters were applied. Heatmap analysis was performed on both 12 and 48 h samples. Under microgravity conditions, both 12 and 48 h showed increased expression of the gene coding Acyl-coenzyme A: isopenicillin N acyltransferase (Acyltransferase) AAT/PenDE-*Penicillium chrysogenum* (GO ID: 0016746). Acyltransferase enzyme is involved in penicillin biosynthesis [43]. Acyltransferase is also known to be involved in various metabolic pathways and secondary metabolite production (KEGG REACTION: R06361). The changes are in agreement with the Venn diagram represented in Fig. 4. Pathogenicity of an organism can either be increased or decreased in the absence of gravity. *Staphylococcus aureus* grown in HARV is less virulent compared to the normal gravity control [2]. In contrary, slight upregulation of the genes involved in pathogenicity was observed in *P chrysogenum* under microgravity. Thus, the obtained result is in good agreement with the previously published report by [44] as *Salmonella enterica* becomes more virulent under microgravity. Figure 3 indicates the number of genes responsive to environmental stimulus and stress tolerance under microgravity and normal gravity. The number of genes differentially expressed is represented in Fig. 5. As the figure suggests, there is an increased response to environmental stimulus and stress tolerance under microgravity compared to normal gravity (Fig. 6).

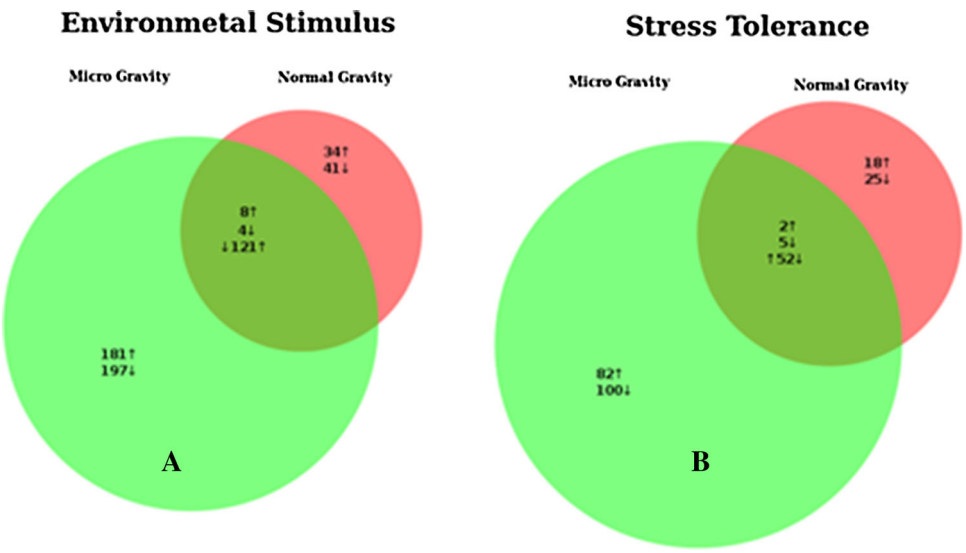
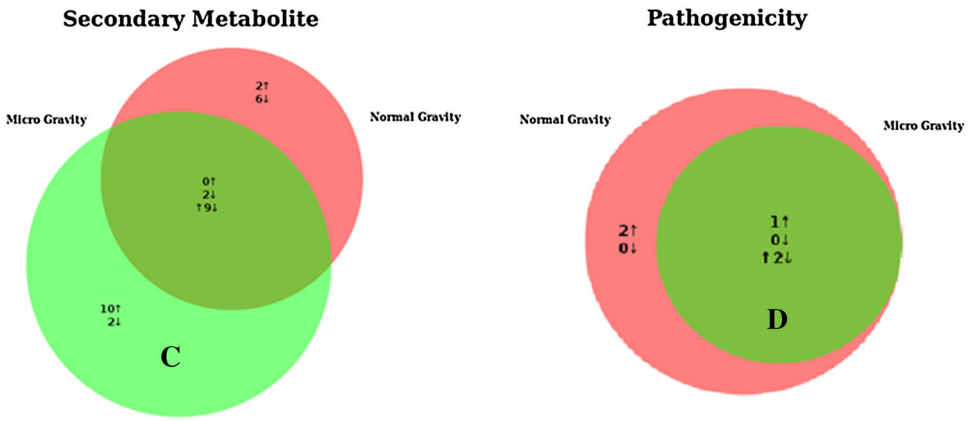


Fig. 3 *Venn diagram* indicating the number of differentially expressed genes under microgravity and normal gravity corresponding to environmental stress and stress tolerance in *P. chrysogenum*



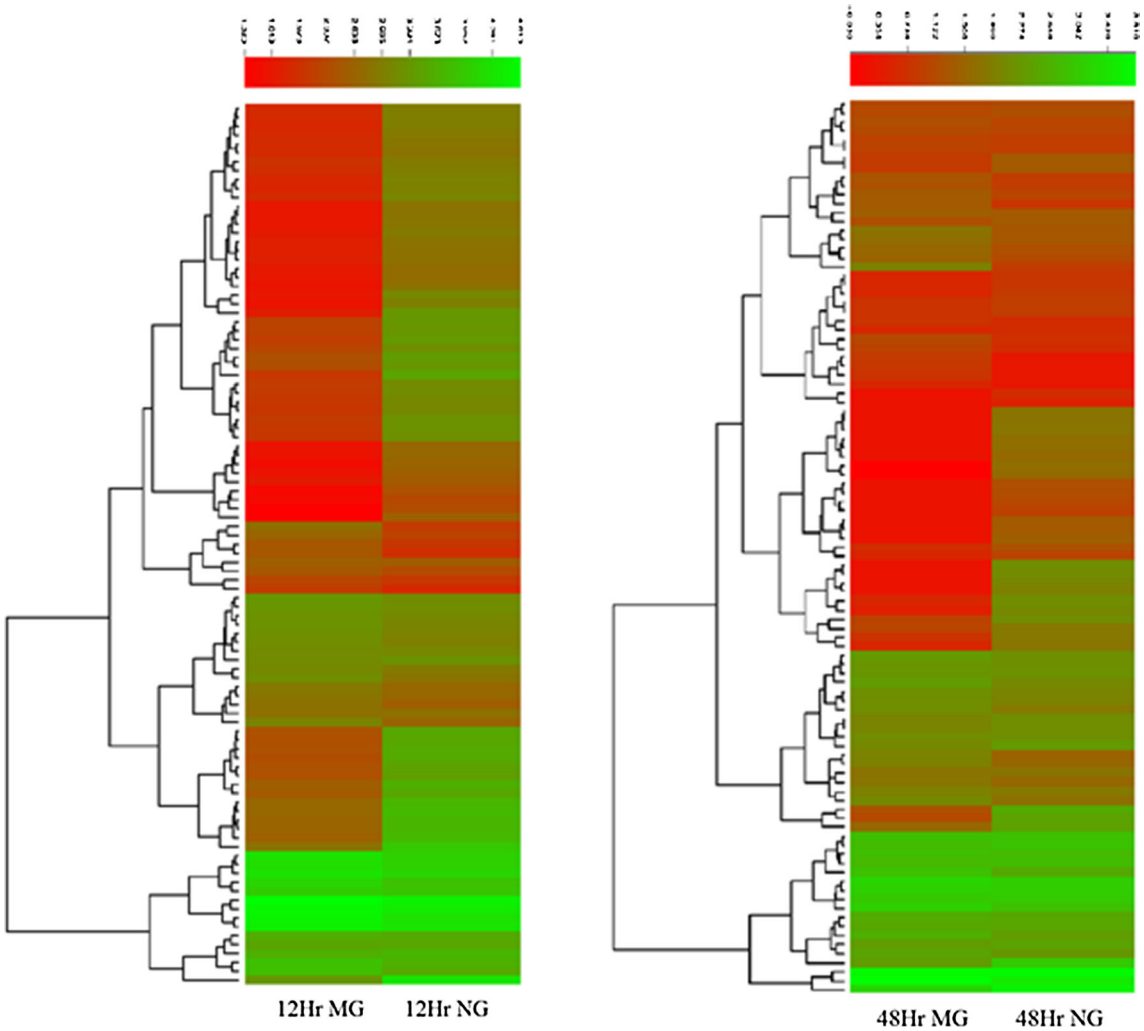


Fig. 4 *Venn diagram* indicating the number of differentially expressed genes under microgravity and normal gravity corresponding to secondary metabolite production and pathogenicity in *P. chrysogenum*

Fig. 5 Heat maps for the global gene expression profile of *P. chrysogenum* under microgravity and normal gravity. Visualization was performed using the plot package. *Each row* represents one gene. *Green* represents highly expressed, *Red* means low expression value

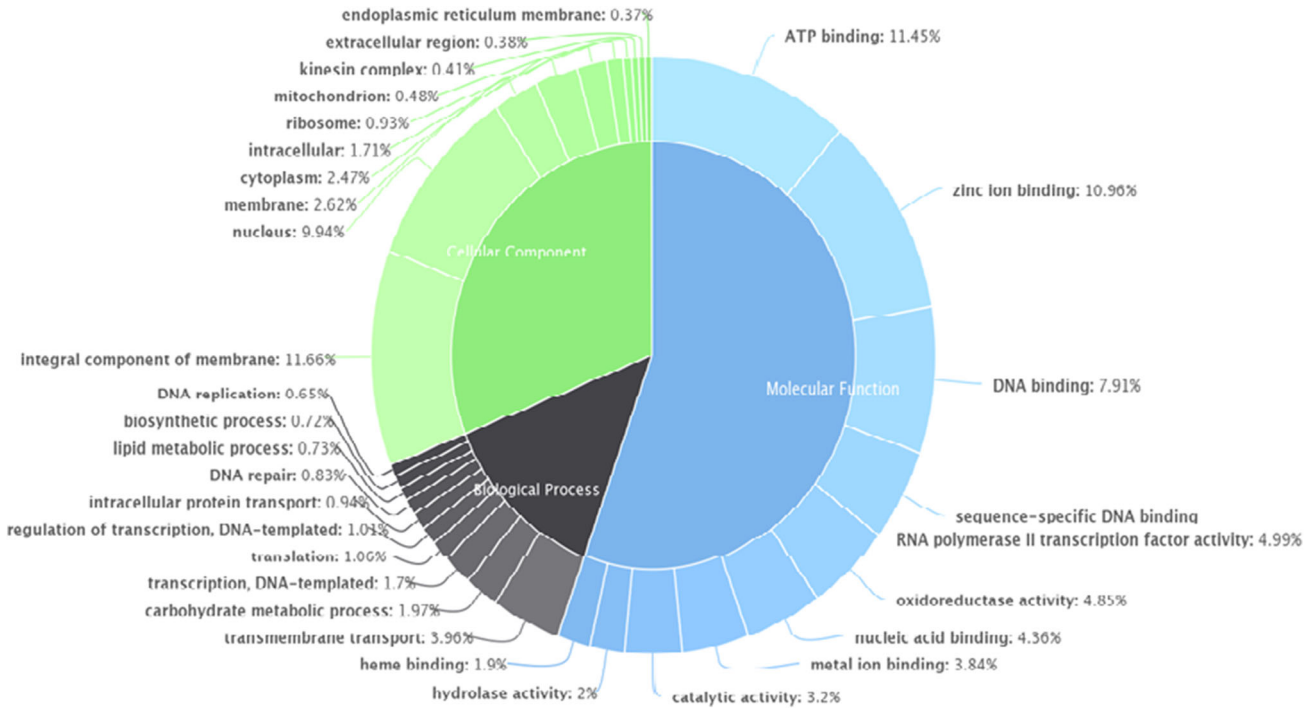


Fig. 6 Changes in cellular components, biological processes and molecular functions of *P. chrysogenum* to microgravity

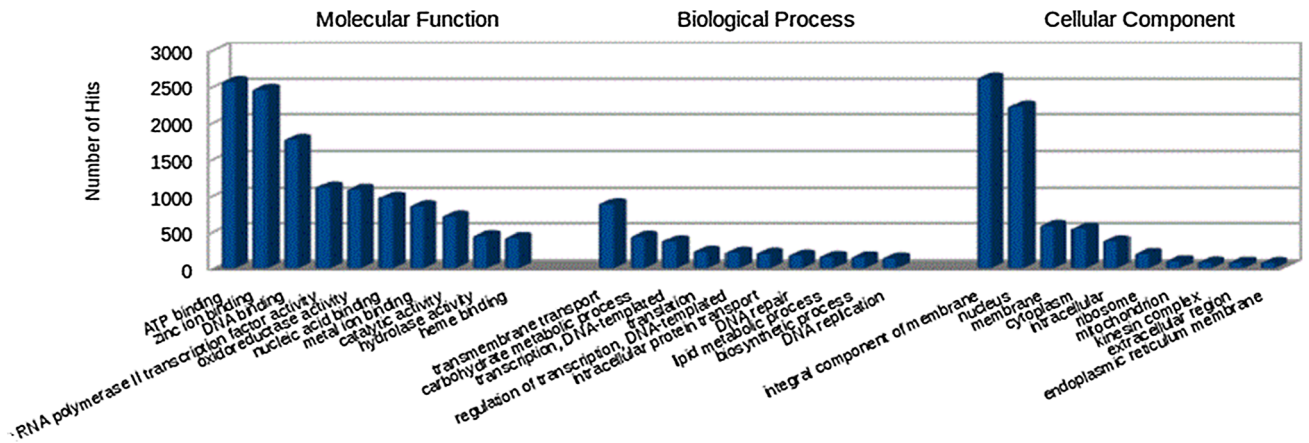


Fig. 7 This chart represents top 10 gene ontologies, including molecular function, biological process and cellular components

Changes in cell wall components

Another significant finding is the over-expression of multidrug/pheromone exporter/ABC transporters (GO: 0005524; GO: 0042626; GO: 0016021) in 12 and 48 h microgravity samples. The ATP-binding cassette (ABC) transporters is one of the largest known protein families found in bacteria, archaea, and eukaryotes. ABC transporters couple ATP hydrolysis to active transport of a wide variety of substrates such as ions, sugars, lipids, sterols, peptides, proteins, and drugs (KEGG PATHWAY: hsa02010). Penicillin secretion is also possibly mediated by ABC transporters [45]. The MFS transporters (GO: 0055085) were also found to be increased at 48MG. Major facilitator superfamily transporters (MFS) are involved in transportation of substrate molecules, including sugars, drugs, metabolites, amino acids, vitamins and both organic and inorganic ions, or small peptides [46]. The major changes observed in the integral cell wall component (11.6 %) are due to the ABC & MFS transporters as shown in Fig. 7.

Metabolic changes under microgravity

The altered carbohydrate metabolism under microgravity by the gene ontology analysis could possibly indicate increased

pathogenicity of *Penicillium chrysogenum* under microgravity. Changes in the carbohydrate metabolism are shown to be directly linked to the virulence of the organisms like *Candida albicans* [47], *Neurospora crassa* [48], *Fusarium verticillioides* [49], and *Magnaporthe grisea* [50]. Figure 7 shows that changes in the gravity vector cause changes in carbohydrate metabolism in *Penicillium chrysogenum* which is noteworthy for health crew of the astronauts.

Further, the result highlights the most interesting genes, grouped into several regulatory processes, including (1) ATP binding, (2) Zinc ion binding, (3) DNA binding and (4) Sequence-specific DNA binding RNA polymerase II transcription factor (5) Oxidoreductase activity. In addition, biological processes, including primary metabolism (carbohydrate biosynthesis) and secondary metabolism (penicillin synthesis) were affected as well. Details of the number of hits by the contigs and its corresponding gene ontology details are shown in Fig. 7. We have also observed upregulation of many genes under microgravity coding for proteins, whose functions are yet to be determined. As future work, current genomic data allowed us to narrow down and look for an increase in specific proteins like Acyl-coenzyme A: isopenicillin N acyltransferase under microgravity.

Most of the previous reports on effects of microgravity elucidated in Fungi, Bacteria, Actinomyces were based on real space conditions. The genomic, transcriptomic and proteomic variations shown were not exclusively due to gravity vector, but there are several other factors which influenced these changes. Space radiation containing high-energy particles (HZE) encountered during space mission has a greater influence on the gene expression changes than microgravity alone [12]. Hence, data obtained from ground-based HARV gives more reliable information about the biological changes in *Penicillium chrysogenum* based on a single parameter, microgravity.

Conclusion

This is the first report on the gene expression changes in the *Penicillium chrysogenum* under microgravity condition. Information from previous reports was significantly scarce to predict the biological pathways affected under microgravity. The results obtained in this study not necessarily reflect the changes found in real space conditions as the effects of radiation and vibrations are avoided in our laboratory setup HARV. However, the basic knowledge obtained through the de novo RNA sequencing study should lay a foundation for future research in the field of space microbiology for the betterment of the astronaut crew health and necessary precautions against the undesirable effects of filamentous fungi under microgravity for the upcoming long-term space missions.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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