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Effects of Hypobaric and Hyperbaric Pressures on Mycelial Growth of Isolated Strain of Wild *Ophiocordyceps sinensis*

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Abstract

The exploration of the effects of pressure leads to new insights into the mycelial growth of *Paecilomyces hepiali* fungal strain. This strain has been generally accepted as true anamorph of wild *Ophiocordyceps sinensis.* It is only found at high altitude area like Himalayan plateau where atmospheric pressure is very low. Most of researches about *P. hepiali* and artificial mycelial cultivation have been done around mean sea level pressure. Then new experimental set up was developed and described. The apparatus permitted growth of mycelia under different pressure levels while other micro environmental conditions were carefully controlled. Potato dextrose broth was used as liquid media. As solid and semi solid media, sorghum base media and potato dextrose agar were prepared. Results of mycelial growth under hypobaric pressures and hyperbaric pressure were compared with mycelial growth of atmospheric pressure and hence growth influence has been shown. Specially, −100 mmHg treated sample showed significantly highest growth in both solid media and semi solid media. In semi solid media, −100 mmHg was not significant with other reduced pressure treatments. Meanwhile, −150 mmHg treated samples showed significantly highest mycelial growth of liquid media and −150 mmHg of pressure adversely affected on water contents of solid growing media. This may be an effect of pressure on enzymatic activities, protein and fatty acid of plasma membrane. As well as, pressure changes equilibrium of biochemical reactions, bond of some molecules and partial pressure of air molecules. Further molecular and biochemical researches are required to evaluate the possible stimulation of mycelial growth through hypobaric and hyperbaric treatments.

Keywords

Hypobaric Pressure, Hyperbaric Pressure, *Ophiocordyceps sinensis*

1. Introduction

The *Paecilomyces hepiali* is a true anamorph of the wild *Ophiocordyceps sinensis* which grows on high elevation, around 3000 m to 5000 m mount peeks [1]. The elevation is conducive to change various types of growth factors such as temperature, Rh, negative ions (Sulfate, nitrate, organics and OH^- were the most common peaks observed in the negative ion spectra) [2]. As Christy mentioned, atmospheric pressure is gradually decreased in order to raise the elevation [3]. The gravity causes the density and pressure of air to decrease exponentially as one which moves away from the surface of the Earth [4]. So, natural habitat of *P. hepiali* exposes to hypobaric pressure compared to less elevated area around mean sea level where many experiments have been done. The importance of the pressure has not been considered by previous researches hence filling the gap was main objective of this study. Natural environmental conditions were facilitated and hypobaric and hyperbaric pressure effects on mycelial growth of *P. hepialid* strain were revealed and also more possible efficient ways to mycelia production for pharmaceutical purposes were detected.

The entire lives on the Earth live under pressure gradient which is affected by rising of elevation. It is generally assumed that the sea level pressure is 760 mmHg (1 atm = 1 $kg{\cdot}cm^{-2}$ = 1 bar = 0.1 MPa). Microorganisms grow optimally within a characteristic range of fundamental physical parameters such as temperature, osmolarity, pH, and pressure. Cells that can grow at the extreme limits of these parameters are known collectively as extremophiles [5]. The biological effects of hypobaric and hyperbaric pressure on living organisms have been explored in many studies. Clearly, pressure plays an important role in the distribution of life in the world's oceans [6]. In this study, the effect of hypobaric pressure and hyperbaric pressure on mycelial growth of *P. hepiali* fungal strain was studied.

The pressure influences the rate and equilibrium position of many chemical reactions and catalyzes reaction forward or backward. Processes accompanied by a decrease in volume such as a C-C bond formation, in which the van der Waals distance between two carbon atoms decreases to the bonding distance are accelerated by pressure. The equilibriums are shifted toward the side of products [7]. A similar phenomenon, termed negative pressure effect has been observed during the crystallization of some synthetic polymers [8].

The concentration of oxygen in sea level air is 20.9%, so the partial pressure of O_2 is 21.136 kPa when the atmospheric pressure is 101,325 Pa. Atmospheric pressure decreases exponentially with altitude while the O_2 fraction remains constant to about 100 km, so partial pressure of O_2 decreases exponentially with altitude as well. Oxygen concentration at low pressure and atmospheric pressure there is a close correlation between a microbe's ability to grow on agar or in liquid media [9]. Previous work demonstrated that hyperbaric air could be successfully applied to yeast cultivation, as a way of improving the oxygen transfer rate to aerobic cultures [10]. It has been revealed that rates of net photosynthesis

at reduced pressure higher than at ambient pressure and some plant growth occurs in low pressures was higher than in normal atmosphere [11] [12]. The CO_2 concentration controls the sporulation and mycelia growth of many fungi and pigment yields and pigment production of *Monascus purpureus* on rice [13].

It has been reported that periodic pressure oscillation significantly enhances cellulase production by filamentous fungi [14] [15]. Low pressure changes fatty acid composition of plasma membrane of cells and decreases in the ratio of unsaturated to saturated fatty acids but increases in the ratio of anteiso- to iso-fatty acids [16]. Moreover hypobaric and hyperbaric treatments have been used to control microbial activities [17] [18] [19]. Even though human body performs best at sea level, *P. hepiali* performs with low pressure environment and hence it naturally propagates sexually and asexually while producing fruit bodies at high altitude. The mycelial growth of *P. hepiali* is determined in order to effect of pressure on different growing media.

2. Materials and Methods

2.1. Study Area and Sample Collection

This study was conducted in the laboratory of Kasuya Research Forest, Kyushu University which is situated at ~20 m above sea level. The atmospheric pressure of study area is assumed to be similar to pressure at mean sea level.

2.2. Fungal Strain

The fungal material used in this study was originally brought from Jilin Agricultural University, China and assigned to accession number KUMB1081 in the mushroom culture bank at the Laboratory of Forest Production Control, Kyushu University. The Internal Transcribed Spacer (ITS) sequence of the genome was exactly match (100%) with NCBI Gene bank entry EF555097.3, *P. hepiali* strain Ph-4Qinghai. It had been recorded as *O. sinensis* Ph-4Qinghai before being updated to *P. hepiali* strain Ph-4Qinghai.

2.3. Media Preparation

Three different types of growing media have been developed by using individual methods and adapted to various pressure experiments. Physical and chemical properties were different among them.

2.3.1. Liquid Media

Most favorable pressure for mycelial growth of *P. hepiali* in liquid media is to be determined; 5 mm diameter agar plugs with actively growing mycelium were inoculated to liquid media by placing a one plug in to the liquid media in conical flask. Potato dextrose broth was used as media and followed general procedure for preparation. Six inoculated conical flasks in each bell jar were then incubated at 25˚C with each of the following different pressure levels. Total biomass measurements were made 16 days after inoculation. Mouths of the conical flasks were covered with sealing tape to reduce evaporation and contaminations.

2.3.2. Semi Solid Media

To determine the most favorable pressure for mycelial growth of *P. hepiali* on semi solid media; 5 mm diameter agar plugs with actively growing mycelium were inoculated on PDA plates by placing a one plug on the centre of the PDA surface [20]. General procedure was used to prepare 9 cm diameter PDA petri dishes. Seven inoculated dishes were kept inside of individual bell jars and then incubated at 25˚C with each of the following different pressure levels. Colony diameter measurements were made after 16 days of inoculation.

2.3.3. Solid Media

The most favorable pressure for mycelial growth of *P. hepiali* on sorghum base media is to be determined; 5 mm diameter agar plugs with actively growing mycelium were inoculated on sorghum base media by placing a one plug on the centre of the sorghum filled 9 cm glass petri dishes. Sorghum has actual nutritional value, because of its content of protein, vitamins, both fat-soluble (D, E and K) and of B group (except for B12), as well as minerals, such as iron, phosphorus and zinc, vitamin E , low contents of carotenoids [21]. Sorghum grains were precooked and washed until excess starch drain away from media prior to placing in the glass petri dishes. Seven inoculated plates in each bell jar were then incubated at 25˚C with each of the following different pressure levels. Colony diameter and moisture content measurements of the media were taken 16 days after inoculation.

2.4. Experiment Set Up

In general natural habitat of *P. hepiali* is surrounded with low pressure environment in order to the elevation. So, treatments have mainly been focused on hypobaric pressures rather than hyperbaric pressure. An air pump (**Figure 1(d)**), pierced rubber cap, air control valve, connecting tubes, bell jars and digital manometer were used to generate particular micro environments. As shown in **Figure 1**, series of three bell jars have been connected with air inlet of the air pump. It sucks inside air of bell jars for obtaining three different hypobaric pressures. The outlet of the air pump compresses air in to bell jar and one hyperbaric pressure compartment was generated. Weight of bell jar was not enough for holding the air inside of the chamber which is responsible for pressure retention. So, ropes and adjusting screws (**Figure 1(e)**) were used to compress glass base toward bell jar. Another bell jar was connected with air flow as control without any pressure change. Bell jar units were connected with adjusting air valves and adjacent bell jars to manipulate air pressure and maintain the air flow. The main air inlet of apparatus has been connected with conical flask filled with water (**Figure 1(a)**) to humidifying the airflow.

Those bell jars provided favorable general growth conditions while maintaining all those factors constant or at desired levels which modify the action of air pressure [22]. Five different treatments including control are shown in **Table 1**.

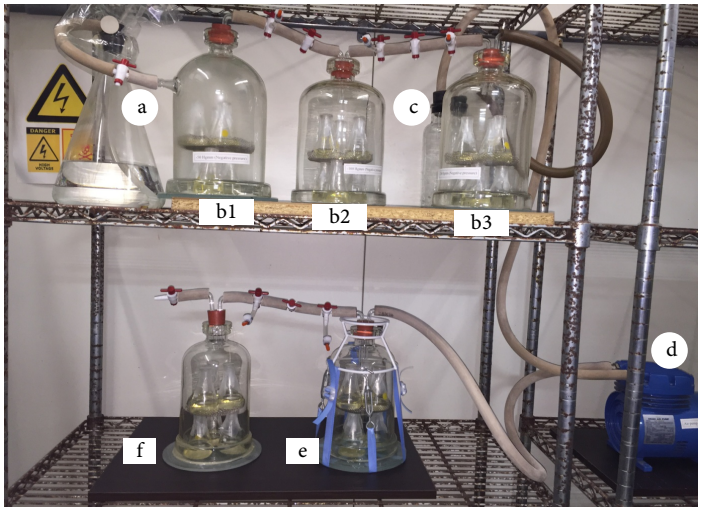

Figure 1. (a) Humidifier unit; (b) Hypobaric chambers: (b1) −50 mmHg, (b2) −100 mmHg, (b3) −150 mmHg; (c) Pressure stabilizer; (d) Air pump; (e) Hyperbaric (50 mmHg); (f) Control.

Table 1. Digital manometer readings and actual pressure of five different treatments including control, one hyperbaric and three hypobaric treatments are presented.

	Hypobaric pressures			Control	Hyperbaric pressure
Pressure gage readings (mmHg)	−150	−100	−50	0	50
Atmospheric pressure (mmHg)	760				
Actual pressure (mmHg)	610	660	710	760	810

2.5. Measurements and Observations

Digital manometer (Fuso-8230) was used to evaluate chamber pressure of bell jars. Colony diameters of PDA media and sorghum base media were taken by using a Vernier caliper. Two perpendicular colony diameter measurements were made. The vacuum suction filter device separated biomasses from liquid media by using filter papers which have 1 μm pore size. Samples were dried for 24 - 48 hours at 80˚C (constant weight), cooled in a desiccator jar, and reweighed (dry weight) [14]. Though PDA media had only a surface growth (horizontal), vertical growth was also observed in sorghum base media and liquid media.

2.6. Statistical Analysis

Identification of statistical differences within treatments was done by Analysis of Variance (ANOVA) followed by Tukey's post hoc test. All the analyses were done with 0.05 significance levels. The analyses were done by using Minitab 17 statistical software (Minitab Inc.) and Microsoft Excel. All graphs are presented with standard error bars.

3. Results and Discussion

The mycelial growth of every type of media has been diferentciated by hyperbaric

and hypobaric pressures treatments used in this study. Table 2 shows the statistic performs done by each pressure treatment on individual media.

3.1. Effect of Pressure on Mycelial Growth in Liquid Media

The growth of mycelial biomass of potato dextrose broth has been induced by both hyperbaric and hypobaric treatments. Out of them −100 mmHg and −150 mmHg treated samples show significantly higher mycelial growth compared to atmospheric pressure. Figure 2 shows that −150 mmHg treated samples have acquired highest fungal biomass. Biomasses of −50 mmHg and 50 mmHg treated samples have no any significant. Liquid media allows multidirectional mycelial growth by providing nutrient, air and water from every direction.

A barometric pressure of 30 mb is required for the retention of free water. It has been suggested that the pressure less than 4 mb, the moisture is present as a vapor above the freezing point and consequently it is not available for utilizing by living cells [23]. Even though reduced pressure enhances transpiration [24] and water evaporation from media, the amount is considerably low compared to water content in liquid media hence, there is no water limitation for fungal metabolism in liquid media compared to sorghum base media.

3.2. Effect of Pressure on Mycelial Growth on Semi Solid Media (PDA)

The PDA generally performs as good nutrient and water supplement for mycelial growth. It has semi solid filled structure hence molecular movement is very low across the media and also free moving of air molecules and other supplements is controlled and holds less useable air portion. So, mycelia are allowed only surface growth and unable to penetrate across the media. Figure 3 represents the mycelia which were grown on semi solid media show a significant hypobaric

Table 2. This graph represents five different treatments on three different growing media. The values in columns of solid and semi solid media represent every mean ± SD of colony diameter of seven replicates measured on the 17th day after inoculations and also moisture percentage of solid media. The mean ± SD of bio mass of six replicate measured on 17th day after inoculation is represented in column of liquid media. Values in the same column with different letters differ significantly according to Tukey's test ($p < 0.05$).

Pressure level mmHg	Growth parameters			
	Bio mass of liquid media (n = 6) mg	Colony diameter of semi solid media (n = 7) mm	solid media	
			Colony diameter (n=7) mm	Moisture percentage (n = 7)%
−150	199.2 ± 13.78 a	67.9 ± 3.21 a	54.9 ± 3.75 d	23.6 ± 2.5 d
−100	182.8 ± 23.13 ab	72.4 ± 3.44 a	75.2 ± 5.72 a	53.2 ± 2.4 c
−50	154.6 ± 12.94 bc	68.1 ± 3.16 a	66.6 ± 4.4 b	63.4 ± 2.2 b
0	141.5 ± 15.35 c	61.2 ± 3.83 b	58.1 ± 2.97 cd	66.1 ± 2.1 a
50	158.0 ± 17.25 bc	62.1 ± 3.74 b	63.2 ± 3.54 bc	67.2 ± 2.1 a

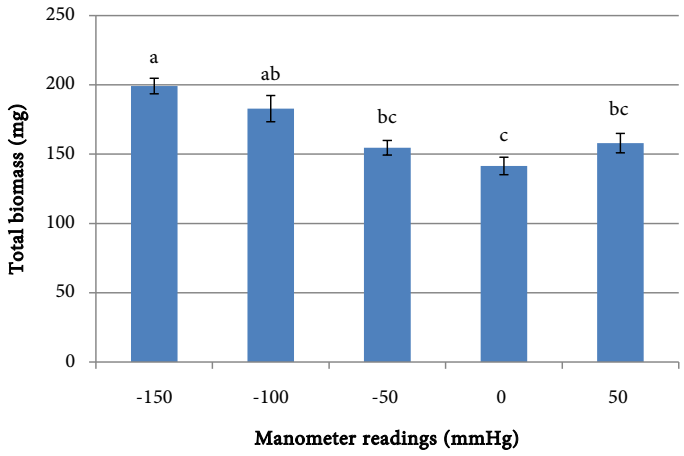

Figure 2. Mycelial biomasses of *P. hepiali* in liquid media with various pressure levels (digital manometer readings) are represented. Each bar shows the mean value of six replicates measured on the 17th day after inoculation. The error bars represent standard error.

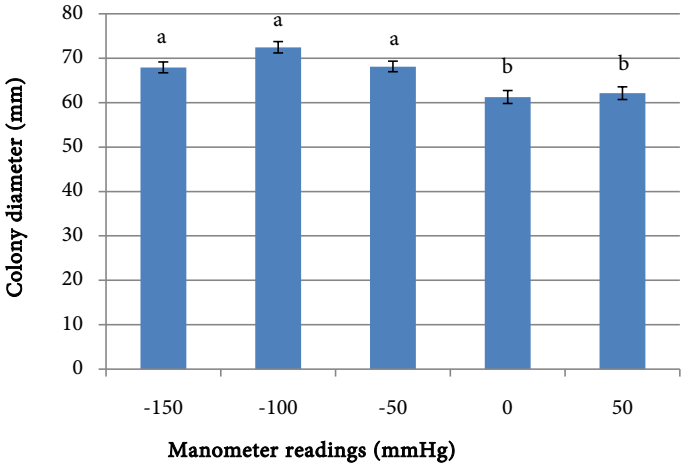

Figure 3. Mycelial growths of *P. hepiali* in semi solid media with various pressure levels (digital manometer readings) are represented. Each bar shows the mean value of seven replicates measured on the 17th day after inoculation. The error bars represent standard error.

pressure influence compared to atmospheric pressure. Mycelial growth has been increased in semi solid media and −100 mmHg treated samples have acquired highest growth and no any significant growth shown by hyperbaric pressure treated samples.

The pressure has effect on aqueous solubility of substances. Outer surface area of mycelia which has interaction with pressure and airborne molecular and, bottom part which plays an important role in nutrient absorption is comparatively less than other types of media which has multi directional mycelial growth. Hypobaric pressure grabs some water molecules from media to regulate spatial pressure of water. Even though phenomena removes water molecules, semi solid media has sufficient water storage where only 39 g of PDA powder has been

dissolved in 1000 ml of distilled water. Media able to maintain sufficient water supply for mycelial growth.

3.3. Effect of Pressure on Mycelial Growth on Solid Media

Sorghum grain media provides balanced supplements [21] for general mycelial growth. Considerable spaces were available between particles inside the media hence surface mycelial growths as well as multidirectional downward growths have been observed. The total surface area which was exposed to pressure effects was greater than mycelial colony in semi solid media. Figure 4 shows that sorghum base media has significant hypobaric pressure influence compared to atmospheric pressure. Though −100 mmHg treated samples show significantly higher growth, −150 mmHg treated samples provide unexpected growth retardation. Analysis of the water content of media showed that less water content has been remained in −150 mmHg treated samples compared to other treated samples.

Evaporation of water from media in hypobaric chamber is greater than hyperbaric chamber and control. The water availability for mycelial growth has been gradually decreased in solid media compared to semi solid and liquid growing media. The extreme sensitivity of hyphal extension to turgor pressure changes, suggested that one possible explanation of growth retardation by water stress may be inability of the fungus to maintain an adequate turgor pressure at low water potentials [25]. Therefore, even greater limitations of productivity were expected after low pressure treatment [22].

The previous researches have been explained that alterations of protein structure/dynamics at moderate pressure might also play an important role in determining binding/catalytic efficiencies of enzymes. Indeed, biological activity normally requires structural flexibility, and therefore it has been proposed that

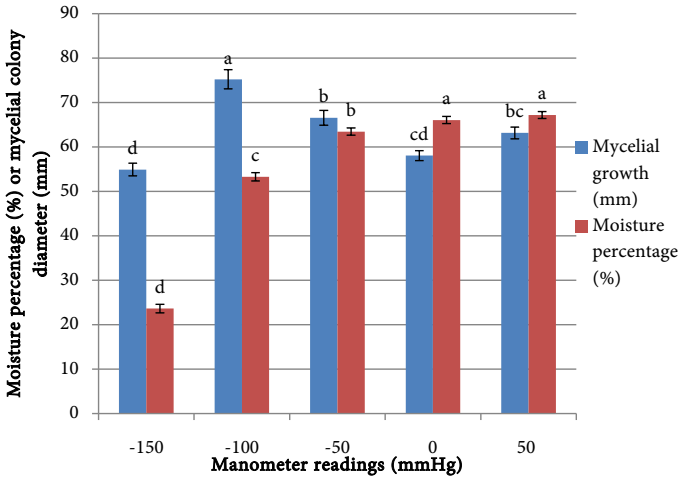

Figure 4. Mycelial growths of *P. hepiali* in solid media with various pressure levels (digital manometer readings) and moisture contents of particular media are represented. Each bar shows the mean value of moisture content and mycelial colony diameter of seven replicates measured on the 17th day after inoculation.

inhibition of biological functions at moderate pressures could be due to the reduced flexibility of biological macromolecules, globular proteins in particular [26]. Pressure has ability to affect on noncovalent bonds such as hydrogen, ionic, and hydrophobic bonds [27]. It has been revealed that vegetative cells, including yeasts and moulds, are rather pressure sensitive. Changes in external factors, such as pressure, can perturb the subtle balance of intramolecular and solvent-protein interactions [28]. The effect of pressure on protein stability is determined by changes in volume, expansibility and compressibility associated with the unfolding transition of the protein at the experimental temperature and pressure [29]. Analysis of membrane fatty acid composition of cells grown at 1013 mbar versus 50 mbar revealed a decrease in the ratio of unsaturated to saturated fatty acids but an increase in the ratio of anteiso- to iso-fatty acids [16].

Most of enzymes are protein and affected by pressure. Mycelia secrete digestion enzymes which involve to breaking down large polysaccharide, lipid and proteins. Pressure controls the rate of rebinding through the activation volume and through redistribution of substate populations of protein reactions [30]. Low pressure might be positively affected on enzymes and enhance biochemical reactions. Cellulase is one major enzyme of *P. hepiali* which is used to broken down cellulose in media. Pressure significantly enhanced cellulase production by filamentous fungi [14] [15]. However, total surface area where substrate and mycelial enzyme reaction are taken place is lowest in PDA media. So, enzymatic reaction might be lower than other media.

Respiration of every living creature is mainly related with airborne molecules. Pressure affects for concentration and solubility of air molecules in liquid media and also changes Oxygen utility at high elevated area. The distribution of a chemical among air and water is largely dependent on some key physical-chemical properties which include the vapor pressure, water solubility [31]. The CO_2 concentration influences the O_2 consumption as well [32]. Exchanging air molecules which are nearby membrane or dissolved in water are done by molecular diffusion across the membrane. There are some diffusion limitations, caused by the diffusion barriers [33]. It is a combined effect of respiration and gas diffusion: the cytochrome *c* oxidase, which is believed to be the rate- determining enzymatic reaction, is saturated at O_2 levels lower than 5% [34]. Low pressure reduces partial pressure individual airborne molecules in chambers. It changes solubility of reactant and output of biochemical reactions.

The volume of reaction and activation can be determined from the pressure dependence of the equilibrium constant and rate constant, respectively. The volume of reaction also corresponds to the difference between the partial molar volumes of reactants [35]. The reaction rate increases in the direction where there are fewer moles of gas and decreases in the reverse direction. The *Saccharomyces cerevisiae* at a fermentation temperature of 35˚C, The vacuum fermentor eliminated ethanol inhibition by boiling away ethanol from the fermenting beer as it was formed [36]. Similarly, some chemical compounds might be removed from media by hypobaric pressure and enhances anabolism of *P. hepiali*

mycelia. Artificial mycelial production is one of gaining industry but their researches have mainly been focused on nutrient media composition and typical physical environmental condition [20]. This study has filled that gap and improved the efficiency of mycelial production and it can be implemented for commercial process.

4. Conclusion

The *Paecilomyces hepiali* performs with low pressure environment at high altitude. This study showed some growth influences, which have been done by pressure. Specially, −150 mmHg treated samples have acquired highest growth in liquid media. Meanwhile, −100 mmHg treated samples showed significantly highest mycelial growth of semi solid and solid media. The water content of growing media has been adversely affected by low pressure. Consequently, there is still no practical application of pressure treatment as a growth enhancer, but it is still an interesting area for further exploration.

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