CULTURING AND GROWTH REQUIREMENT OF *ASPERGILLUS NIGER*

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ABSTRACT

Aspergillus niger, a common soil borne fungus is industrially important and utilized in the field of biotechnology and food microbiology. It is enormously cultured in large scale due to its positive significance in production of biofuels, chemicals and enzymes such as alpha-amylase, lipases, and proteases in the production of citric acid. *Aspergillus niger* is easily isolated from common thing such as dust, paint, grains and soil. Commonly in laboratories, *Aspergillus niger* is isolated via chemostat cultures which can test positively or negatively for the fungi. This review paper describes methods used for cultivating *Aspergillus niger* for enzymes and biofuel production. It addresses the general considerations pertaining the growth requirements mechanisms i.e. the morphological characteristics, environmental conditions, mass transfer, oxygen transfer and media components, along with important physiological parameters that lead to maximum sporulation in fungi. Though Potato Dextrose Agar (PDA) is the most commonly used media, but there is need for more economical way of cultivation. In contrast, a variety of optimized methods can offer substantial cultivation, but few feature the likelihood for levels of productivity that offset their high cost. One of the greatest challenges is to modify media in order to benefit from economy of scale and produce meaningful quantities of enzymes and biofuels. This paper also highlights some of the growth morphology, mass transfer, oxygen transfer, vegetative growth and asexual development.

Keywords: *Aspergillus niger*, Growth, Morphology, Media, Mass transfer, oxygen transfer.

1. INTRODUCTION

Aspergillus is a genus consisting of several hundred-mold species found in various climates worldwide. The Italian priest and biologist Pier Antonio Micheli first catalogued *Aspergillus* in 1729. Viewing the fungi under a microscope, Micheli was reminded of the shape of an aspergillum (holy water sprinkler), from Latin spargere (to sprinkle), and named the genus accordingly (Bartniki, 1968). *Aspergillus niger* was described in 1867 in a manuscript entitled "*Physiologie des mucédinées*" by the French botanist Philippe Edouard Leon Van Tieghem. (Jan and Han, 2013).

Aspergillus niger is a haploid filamentous fungi and is a very essential microorganism in the field of biology. In addition to producing extracellular enzymes and citric acid, *Aspergillus niger* is used for waste management and biotransformation. The fungi most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Schuster et al., 2002).

Aspergillus niger is relatively harmless compared to other filamentous fungi. Despite this, there have been some medical cases that have been accounted for, such as lung infections or ear infections in patients that have a weakened immune system, or an immune system that has been impaired by a disease or medical treatment. In the case of ear infections, *Aspergillus niger* invades the outer ear canal which can cause damage to the skin it came in contact with (May and Adams, 1997). The production of *ochratoxin A* from *Aspergillus niger*, is liable to cause immunutoxicity in animals. The effects on animal include a decrease in antibody

responses, a size reduction in immune organs, and an alteration in the production of cytokine that are proteins and peptides specifically used in signaling (Schuster et al., 2002). Food that has been contaminated by *Aspergillus niger's* toxic metabolite has a major effect on the poultry industry. Different animals such as chicken, turkey and ducks, are very prone to ochratoxin (May and Adams, 1997). *Aspergillus niger* has been a very important microbe used in the field of biotechnology. Many of the enzymes produced by *Aspergillus niger*, such as citric acid, amylases, lipases, cellulases, xylanases and proteases, are considered GRAS (generally recognized as safe) by the United States Food and Drug Administration and is excepted from the Federal Food, Drug and Cosmetic Act food additive tolerance requirements. Even though it is considered GRAS, *Aspergillus niger* still must be treated safely and with care (Van de Vondervoort et al., 2004).

Aspergillus niger produce colonies that are composed of white or yellow felt that is covered by dark asexually produce fungal spores. Mycelial, or thread-like, hyphae are divided by a septum and transparent. Conidiophores (asexually produced fungal spores) of *Aspergillus niger* usually ranges from 900-1600 µm in length and contain globose (globular) vesicles ranging from 40-60 µm in diameter. Each globose vesicle is completely covered with biseriate phialides, which are projections from the conidiophores of *Aspergillus niger*. These phialides come out from brown metulae, the site where a conidiogenous cell is created. The phialides go through a process of blastic basipetal conidiogenesis to create globose mitospores, with a diameter ranges from 3 to 5 µm (Debetes et al., 1990).

Aspergillus niger is a cosmopolitan fungus. It can be isolated from all continents and is not very selective with respect to the environmental conditions. It grows between 6 to 47^{o}C, pH 1.5 and 9.8 and a water activity of $\geq$ 0.77 (Pitt and Hocking, 2009). *Aspergillus niger* thrives in the soil and on decaying plant material but is also abundant in man-made environments. For instance, it can be found and in carpet and mattress dust (Flannigan et al., 2011). Pitt and Hocking (2009) state that *A. niger* is by far the most common *Aspergillus* species responsible for postharvest decay of for instance guava's, litchis, mangoes papaya's, pineapples, pomegranates, apples, pears and grapes. Other food products such as onions, rice, coffee, nuts and sunflower seeds are also substrates for *Aspergillus niger*.

2. MORPHOLOGY AND MASS TRANSFER

In submerged cultivations, depending on the conditions, the morphology of filamentous fungi lies between a compact form, often spherical, named pellet and a free dispersed form named filamentous (Figure 2). In between these two extreme morphologies, filamentous fungi can also grow as loose aggregates named clumps. The parameters determining the type of growth are: the inoculum level (Papagianni and Mattey, 2006), the pH (Grimm et al., 2005), the concentration of trace elements (Majolli and Aguirre, 1999; Pera and Callieri, 1997), the agitation (Gomez et al., 1988; Grimm et al., 2005; Papagianni et al., 1998) and the aeration (Cui et al., 1998; Grimm et al., 2005). Moreover, the hyphal morphology itself can vary from linear filament to highly branched structure. The branching intensity decreases in response to low concentration of oxygen (Wongwicharn et al., 1999), at low agitation (Amanullah et al., 2002) and at low growth rate (Wiebe and Trinci, 1991).

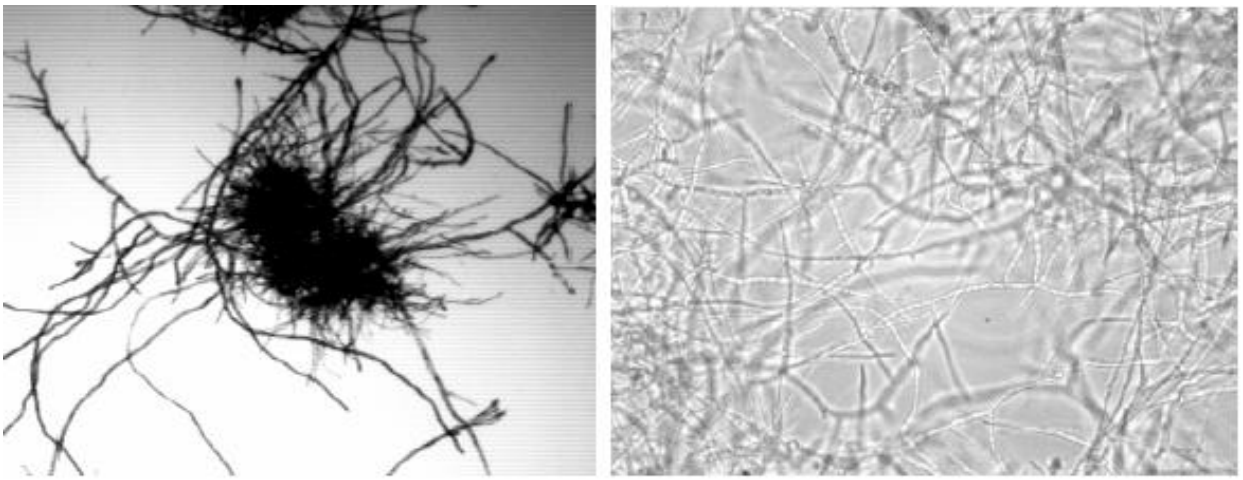

Figure-2: The two extreme shapes of *Aspergillus spp.*: pellet (left) and filamentous (right) (source: Audrey, 2007)

The pellet form is use mainly in the production of citric acid (Bodie et al., 1994). This shape is initiated by an agglomeration of spores or hyphae (Dynesen and Nielsen, 2003). One of the disadvantages of this growth is the difficulty to control the size of the pellet. As the pellet size increases, the diffusion of substrate within the pellet decreases (Kobayashi et al., 1973), this reduces the productivity of many processes (Abarca et al., 2004). The filament form is use often for the production of homologous and heterologous proteins or secondary metabolites (Gyamerah et al., 2002; Paul et al., 1994; Shiba et al., 2001). However, the growth as filament and the entanglement of hyphae result in a formation of network (Figure 2). As the biomass concentration increases, the mycelial branched network increases the viscosity of the medium, leading to a non-Newtonian fluid behavior, having relatively low viscosity in region of high shear and high viscosity as the shear decreases. In stirred tank, the most important type of bioreactor used in bioindustry, such fluid behavior leads to a non-homogeneous broth, well mixed near the impellers, where the shear is high, but with limiting mixing elsewhere (Reuss et al., 1982; Li et al., 2002). At high biomass concentration, the presence of slow moving or stagnant broth areas is observed near the fermentor wall (Wernau, 1985). In these conditions, the entrance of titrant controlling the pH and the entrance of the pH probe must be carefully position, in order to avoid large pH deviations. The extracellular pH has indeed a lot of importance in *Aspergillus* fermentation. First, it influences the production of acids: while citric acid can be produce at low pH, production of oxalic and gluconic acids are optimal at pH 5-6 (Ruijter et al., 2002). Secondly, pH has been reported to have an influence on the morphology of filamentous fungi: Pirt and Callow (1959) and Miles and Trinci (1983) showed a relation between the extracellular pH and the branching intensity as well as the hyphal diameter of *Penicillium chrysogenum*, which is another filamentous fungi from the same subfamily as *Aspergillus spp.* However, Van Suijdam and Metz (1981) reject this correlation. This may be due to the difference in the mode of cultivation: chemostat for Pirt and Callow and Miles and Trinci, and batch cultivations for van Suijdam and Metz (1981).

Transport processes usually affect the productivity of bioprocesses and the efficiency of bioreactors. Thus, mass transfer phenomena belong to common problems,

which a bioreactor engineer encounters during fermentation practice and bioreactor design. Gas–liquid (G–L) transfer is often a limiting factor of many aerobic fermentations. This is caused mainly by a low solubility of oxygen in fermentation media in comparison with the solubility of carbon sources and other nutrients (Jaroslav et al., 2002).

In recent years, airlift bioreactors (ALRs) are been extensively investigated as a possible alternative to stirred tank bioreactors, which are most widely used for fermentation purposes. The ALRs possess features, which make them more advantageous for various biotechnological applications. A special property of ALR is a liquid circulation loop created by the interconnected aerating (riser) and recirculating sections (downcomer). Despite an absenting mechanical agitator, this well-defined flow pattern with high liquid velocities in line with efficient mixing and low uniformly distributed shear stresses can create an optimal environment for many productive microorganisms (Jaroslav et al., 2002).

Mathematical model for mass transfer
Gas phase is assumed to be perfectly mixed.
For liquid phase;

$$K_{La} = \frac{r\ (mo/mi)}{(co^* - co)} \qquad \text{.....(1)}$$

Where
K_{La} = overall volumetric mass transfer coefficient (h^{-1})
r = production rate ($gdm^{-3}h^{-1}$)
Mo = molar weight of the atom of oxygen ($gmol^{-1}$)
Mi = molar weight of intending product ($gmol^{-1}$)
Co* = saturation oxygen concentration ($mgdm^{-3}$)
Co= actual dissolved oxygen concentration ($mgdm^{-3}$)

3. OXYGEN TRANSFER

Here also the position of the feed port has to be considered carefully in order to limit the gradient of substrate. Mixing problems affect as well the transfer of gas, especially oxygen, which is problematic for aerobic fermentation processes. The maximal dissolved oxygen concentration in a liquid is limited to 0.26 mmol O_2/L at 25°C. This maximal concentration is affected by the existence of stagnant films causing resistance to the transfer at the gas/liquid interface and at the liquid/cell interface (Figure 3). The most important resistance in Newtonian media is generally considered to be the liquid film resistance around the bubble (Kobayashi et al., 1973). However, in non-Newtonian cultures such as filamentous fungi broths, the main resistance shown to be downstream of the air/liquid interface (Li et al., 2002). The thickness of these films depends on the degree of turbulence and on the physical properties of the medium: it increases with viscosity (Barberel and Walker, 2000), causing a decrease of oxygen transfer. Moreover, the mass transfer is proportional to the **kla** coefficient, where "kl" is the mass transfer coefficient and "a" is the gas/liquid interfacial area per unit liquid volume. Thus, the mass transfer from the gas phase to the liquid phase also decreased by the formation of large coalescent air bubbles, which are formed due to bad mixing and high viscosity and that reduce the exchange surface (Figure 3). The repartition of air bubbles is affected as well by the bad mixing, which means that oxygen transfer is mainly taking place near the impeller region, leading to a heterogeneity of dissolved oxygen levels in the fermenter (Manfredini et al., 1983). Furthermore, the high viscosity and the low mixing also limit the transfer of oxygen within the liquid phase.
Different parameters can be adjusted for better mixing and transfer such as the number, shape and size of the impellers

(reviewed by Nordkvist, 2005), as well as the position of impellers and the impellers speed (Loucaides and McManamey, 1973). However, increasing the agitation is costly for industrial fermentation and does not always lead to an increase in productivity. Indeed high agitation level affects the morphology by fragmenting the hyphae and may lead to a decrease of productivity (Makagiansar et al., 1993; Smith and Lilly, 1990). Moreover, in 2002, Li et al. pointed out that in non-Newtonian broth, better mixing does not always lead to better oxygen transfer and that oxygen transfer depends not only on the agitation power but also on how this power is apply to the culture. Another solution to improve mass transfer is the use of pulsed feeding. Most fungal industrial bioprocesses are carried out using fed batch cultivations and studies have shown that the advantages of pulsed feeding leading to smaller fungal elements, to a lower medium viscosity and thereby to a better oxygen transfer (Bhargava et al., 2003).

One of the most areas in designing and scaling-up of fermentation involves oxygen transfer from a gas phase to the liquid phase. Yet little information is available on the problem in the industrially important cases involving non-Newtonian broths. Most of the fungal enzymes broths show pseudoplastic behavior and the mass transferring rates may be critical in determining vessel productivities (Sung-Hoon et al., 1993).

4. MIXING TIME

Norwood and Metzner proposed that the Newtonian mixing time versus Reynold's number plot might be a suitable for estimating mixing times for pseudoplastic fluids. That is,

$$\Theta_M = \alpha_m \, . \, N_{Re}^{\beta m} \quad \text{.....(2)}$$

Problems coupled with oxygen (O_2) supply especially occur in viscous fermentation broths and in cultures, which contain a large amount of cells with high oxygen consumption. These problems can be solved by different ways. For instance, higher airflow rates is applied; nevertheless, this usually does not help in a bioreactor with a viscous non-Newtonian broth (Godo et al., 1999). A more intensive oxygen transfer from the gas to the liquid phase can be achieved by increase of the partial pressure of oxygen in the inlet gas stream, e.g., using pure oxygen. However, this solution is mostly financially unfavorable in large scales. One of further possibilities is adjusting of the existing bioreactor or use of a more suitable type of bioreactor (Jaroslav et al., 2002).

Due to high oxygen demand of the fungi *A. niger*, the oxygen transfer from the gas phase into the liquid phase was found to be a limiting step of the bioconversion. For this reason, a great attention has to be paid for the optimization of the bioreactor operation (mixing, aeration and bioreactor design) in order to enhance the oxygen transfer rate (Jaroslav et al., 2002).

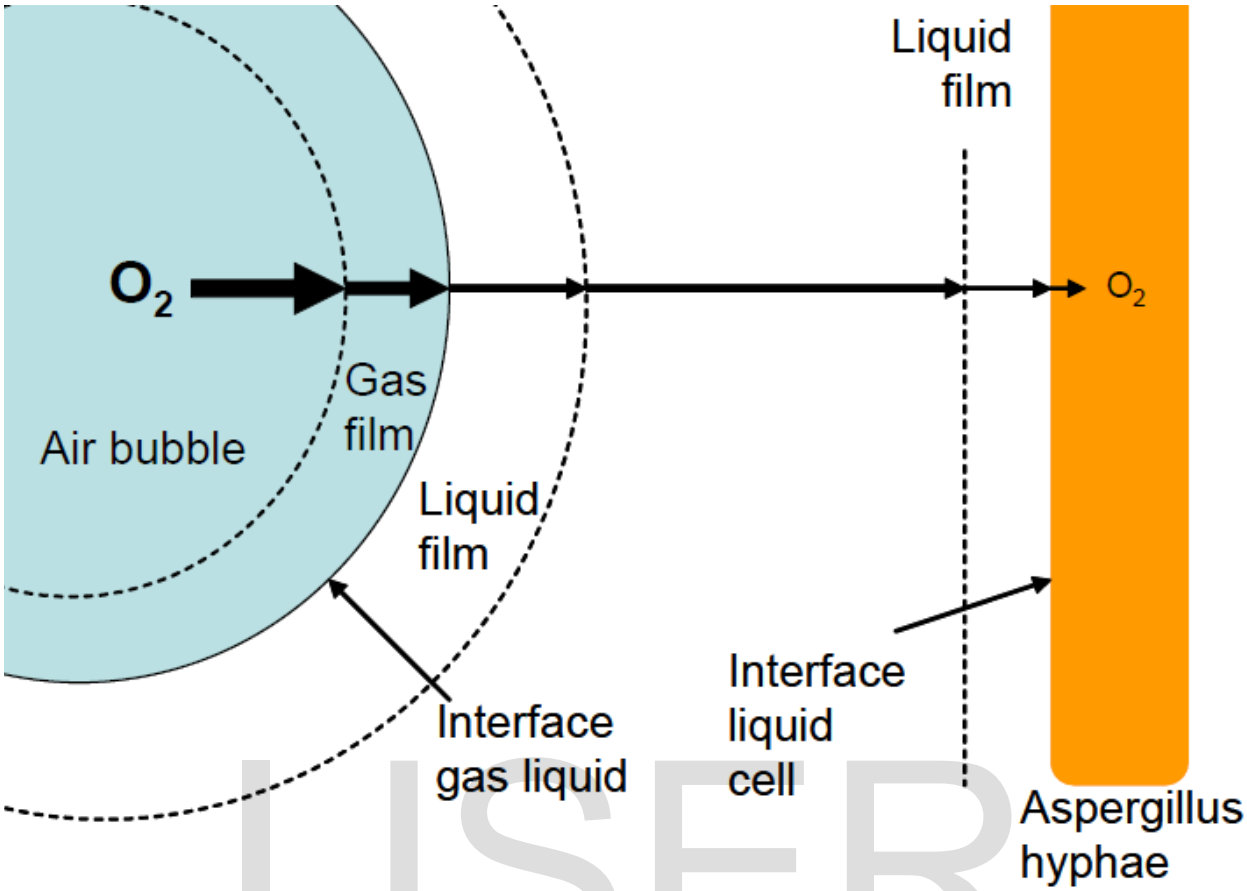

Figure-3: Oxygen transfer from the air phase to *Aspergillus* cell. The thickness of the arrow indicates the concentration of the oxygen and shows the resistance encountered during the transfer. (Source: Audrey, 2007)

Furthermore, mixing problems also lead to heat transfer problems. Heat transfer usually achieved by intern loops or by an external jacket. The existence of slow moving or stagnant areas near the fermentor wall is a problem for temperature control, especially when the cooling system is located nearby the reactor wall. Bad mixing could affects the repartition of products and nutrients, in particular during continuous cultures and fed batch cultivations.

Another way to improve mixing and transfer is the use of a rotary jet instead of impellers to mix the reactor (Nordkvist, 2005). In such a system, the broth is drawn from the bottom of the fermentor, circulated by a pump and reinjected in the fermentor by a rotary jet located in the fermentation medium. Liquid feed, titrants and gas can be added in the loop, thus limiting the formation of gradients. Heat exchange can also be performed in the loop via a heat exchanger. If this system presents many advantages (Nordkvist, 2005), it may, however, be difficult to apply to filamentous fungi fermentation as the shear stress in the loop and at the exit of the rotary jet may damage the morphology of the fungi and thereby may affect the productivity.

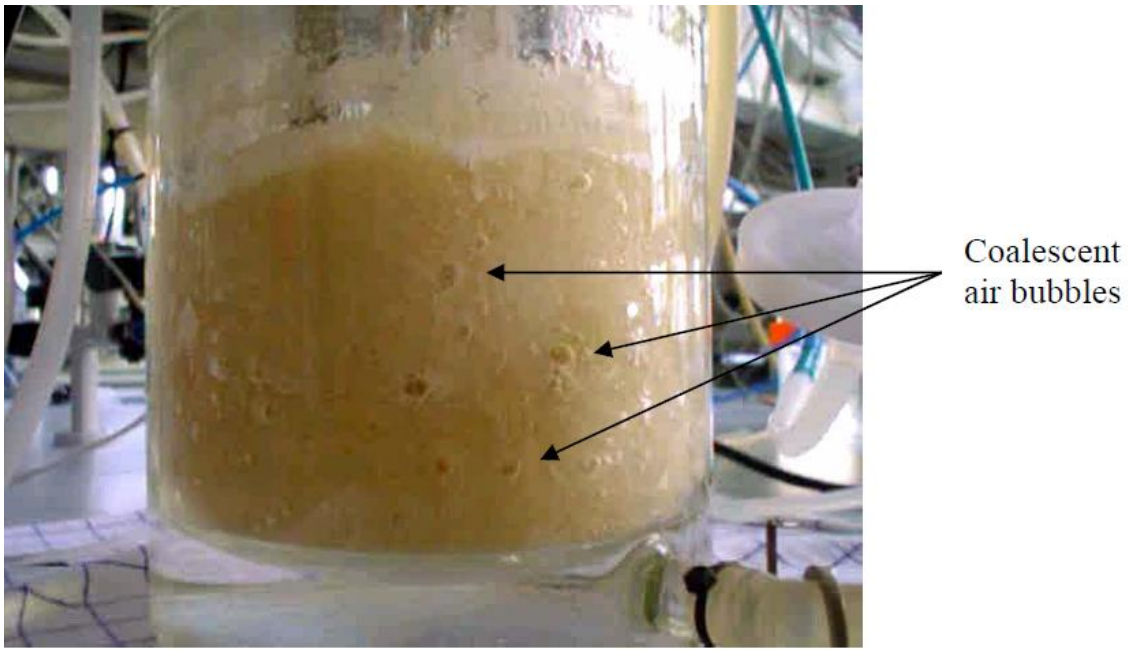

Figure-4: Picture of a highly viscous *Aspergillus niger* broth.

Source: Audrey, 2007

5. VEGETATIVE GROWTH

In nature, Aspergilli grow within and on solid substrates. A colony can result from a single sexual or asexual spore but it may also arise after conidia and/or germlings that are in close vicinity to each other have fused. It has been described that fusion in *A. oryzae*, *A. sojae* and *A. tamarii* most often occurs within conidia (>80%), while fusions between conidia and germlings and fusion of germlings are much less frequent (Ishitani & Sakaguchi 1956). Colonies can reach a diameter in the (sub-) millimeter (microcolonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, microcolonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, *Aspergilli* are routinely grown on agar media or in liquid media. On agar medium, *Aspergilli* form radial symmetrical macro-colonies. The mycelium of *A. nidulan*s (Lee & Adams 1994) and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively.

6. ASEXUAL DEVELOPMENT

After a period of vegetative growth, air-exposed colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae. One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2–3 μm. The second type of aerial hyphae has a diameter of about 4–5 and 6–7 μm in the case of *A. nidulans* and *A. niger*, respectively.

7. EFFECTS OF SOME ECOLOGICAL FACTORS ON THE RATE OF GROWTH OF ASPERGILLUS NIGER

Environmental factors such as humidity and temperature plays an important role in dispersing fungi spores in air for short and long distances and when spores deposited a solid or liquid surface and if conditions of

moisture and food are appropriate, they germinate (Bennett, 2010; Goncalves *et al*., 2010). Normal indoor conditions such as humidity and temperature provide a suitable environment for the growth of a wide range of fungal spores (Ababutain, 2011; Li and Kendrick, 1995). Few researchers on airborne fungi reported the observation of an increase in fungi spore concentration at air temperatures between 15 and 25°C and relative humidity at 60-70% (Segvic and Pepeljnjak, 2006). Relative humidity and temperature extremities may result in decreased airborne fungi spore concentration (Levetin and Horowitz, 1978).

Effect of Temperature

Ibtisam (2013) showed that the temperature of 30ºC favored colony diameter growth for aspergillus *niger* and so considered the optimum growth temperature. The temperature ranges for *A. niger* was wide from 10-40°C. At the temperature of 10°C, *A. niger* is unable to form spores and only the mycelium growth has appeared.

Table-1: Effect of incubation temperature on the linear growth (cm) ± SD of *A. niger* (Ibtisam, 2013).

Temperature	*A. niger*
5	0.000000
10	1.20±0.17
15	2.10±0.10
20	3.17±0.49
25	5.33±0.30
30	8.36±0.15
35	7.80±0.53
40	5.03±0.15
45	0.0000000

Effect of Relative Humidity

The growth of *A. niger* on different relative humidity is shown in the table below:

Table-2: Effect of Relative Humidity on the linear growth (cm) ± SD of *A. niger*. (Ibtisam, 2013).

Relative humidity	A. *nigar*
100	7.83±0.06
95	7.90±0.10
90	8.20±0.20
75	8.50±0.00
65	7.73±0.25
50	7.33±0.15
35	7.23±0.25

8. EFFECTS OF MEDIA TYPES ON THE RATE OF GROWTH OF ASPERGILLUS NIGER

Meera et al., (2012) carried out an experiment to determine the rate of growth of *A. niger* on four different mediums:

- Potato Dextrose Agar, PDA
- Optimised PDA Containing Sucrose + Peptone
- Czapek's Dox + Yeast Extract Agar (CYA)
- Lignocellulose Agar (LCA)

They observed that the hyphae of *Aspergillus niger* were septate and hyaline. Conidial heads were radiate initially, splitting into columns at maturity. The species was biseriate (vesicles produced sterile cells known as metulae that support the conidiogenous phialides). Conidiophores were long, smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle. Metulae and phialides covered the entire vesicle. Conidia were brown to black, very rough and globose.

Rate of Growth of Aspergillus Niger in Simple PDA Medium

Amongst fungal polyculture, spiral patterns of growth were sometimes encountered in the colonies of *A. niger*. Such patterns arise from various causes. For example, an endogenous rhythm of sporulation in *Aspergillus niger* produced a colony which forms an Archimedes spiral. It looked like a black powdered dusting left on PDA. The fungus started to develop in small circular patterns on the media and appeared as black spotty dots. On PDA, the radially expanding colonial growth form of the fungal mycelium was most evident, extending from an inoculum, on, within and sometimes above the substrate, forming a near spherical three-dimensional colony.

Rate of Growth in Modified /Optimised PDA Containing Sucrose+Peptone

At high nutrient level PDA+dye+sucrose+peptone), the *Aspergillus niger* colonies formed thick layers due to the high nutrient influx. It appeared roughening in the colony interface at relatively high nutrient levels. At high nutrient level, the hyphae produced densely inside the colony. With increasing nutrient content, colony shapes became similar to the compact morphology. Increasing nutrient content increased colony and mycelial density. At low nutrient level, the colonies of *Aspergillus niger* were seen in the form of thin mycelial layers. Hyphae created homogeneously inside the colonized area. Dense *Aspergillus* colonies were observed up to 10th day of inoculation. As the nutrient content in PDA depleted up to 10th day, sporulation triggered. Hence on the 10th day fungal colonies exhibited maximal spores.

During development, *A. niger* extended its hyphae into the medium. The various forms and patterns of fungal growth were referred to as a phenomenon of biological self-organization. Each colony was seen as a uniform multicellular structure developing radially by growth and branching of mycelium. The colony was able to consume substrate (carbon source, i.e. sucrose) and to produce diffusible metabolites that could suppress other microbial development viz. bacteria. Hence fungal colony initiation led to suppression of bacterial growth due to production of mycotoxins (aflatoxins) in the PDA. The final stage of fungal morphogenesis was observed as the formation of spores. Different growth patterns were seen arising in colonies of *A. niger* while they were cultivated on PDA+sucrose+peptone.

Rate of Growth in Czapek's Dox + Yeast Extract Agar (CYA)

Observation shows that poor or moderate fungal sporulation occurred in CYA.

Okunowo *et al.*, (2010) also observed least sporulation and minimum mycelia growth of a fungus on Czapek's Dox agar, which may be due to the presence of chloride ion in the test medium. Thus, Czapek's dox agar suppresses the fungal sporulation due to presence of chloride ions.

Rate of Growth in Lignocellulose Agar (LCA)

In the present study, *Aspergillus niger* showed heavy fruiting bodies formation in LCA. Osono and Takeda, (1999) stated that LCA because of its low glucose content suppresses the overgrowth of fast growing species. Thus, the fast growing *Aspergillus niger* in nature is suppressed by LCA medium in comparison to PDA. However, the sporulation enhanced as compared to CYA. Hence, this medium is useful for fungal identification.

Table-3: Growth rate of *Aspergillus niger* as colony forming units (CFUs/ml) in various types of nutrient media (Meera et al., 2012).

S. No.	Media type	Colony characteristics	Growth rate as CFUs/ml
1.	Simple PDA	velvety texture, white mycelium with typical black spores, Yellow reverse of petriplate, zonation is Heavily furrowed on the reverse, Heavy sporulation	$10x10^3$
2.	PDA+Sucrose+Peptone	Texture dense velvety, mycelium entirely covered by black spores, yellow to orange reverse of petriplate, extremely furrowed zonation on the reverse, sporulation triggered	$22x10^3$
3.	Czapek's Dox + Yeast Extract Agar (CYA)	Powdery texture, mycelium White with black spores , reverse of petriplate Yellow, zonation as single concentric ring at periphery and radial furrow at the centre, sporulation moderate	$7x10^3$
4.	Lignocellulose Agar (LCA)	Powdery texture, Hyaline mycelium with black spores , reverse of petriplete was Colourless, zonation was light and concentric, Heavy sporulation	$8x10^3$

CONCLUSION

The morphological characteristics, environmental conditions, mass transfer, oxygen and media components are important criteria for fungal culture and study, along with important physiological parameters that lead to maximum sporulation in fungi. In the present investigation, type of culture media and their chemical compositions significantly affect the mycelial growth rate and conidial production in *Aspergillus niger*. Thus, the findings in this work revealed that different types of culture media differentially influenced the growth, colony character and sporulation of the test fungi (Aspergillus niger). Out of three test media employed in the Meera's study, optimized PDA proved to be most suitable for heavy sporulation, with a colony density of $22x10^3$ CFU/ml within the study hours, as compared to $7x10^3$, 8x10 and $10x10^3$ for CYA, LCA and PDA respectively. Therefore, in conclusion the optimized media is the best for growing

Aspergillus niger, or any fungi with similar morphological characteristics.
Manufacturers of biofuels and enzymes such as alpha amylase, glucoamylase, glucose Oxidase etc. should explore more standard methods of culturing *Aspergillus niger*.

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