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Thermomyces lanuginosus: A True Representative of Thermophilic, Fungal World!

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ABSTRACT

***Miehe* was first to isolate *Thermomyces lanuginosus* with four other species of thermophillic fungi. Tsiklinskaya, in 1899 found *Thermomyces lanuginosus* (*Humicola lanuginosa*) a chance contaminant on potato inoculated with garden soil. This fungus is explicitly associated with organic substrates such as compost resources, paddy straw , wheat straw, manures of birds and mammals droppings, dried and dead materials of plant like litter fall as leaves, twigs, stems and root, and municipal refuse where the process of decomposition by the mesophilic paves way for its colonization. *Humicola lanuginosus (Thermomyces lanuginosus)* grows from 30 to 52-55°C. The fungus possesses both intra- and exocellular thermozymes like xylanase, protease, lipase, amaylase, etc. However, for cellulose degradation it seems to live as a commensal with cellulose-decomposing species, like *Chaetomium thermophile*. Phylogenetically *Thermomyces lanuginosus* is classified as a mitosporic fungus (imperfect fungus), that reproduces asexually by forming aleurioconidia. It is also reported as a member of the order *Eurotiales* in a sister relationship with *Talaromyces thermophilus*. *Thermomyces lanuginosus* is a candidate organism for future of bioenergy based technologies by virtue of its colonization capacity, ability to deconstruct wood and wood based products, and release of cellulase-free xylanase.**

Keywords: Compost, mycoflora, thermal adaptation, enzyme, biofuel alternative

HISTORICAL PERSPECTIVES

From the medieval time agriculture is one of the most important enterprises in the world as it helps to meet the basic needs of human civilization by providing food, clothing, shelter, medicine and recreation. Soil as prime need acts as a store house of water and nutrients for plant growth but all fertile soils need not be productive due to lack of essential nutrients like nitrogen, potassium, cobalt, copper, organic matter, moisture and microorganism, etc. To maintain the fertility and health of soil without deterioration soil conservation practices of various kinds are followed. One such step is composting, the process of decomposition of organic wastes. For the nutrient cycling to occur at the optimum level for plant growth we need to ensure that the soil is balanced in three ways: chemically-nutrients, biologically-microbiological, physically- structure; the components of an organic nutritional system are made up of compost as the foundation of the biological system, over cropping-green manure, foliar sprays and natural, fertilizer as organic (Sharma and Johri, 1992). The heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein warm, humid, and aerobic environment provides the basic conditions for the development of thermophillic microflora (Maheswari *et al.*, 1987). During compost exothermic reactions take place through self-heating of composed flora and fauna in a manner that follow the following schedule. At the beginning of the process the mesophillic saprophytes are dominant and later on increase in temperature ~ 60°C, thermophillic spores are favoured to occupy the substratum (Johri and Satyanarayana, 1984). This unique thermal adaptation as a puzzle of stored agricultural products compelled Miehe (1907, 1930) to study the microflora present therein. He was the first to present extensive work regarding thermophilic microorganisms and isolated four species of thermophilic fungi: *Mucor pusillus*, *Thermomyces lanuginosus*, *Thermoidium sulfureum*, and *Thermoascus aurantiacus*. Discovery of the ubiquitous fungus by Tsiklinskaya in 1899 as *Thermomyces lanuginosus*, or *Humicola lanuginosa* was a chance contaminant on potato inoculated with garden soil. Noack in 1920 isolated thermophillic fungi and was intrigued by the fact that in addition to self-heating masses of hay and compost and heaps of leaves, the thermophillic fungi were present in places where temperatures conducive to their growth occur red only infrequently; this then provided the foundation for pioneering discovery of their physiology and associated importance.

DISTRIBUTION

In an ecosystem temperature is a key component which is responsible for frequent distribution and metabolism of any organism. *Thermomyces lanuginosus* is a thermophillic fungus which normally grows at maximum 60°C and minimum of 20°C but the optimum temperature for growth is 50°C (Wang *et al.*, 2012). *T. lanuginosus* is explicitly associated with organic substrates such as compost resources, paddy straw , wheat straw, manures of birds and mammals droppings, dried and dead materials of plant like litter fall as leaves, twigs, stems and root, and municipal refuse where the process of decomposition by the mesophillic as pioneer community leads to a raised temperature of up to 45°C (Chang and Hudson,1967) and paves way for the growth of resting propagules of thermophillic to active mycelia as it reaches the climax community of the system (Johri *et al.*, 1999; Subrahmanyam, 1999). *T. lanuginosus* has also been studied in other self-heating environments, including self-heating hay, coal soil tips, industrial wood chip piles, stored grains, aerial parts of crops, and freshly harvested grains (Rawat and Johri, 2013). But the occurrence of *T.lanuginosus* is also reported from different types of dry to drenched soil conditions, like from aquatic sediments, water logged mangrove soil, cultivated clay soil to and loamy garden soil to desert soil and rocks (Singh *et al.*, 2003). Isolations have also been reported from air in Indonesia and British Isles and on skin of human patients (Abbas, 2009). Recently *T.*

lanuginosus was reported as a cause of infective endocarditis (Shivagnanam *et al*., 2013). Due to the dissemination of propagules from self-heating masses of organic material thermophillic fungi are found dominant in soils of temperate compared to the tropical countries. The ups and down in moisture content and pH with the decomposition of organic substrates of different material resources in compost plays a significant role for microbial structural diversity and activity. Thermophilic fungi are much more common in acid thermal habitats than the neutral to alkaline pH (Rawat and Johri, 2013). The level of inoculum concentration of paddy straw was found to affect colonization; increased colonizing ability of *T. lanuginosus* was associated with higher inoculums dose. Souza *et al*. (2014) reported the presence of the fungus during peak-heat of phase II in mushroom composting although Zhang *et al*. (2014) hypothesized that *T. lanuginosus* was dominant and abundant during thermophillic phase under higher temperature in lignocellulosic compost. Although CO_2 is not regarded as a nutritional requirement for fungi, but it shows significant role in the growth of *T. lanuginosus* which is severely affected in the absence of CO_2 (Noack, 1920). The concentration of CO_2 inside composts can be as high as 10 to 15 ppm therefore, it is likely that its assimilation plays a nutritional and morphogenetic role in the development of the fungus. *T. lanuginosus* is ubiquitous and has been reported from different substrates by researchers from various geographical locations in USA, UK, Nigeria, Ghana, India, Japan, Australia and Indonesia (Tonouchi, 2009; Hudson, 1992). The available worldwide distribution across the habitat is presented in **table 1**.

Table 1. Geographical Distribution of *Thermomyces lanuginosus* across various habitats.

Habitat	Author
Compost soil	Chadha *et al*., 2004
Cultivated soil	Rajasekaran and Maheshwari, 1993 ; Chadha *et al*., 2004
Paddy straw compost	Satyanarayana and Johri, 1984
Alluvial soil	Johri and Thakre, 1975
Coal spoil tips	Apinis 1963a; Evans 1971; Johri and Thakre, 1975
Hay	Miehe, 1907
Wheat straw compost	Zhang *et al*, 2014
Garden soil	Tsiklinskaya, 1899
Stored grains	Clerk *et al*., 1969
Mushroom compost	Salar and Aneja, 2007; Zhang. M. *et al*., 2014
Coastel Grass land	Apinis, 1963b
Hot springs	Mehta and Satyanarayan, 2013
Vermicompost	Anastasi *et al*., 2004; 2005
Rice field soil	Tonouchi, 2009
Near neutral and alkaline thermal springs	Pan *et al*., 2010
Maize straw compost	Zhang *et al*., 2015
Human skin	Abbas, 2009
--	Satyanarayana *et al*., 1977; Tansey, 1973
Surgical infection	Sivagnanam *et al*., 2013

FUNCTIONAL CHARACTERISTICS

Enzymes are the soul of microorganisms or any living organisms which make them alive and help to complete their nutrition and life cycle. Enzymes of thermophillic fungi have been studied primarily to explore their suitability in bioprocesses as tool for white biotechnology (Littlechild *et al.*, 2013). Thermozymes are able to function at higher temperatures but often show increased stability to solvents, pH and proteolytic degradation. Daniel *et al*. (2008) reported dependency of enzyme stability on maintenance of a functional structure, and the stability of any protein is marginal and equivalent to a small number of molecular interactions. The only difference in thermostability with the mesophillic protein is that the free energy of stabilization is higher in it (Morgan *et al.*, 1972). The thermal stability of all enzymes can be measured in two different ways, either as a function of temperature at which the protein folding is determined and, another as a loss of function. *Humicola lanuginosus (Thermomyces lanuginosus)* grows from 30 to 52-55°C. It is extremely common in all types of self-heating materials including birds' nests and sun-heated soils. It colonizes composts after peak-heating and persists throughout the high-temperature phase. Trent *et al.* (1994) demonstrated survival of conidia and synthesis of heat shock proteins (HSPs) in *T. lanuginosus*, germinated at 50°C and heat shocked at 55°C for 60 min prior to exposure to 58°C.

The fungus *T. lanuginosus* possesses both intra- and exocellular types of thermozymes. However, it cannot degrade cellulose and it seems to live as a commensal with cellulose-decomposing species, sharing some of the sugars released from the plant cell walls by their cellulolytic activities for e.g., the fungus shows profuse growth in mixed cultures with a cellulolytic fungus, *Chaetomium thermophile* (Hedger and Hudson, 1974). In *Thermomyces lanuginosus*, a single transporter was identified for glucose, xylose and mannose, the hydrolytic products of cellulose and hemicellulose. Moreover, it may readily utilize xylan, which is external to cellulose in the plant cell wall and is apparently a more accessible carbon source (Prabhu and Maheshwari, 1999). A great deal has been published on xylanases of *T. lanuginosus* due to its application in biobleaching of pulp in the paper industry and to minimize the need for chlorine for pulp bleaching in the brightening process. The majority of xylan-degrading enzymes from thermophilic fungi are endoxylanases. Xylanases of some strains of *T. lanuginosus* and *T. aurantiacus* are optimally active at 70 to 80°C, contains disulphide bond which attributes as resistant to temperature and also show remarkable resistance to denaturation up to 8 M urea (Tatu *et al*., 1990). Generally proteins have a long shelf-life in the dry state. Though, the lyophilized xylanase of *Humicola lanuginosa* was inactivated after 2 months at 20°C, the purified enzyme in solution did not lose activity. The xylanase of *Thermomyces lanuginosus,* is a polypeptide of 225 amino acids with high homology to other xylanases (Schlacher *et al*., 1996; Gruber *et al*., 1998). Xylose, the pentosan unit of xylan and paper of inferior quality has been found an excellent carbon source and inducer for xylanase in *Humicola lanuginosa* (Anand *et al*., 1990), and some other fungi (Krishnamurthi, 1989; Maheshwari and Kamalam, 1985). Interestingly, the best xylanase-producing strains of *Thermomyces lanuginosus* secreted small amounts of xylan-debranching enzymes in crystallized form as glycoprotein of 21 to 78 kDa mol.wt with which polyclonal antibodies showed antigenic cross- reactivity and did not produce β-mannan- and arabinan-degrading enzymes (Naren, 1992). Like xylanase, thermostable chitinases maintain their structure and exhibit very high specificity and efficiency even at extreme conditions and hence it may have significant industrial value. Chitinase from *T. lanuginosus* predicted 3D model of chitinase I and II showed the characteristic (α/β)8 TIM-barrel conformation (Khan *et al.,* 2015).

An induced production of thermostable alkaline proteases was observed only in two thermophillic fungi i.e., *Humicola lanuginosa* and *Malbranchea pulchella* based on casein agar hydrolysis by culture filtrates in the presence of 2-8% casein as an external substrate (Ong and Goucher, 1973; 1976; Stevenson and Goucher, 1975). Lipase was isolated by Arima *et al* (1972) using *Humicola lanuginosa* strain Y-38 from compost employing soybean oil, starch, corn steep liquor, and antifoaming agent as substrate in the medium. Lipases have wide applications in the food industry and are also used as biocatalyst in stereo selective transformations (Jaeger and Reetz, 1998). The lipase produced by *H. lanuginosa* is a glycosylated hydrolase that consists of 269 amino acids (Kumar *et al.*, 2015). This has been used in detergent formulation along with other microbial enzymes such as protease, amylase, and cellulase. Lipase produced by *H. lanuginosa* is structurally similar to *Rhizomucor miehei* lipase. The efficiency and thermostability of lipase was found to differ with strains of *Humicola lanuginosus*. Optimum conditions for the production of lipase by this fungus is pH between 7 and 8, temperature 45°C and an incubation period of 30 h (Arima *et al.*, 1972).

Thermomyces lanuginosus also shows considerable glucoamylase and α- amylase activities simultaneously in the presence of starch. (Rao *et al.*, 1979, 1981; Taylor *et al.*, 1978). Thermostable starch degrading enzymes play an important role in the industrial production of glucose from starch; this has significance in food, feed, pharmaceutical and chemical industries. Detailed characterization of amylase of *Thermomyces lanuginosus* has been reported by several worker (Adams, 1994; Adams and Deploey, 1976; Barnett and Fergus, 1971; Bunni *et al.*, 1989; Fergus, 1969; Jaychandran and Ramabadran, 1970; Sadhukhan *et al.*, 1992). Diversity of amylase enzyme is common in fungi. Out of seven different tested strains of *T. lanuginosus* only one similar form of the enzyme was detected (Mishra and Maheshwari, 1996). Glucoamylase is an exo-acting enzyme which hydrolyzes alpha-1, 4-glycosidic linkages and, less frequently, alpha-1, 6- glycosidic linkages from the nonreducing end of starch, producing beta-D-glucose as the sole product. Glucoamylases of *T. lanuginosus* had dissimilar carbohydrate contents of different molecular masses but with similar thermostabilities (Mishra and Maheshwari, 1996). Moreover being a stronger thermophile, *Humicola lanuginosus* produces less stable cytosolic enzyme malate dehydrogenase (Wali *et al.*, 1979; Wali and Mattoo, 1984).

Thermomyces lanuginosus trehalase is found as a monomeric protein of 145 kDa responsible for degrading the non - reducing disaccharide trehalose which is accountable for the stability of cell membranes and acts against drying and thermal denaturation; variable levels of this enzyme have been reported in *Thermomyces lanuginosus* (Colaco *et al.*, 1992; Crowe *et al*. 1984; Bharadwaj and Maheshwari, 1999) Trehalase from this fungus had acidic pH optima, between 5.0 and 5.5., they were glycoproteins, with a carbohydrate content of 20% and were optimally active at 50°C. Thermophillic invertase, an atypical enzyme in its behaviour from *T. lanuginosus,* is an inducible thiol protein which depends on the maintenance of a catalytically important sulfhydryl group(s) in the reduced state (Shenolikar and Stevenson, 1982). In addition to this, an inducible, dimeric, glycoprotein beta-galactosidase of molecular mass 75 to 80 kDa, stable at 56ºC was recovered (Fischer *et al.*, 1995). Further, *Humicola lanuginosa* was examined for activity of the total tRNA synthetase preparation by aminoacylation activity which gets reduced to half at 50ºC. (Joshi and Cherayil, 1987).

PHYLOGENY

A number of phylogenomic and multigene studies have led to an improved understanding of fungal phylogeny (Fitzpatrick *et al*., 2006; James *et al*., 2006; Robbertse *et al*., 2006) that has resulted in the adoption of a vastly improved classification of the fungi (Hibbett *et al.*, 2007). However, these studies are not completely congruent and the classification of certain taxonomic groups remains problematic. The phylogenetic analyses are complemented by experimental growth, temperature relationships for fungal species reported to be thermophilic. Using the criterion that a thermophilic fungus is one that grows faster at 45°C than at 34ºC, phylogenetic analyses suggestes that the known thermophilic fungi belong to the orders *Sordariales*, *Eurotiales*, *Mucorales*, and *Onygenales*. Cooney and Emerson (1964) discussed comprehensive account of the taxonomy, biology and activities of thermophilic fungi. *Thermomyces lanuginosus* is classified as a member of *Deuteromycetes* (imperfect fungus), that is unicellular or septate and reproduces asexually by forming aleurioconidia (Singh *et al*., 2003). However, Salar and Aneja (2007) have reported *T. lanuginosus* as a member of the order *Eurotiales* in a sister relationship with *Talaromyces thermophilus*. Seven species of *Euriotiales* regarded as thermophiles include: *Thermoascus aurantiacus, T. crustaceus, Talaromyces thermophilus, T. leycettanus, T. byssochlamydoides, T. emersonii*, and *Thermomyces lanuginosus*. According to Morgenstern *et al*. (2012) the species pairs *Talaromyces thermophilus* and *Thermomyces lanuginosus, Talaromyces byssochlamydoides* and *T. emersonii,* receive strong support as being monophyletic (each 100 % BSS).

Synonyms of *Humicola lanuginosa* include, *Sepedonium lanuginosum* Miehe (Griffon and Maublanc, 1911), *Monotospora lanuginosa* (Griffon and Maublanc, 1911; Mason, 1933), *Acremoniella* sp. (Rege ,1927; Mason 1933), and *A. thermophila* (Mason, 1933). *Thermomyces lanuginosus* as the first assessed thermophilic fungus is supported by majority of the investigators and its nomenclatural history has involved genera such as *Acremoniella*, *Humicola*, *Monotospora* and *Sepedonium* (Salar and Aneja, 2007).

In the *Eurotiales*, thermophiles are not abundant but occur in different positions of the phylogeny, indicating a potentially complex evolutionary history for this trait. Since they can propagate under the conditions where other organisms either cannot grow or grow little, microorganisms living in extreme environments always have been considered as a popular research subject by scientists. In particular, the tolerance of these microorganisms' cell components to high temperature has caused thermophilic fungi to be used extensively in different types of biotechnological applications.

BIOFUEL SCENARIO

Environmental awareness and abatement scenario is opening new vistas of energy generation which is based largely through biological agents. In this scenario, microbial world is viewed with favour since fungi are usually rich in hemicellulosic enzymes and availability of agroresidues is considerable. Global demand estimates for 2022 show a change in the conventional v/s biofuel scenario (**Table-2**).To reach this goal, attention will have to be diverted towards lignocellulosic residues that can yield up to app. 442 billion L per year of EtoH from agroresidues (**Table-3**). A number of thermophillic fungal species were attractive candidate on account of cooperative cellulolytic machinery. The inherent process based problems of degradation, temperature requirement (40-50°C), and longer reaction times did not favour mesophilic species of *Aspergillus, Penicillium,* and *Trichoderma*. Some or most limitation of the first step cellulolytic hydrolysis therefore turned towards the well established thermophilic fungus systems of *Chaetomium thermophilum*, *Malbranchea pulchella*, *Myceliophthora thermophilia*, *Thielavia terrestris*, *Thermomyces lanuginosus,* and *Thermoascus aurantiacus.*

Table 2. Global fuel and Biofuel Demand in 2022 (Worldwatch Institute, 2016)

Billion	***Gallons***	
Country	***Fuel***	***Biofuel***
India	34.0	6.8
Brazil	16.0	8.0
USA	180.0	36.0
EU-27	95.0	6.7
China	20.0	3.0

Table 3. Lignocellulose Residues;Potential for Ethanol

Waste	Annual Production (Trillion G/Yr)	Potential Ethanol (Billion L/Yr)
Corn Stover	203.62	58.6
Rice Straw	731.34	204.6
Wheat Straw	354.35	103.8
Bagasse	180.73	51.3
Barley Straw	58.45	18.1
Oat Straw	10.62	2.78
Sorghum Straw	10.32	2.79
TOTAL	1549.42	442.0
Municipal Solid wastes Animal wastes (Dashtam *et. al.*, 2009..*Int.J.Biol.Sci*.**5** :579)		

The major attention as biofuel alternatives is likely to be achieved through, cellulosic route (60% GHG), biomass-based diesel (50% GHG), advanced biofuels (50% GHG) and total renewable fuel (20% GHG) (doi.10.1155/2010/541698). The biofuel sector has found promise at industrial scale with the participation of university industry as a major initiative. The programme entitled C BioN (www.cellulosic-biofuel.ca) is characterized by operation of independent clusters to investigate biomass availability and pre-treatment step; enzymes involved; designer plant cell walls with greater susceptibility to hydrolysis; expression of enzymes in plants; fermentation efficiency; and, environmental, ecological, and legal issues. This so called Genozymes Project has decided to sequence genomes of nearly 20 true thermophilic fungi of which *Thermomyces lanuginosus* stands in secreting a cellulose- free xylanase. It was only five years ago that xylanases of *Myceliophthora* and *Thielavia terrestris* were cloned in *Aspergillus niger*; and proteins stable from 40 to 70°C were recovered (Berka *et al*., 2011). A comparison of genomes of lignocellulytic thermophillic fungi with mesophilic *Aspergillus niger, Neurospora crassa* and *Trichoderma ressei* is presented in **Table-4**. A quick glance at the data shows the relatively small genome (23.3 Mb) of *Thermomyces lanuginosus*; The number of coding genes is only 5,105.The number of carbohydrate active enzymes is placed at 224 and the nearly cellulase-free xylanase preparation places it as the best cellulase-free xylanase producer (Mchunu *et al*., 2013).This thermophillic fungal species appears to achieve thermal adaptation through ubiquitin degradation pathway; possesses an active histone acetylation/deacetylation machinery besides high number of methylases and is capable of poly ADP-ribosylation. The presence of cellulase-free xylanases and variety of adaptive strategies permit it to grow on dead wood and its deconstruction.

Table 4. Genomics of Thermophilic Fungi

Fungus Name	Size (Mb)	No. of coding genes	G+C (%)	Reference
Aspergillus niger	33.91	14,165	50.40	Nat.Biotech,2007
Myceliophthora thermophilia	38.7	9,110	51.40	Nat.Biotech,2011
Neurospora crassa	40.0	12,188	48.20	Ncbi.nlm.nih.gov/genome
Rhizomucor miehei	27.6	10,345	43.83	BMC Genomics 2014
Thermomyces lanuginosus	23.3	5,105	52.14	Genome Ann.2013
Thielavia terrestris	36.9	9,813	54.90	Nat.Biotech 2011
Chaetomium thermophilium	28.3	7,227		Nuc.Acid.Res.2014
Trichoderma recei	33.9	9,129	52.00	Nat.Biotech.2008

Thermomyces lanuginosus is thus not only a true representative of small world of thermophilic fungi but also a candidate organism for future of bioenergy based technologies by virtue of its colonization capacity, ability of deconstruct wood and wood based products, and release of cellulase-free xylanase. Thermophilic fungal world has suddenly come up in the forefront of biotechnological developments that are likely to add to the enzymes that are already under industrial applications viz, lipase and protease.

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