

# Mycelial growth and microscopic characteristics of *Thermomyces lanuginosus* Tsiklinskaya in YpSs medium with different carbon sources

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**ABSTRACT** - (Mycelial growth and microscopic characteristics of *Thermomyces lanuginosus* Tsiklinskaya in YpSs medium with different carbon sources). Most thermophilic moulds are known to be lipase producers and the potential for esterification by reversal of lipase hydrolysis has long been recognized. We evaluated the mycelial aspects of *T. lanuginosus* growing in Petri dishes in YpSs (yeast extract + starch + agar) medium control and four other media based on YpSs containing different carbon source. These raw materials evaluated as carbon sources suppliers for microorganism were pentaerythritol, oleic acid and pentaerythritol dioleate are commonly used in several classic industrial esters synthesis. *Thermomyces lanuginosus* grewed in all studied media and kept its reproduction capacity, showing that this fungus is a promising alternative for future studies of live microorganism's biocatalysts.

**Key words:** carbon source, culture media, thermophilic fungus

**RESUMO** - (Crescimento micelial e características microscópicas de *Thermomyces lanuginosus* Tsiklinskaya em meio de cultura YpSs com diferentes fontes de carbono). Muitos fungos termofílicos são conhecidos como sendo produtores de lipases e o potencial dessas enzimas nas reações de esterificação (reação inversa à hidrólise), há muito tem sido reconhecida. O aspecto do micélio e diversas observações microscópicas foram efetuadas em *Thermomyces lanuginosus* cultivado em Placas de Petri com meios de culturas YpSs (extrato de levedura + amido + agar) e mais quatro variações na fonte de carbono neste meio. As matérias-primas usadas e avaliadas foram pentaeritritol, ácido oléico e dioleato de pentaeritritol como possíveis fontes de carbono disponíveis ao microrganismo são comumente encontradas na indústria química como intermediários clássicos na síntese de diversos ésteres. Com o crescimento de *Thermomyces lanuginosus* em todos os meios propostos e a manutenção da capacidade de reprodução mostramos ser esse fungo altamente promissor em futuros trabalhos de biocatálise com microrganismos vivos.

**Palavras-chave:** fonte de carbono, fungo termofílico, meio de cultura

## Introduction

Thermophilic fungi are microorganisms that have a minimum temperature of growth at or above 20 °C and a maximum temperature of growth extending up to 60-62 °C (Maheshwari *et al.* 2000). Although nearly all of the thermophilic fungi appeared abundantly on natural substrata, it was not always easy to get them established on laboratory media. Several reports postulated that nearly half of the species of thermophilic fungi required yeast extract for growth in a solid medium (Cooney & Emerson 1964, Fergus 1969a, b). Thus, for growth in a solid medium the thermophilic fungi required water, aeration and

nutritional factors like carbon and vitamins sources (Cooney & Emerson 1964, Maheshwari *et al.* 2000).

The yeast-starch agar medium (YpSs) was particularly favorable to thermophilic fungi and it is based on salts, soluble starch and yeast extract (Cooney & Emerson 1964). The carbon source could be changed by others raw materials, even those commonly used in several chemical ester synthesis.

As enzymatic synthesis would be attractive as compared to chemically catalyzed routes in terms of energy and processing costs, several approaches has been developed by esterification using hydrolases (lipases) as a catalysts (Roberts *et al.* 1995). It is generally accepted that an enzymatic reaction is

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virtually reversible and, hence, the equilibrium can be controlled by appropriately selecting the reaction conditions (Roberts *et al.* 1995). As biotechnology is increasingly incorporated into many industrial processes, there is a need to explore biotechnological approaches to the manufacture of new products showing industrial interest. *Thermomyces lanuginosus* lipase (Lipolase; Novo Industri A/S) is already being used in detergent formulation in conjunction with other microbial enzymes (e.g., protease, amylase, and cellulase). In addition, *T. lanuginosus* lipases have applications in the food industry and are used for the biocatalysis of stereoselective transformations (Jaeger & Reetz 1998).

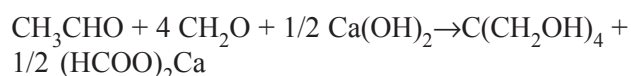
In view of the intensive investigations that have been made of thermophilic algae and bacteria, the lack of knowledge about thermophilic fungi is quite surprising, also in terms of their morphology, specially when they are exposed to industrial raw materials (Maheshwari *et al.* 2000). Alexopoulos *et al.* (1996) stated that a character is any attribute or feature of an organism that can serve as a basis for comparison with other organisms. The different expressions of a character are known as character states. Thus, these characters, mainly reproductive structures like conidiophores and conidia to anamorphic group, were useful to know if they were keeping when microorganisms growth on uncommon substrate.

The aims of this work were to evaluate the growth and the morphological characters in *Thermomyces lanuginosus* exposed to raw materials used in ester synthesis like pentaerythritol, oleic acid and the ester formed by the esterification between pentaerythritol and oleic acid, as the only carbon source available in the media.

## Material and methods

*Thermomyces lanuginosus* IOC 4145 used in this study was originated from culture at UNESP/IBILCE - São José do Rio Preto, São Paulo. Colonies were obtained from stock cultures maintained on YpSs at 50 °C with periodic replication. The fungus was grown on five different kinds of yeast extract + starch + agar media, being the first one, the YpSs standard composition (Emerson 1941). The others versions, v. I, v. II, v. III, and v. IV (the last one without carbon source) were alternative media containing each one, a different carbon source (table 1). The following media components were tested:

Pentaerythritol (v. I) - Produced by Copenor. It is a polyol used in the fabrication of several esters and polyesters. It can be prepared by condensation of acetaldehyde and formaldehyde in a basic environment. The process occurs by successive aldol condensations followed a Cannizzaro reaction (Carey & Sundberg 1990).



Oleic Acid (v. II) - Produced by Braido. It is a monounsaturated fatty acid found in various animal and vegetable sources. It has the following empirical formula: C<sub>18</sub>H<sub>34</sub>O<sub>2</sub>. Its IUPAC name is cis-9-octadecenoic acid, the lipid shorthand name is 18:1 cis-9. It is produced by the hydrolysis of bovine tallow and found several applications in esters synthesis (Carey & Sundberg 1990).

Pentaerythritol dioleate (v. III) - The last one was obtained in the laboratory by the acylation of pentaerythritol (1 mol) by reaction with oleic acid (2 mols). To drive the reaction forward, the glycol is

Table 1. The standard YpSs medium composition (yeast-starch agar) and its four variations based on carbon source substitution.

Media	Raw materials										
	Powdered yeast extract (g)	MgSO <sub>4</sub> .7H <sub>2</sub> O (g)	K <sub>2</sub> HPO <sub>4</sub> (g)	Solubre starch (g)	Pentaerythritol (g)	Oleic acid (g)	Pentaerythritol dioleate (g)	Triton X-100 (g)	Tween 40 (g)	Agar (g)	Distilled water (ml)
YpSs(Emerson 1941)	4.0	0.5	1.0	15.0	-	-	-	-	-	20.0	1000
YpSs (v. I)	4.0	0.5	1.0	-	15.0	-	-	-	-	20.0	1000
YpSs (v. II)	4.0	0.5	1.0	-	-	15.0	-	1.0	1.0	20.0	1000
YpSs (v. III)	4.0	0.5	1.0	-	-	-	15.0	1.0	1.0	20.0	1000
YpSs (v. IV)	4.0	0.5	1.0	-	-	-	-	-	-	20.0	1000

used in large OH equivalent excess, and it is also necessary to remove water as it is formed (Carey & Sundberg 1990). As like as oleic acid pentaerythritol dioleate shows no solubility in water, to elaborate YpSs media with these carbon sources an emulsion must be consider. It is seen to consist of a liquid medium in which are suspended minute droplets of a second liquid. The medium is water and the droplets are oil (“oil-in-water”). The addition of 2 drops of Triton X-100 ( $\alpha$ -[4-(1,1,3,3-tetramethylbutyl)phenyl]-w-hydroxypoly(oxy-1,2-ethanediyl) and Tween 40 (polysorbate) in 50 ml of YpSs v. II and YpSs v. III, created stables emulsions like water/oleic acid and water/pentaerythritol dioleate.

Inoculum and growing conditions - the fungus was inoculated for six days in YpSs standard, and several dishes (10 mm diameter) were token and re-introduced each one in a new Petri dish containing YpSs, YpSs v. I, YpSs v. II, YpSs v. III and YpSs v. IV. The cultures were inoculated at 50 °C in Petri dishes for six days.

To evaluate any possible variation regarding hyphae, conidiophores, and conidia morphology in the cultures of *Thermomyces lanuginosus*, the fungus was compared in all media tested by taxonomic literature (Cooney & Emerson 1964, Pugh *et al.* 1964, Awao & Mitsugi 1973, Nicoli & Russo 1974, Domsch *et al.* 1993). A triplicate criteria was adopted for each medium. All slides were staining with lactophenol cotton blue, a dye utilized for fungi to observe somatic and reproductive structures (Kirk *et al.* 2001).

The pH values and its effect on growth at 50 °C were determinated directly on these five media. Every day during six days, measurement of colony diameter in Petri dishes was the method chosen here to assess growth and the pH was measured by a pHmeter Digimed, model DMPH-1, after the dilution of each used medium with deionized water 1:1 w/w.

## Results and Discussion

The mycelial growth (diameter increase) of *Thermomyces lanuginosus* was daily monitored during six days at 50 °C and the summary data in table 2 show several aspects of the microscopic characteristics of the culture. This fungus can grows in all tested media maintaining the characteristics of the species (Cooney & Emerson 1964, Pugh *et al.* 1964, Awao & Mitsugi 1973, Nicoli & Russo 1974, Domsch *et al.* 1993).

When an emulsion is examined under a

microscope, we observed few conidia and detached from conidiophores in both cases (v. II and v. III) when compared with YpSs standard and YpSs v. I (table 2). When no theoretical carbon source is added to the medium (YpSs v. IV where yeast extract is consider as a vitamin source), the sporulation is very intense and growth is very similar compared to the standard medium YpSs.

Depending on the hydrophobic character of the evaluated carbon source, to prepare a stable mixture in water of such “insoluble” raw material, making it available to the microorganisms, it is necessary to incorporate a surface active agent called an “emulsifier”, which keeps one liquid more or less permanently dispersed in the other. This condition is called emulsion by the action of an emulsifying agent (Roberts *et al.* 1995). There were few conidia in both cases compared with YpSs standard and YpSs v. I.

Although *Thermomyces lanuginosus* is capable of sporulate in all YpSs media evaluated, some macroscopical differences were observed in Petri dishes. The colonies appear white and felty at first, and also very thin. But soon, (regarding *Thermomyces lanuginosus* inoculated in YpSs), they turn grey or greenish grey, beginning at the center of the colony. It is also possible to see the presence of some exudation drops. Gradually the colony turns purplish brown, and at this time the agar substratum stains a deep pink or wine color, due to diffusible substances secreted by the colony. With different carbon sources, even with less growth, the presence of a wine color was noted only in YpSs v. II (oleic acid as carbon source).

The data in table 3 show a considerable growth of *Thermomyces lanuginosus* in YpSs v. IV, a medium without a specific carbon source. The necessity of this fungus regarding the presence of yeast extract was clearly noted such as reported by Cooney & Emerson (1964), Fergus (1969a, b). All media tested without addition of this component showed no growth. The conidia obtained from YpSs, YpSs v. I, YpSs v. II, YpSs v. III and YpSs v. IV, were replicated in the same media, showing always the same mycelial growing.

Based on a triplicate, pH values are shown in table 4. Considerable changes in pH were observed in YpSs v. II. Based on an estimated log octanol/water partition coefficient of 7.73 and a regression derived equation, the Koc for undissociated oleic acid can be estimated to be approximately 38,000. This Koc value indicates that oleic acid will be immobile in soil. Due

Table 2. The microscopic characteristics of *Thermomyces lanuginosus* in standard YpSs medium and its four variations based on carbon source substitution.

Media	Macroscopic characteristics				
	Hyphae	Conidiophores	Conidia	Conidia diameter (µm)	Separated conidia
YpSs (Emerson 1941)	Septate, branched	Short, unbranched or rarely branched	Single on each conidiophore, spherical, hyaline and smooth-walled when young	5 to 7	None
YpSs (v. I)	Septate, branched	Short, unbranched or rarely branched	Single on each conidiophore, spherical, hyaline and smooth-walled when young	5 to 7	None
YpSs (v. II)	Septate, branched	Short, unbranched or rarely branched	Single on each conidiophore, spherical, hyaline and smooth-walled when young	5 to 7	Present
YpSs (v. III)	Septate, branched	Short, unbranched or rarely branched	Single on each conidiophore, spherical, hyaline and smooth-walled when young	5 to 7	Present
YpSs (v. IV)	Septate, branched	Short, unbranched or rarely branched	Single on each conidiophore, spherical, hyaline and smooth-walled when young	5 to 7	None

to the  $pK_a$  value of 5.02, oleic acid will be 99% dissociated at pH 7 but it was very interesting to observe the fungus ability to adjust the hydrogen-ion concentration. Most fungi grow best at approximately pH 7 but tolerate a wide range from 3-10 (Kirk *et al.* 2001). An initial pH of 6.5 to 7 has been used for most organisms, although in most cases the final pH shifts towards alkaline range (Liu *et al.* 1973). This effect was confirmed for *Thermomyces lanuginosus* too. Several studies have dealt with the physiological and environmental factors affecting synthesis and production of extracellular proteases by thermophilic fungi. Although there are distinct differences between

organisms, synthesis of many proteases is governed, at least in part, by the type and concentration of the available individual nutrients (Somukuti & Babel 1968). The production of extracellular protease by thermophilic *Achaetomium macrosporum* J.N. Rai *et al.*, *Chaetomium thermophilum* La Touche, *Thermomyces lanuginosus*, *Sporotrichum thermophile* Apinis and *Torula thermophila* Cooney & R. Emers. was enhanced by the addition of casein, gelatin, glucose and maltose (Somukuti & Babel 1968). The proteases produced by *Thermomyces lanuginosus* are considering as alkaline protease type (Satyanarayana & Johri 1983).

Table 3. Measurement of mycelial growth media (diameter) of *Thermomyces lanuginosus* inoculated in standard YpSs (yeast extract + starch + agar) medium, and its four variations based on carbon source substitution. Diameters (mm) were based on a triplicate.

Media	Period of observation					
	1 <sup>st</sup> day (mm)	2 <sup>nd</sup> day (mm)	3 <sup>rd</sup> day (mm)	4 <sup>th</sup> day (mm)	5 <sup>th</sup> day (mm)	6 <sup>th</sup> day (mm)
YpSs (Emerson 1941)	5	11.5	24.5	35	>35	>35
YpSs (v. I)	5	10	19.5	23.5	29.5	35
YpSs (v. II)	1.5	4	6.5	8	12	16.5
YpSs (v. III)	2	6	15.5	21	27	30
YpSs (v. IV)	4	9	19	22	28.5	35



Table 4. Measurement of pH media of *Thermomyces lanuginosus* inoculated in standard YpSs medium and its four variations based on carbon source substitution. Data based on a triplicate.

Media	pH measurements		
	Initial pH 50 °C	Initial pH 50 °C diluted 1:1 (w/w)	Final pH 50 °C diluted 1:1 (w/w)
YpSs (Emerson 1941)	7.16	7.31	7.87
YpSs (v. I)	7.27	7.34	7.85
YpSs (v. II)	6.11	6.21	7.04
YpSs (v. III)	7.17	7.28	7.47
YpSs (v. IV)	7.21	7.33	7.71

Therefore, *Thermomyces lanuginosus* survived and showed a considerable growth in all media tested, specially in YpSs v. III that contains an ester and also a residual oleic acid. Some macroscopical differences were noted regarding the cultures morphology, but no significant differences were observed in a microscopical study, showing stability in the conidiogenesis. However, the capacity of this fungus to grow when the YpSs medium had its carbon source substituted by glycols, fatty acids and esters synthesized in glass kettles was confirmed.

In order to evaluate an eventual improvement of lipase production, or even the capacity of *Thermomyces lanuginosus* to grow with different carbon sources, additional studies of the effects of sterols, vitamins, and other nutrients on the mycelial growth and sporulation of this fungus would be useful to confirm thermophilic microorganism as a promising alternative for immobilized biocatalysts.

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