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Cellulolytic Activities of Thermophilic Thermoascus aurantiacus MW559792 and Mycothermus thermophilus MZ723073

EL-Sayed M. EL-Morsy*[1], Mohammad M. El-Metwally[2], Sally A Shabara[1] and Marwa T Mohesien[1]

[1] Botany and Microbiology Department, Faculty of Science, Damietta University, New Damietta 34517, Egypt
[2] Botany and Microbiology Department, Faculty of Science, Damanhour University, Egypt

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* Corresponding author's E-mail: el_morsy@du.edu.eg

Abstract

Seven thermophilic fungal species were isolated from various thermogenic habitats on modified Czapek's Dox agar medium with CMC replacing glucose and identified based on their morphological characters and sequence homology of their rRNA gene sequences. Congo red test was used to display their cellulolytic activity. The cellulolytic activities (FPase, CMCase and β-glucosidase) of five selected species were performed on solid state fermentation (SSF). *Thermoascus aurantiacus* MW559792 and *Mycothermus thermophilus* MZ723073 were selected for optimization experiments based on their maximum productivity of cellulases. Culture conditions were optimized under SSF experiment. Of the five lignocellulosic wastes used as carbon sources, rice straw was the most favorable for maximum production of FPase (1.78, 1.45 U/g), CMCase (6.18, 4.09 U/g) and β-glucosidase (31.93, 16.23 U/g) from *T. aurantiacus* and *My. thermopilus*, respectively. The suitability of the lignocellulosic substrates was in the following order: rice straw> maize straw > wheat straw>corn cob> rice hulls in case of T. *aurantiaccus*, and rice straw> wheat straw>maize straw>corn cob>rice hulls in case of *My. thermophilus*. Peptone was the most suitable nitrogen source for maximum FPase (1.88, 1.46 U/g), CMCase (7.02, 4.89 U/g) and β-glucosidase (28.60, 19.91 U/g) activities by *T. aurantiacus* and *My. thermophilus* respectively. Nitrogen sources availability was in the following order: Peptone > Yeast Extract>$(NH_4)_2SO_4$> Beef Extract> NH4NO3> NaNO3> KNO3. The maximum cellulolytic activities of *T. aurantiacus* and *My. thermophilus* were also achieved at pH 5, 50°C and 45°C for 4 to 6 days of incubation respectively. On that bases *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 were suggested to use industrially for bioconversion of lignocellulosic wastes at the saccharification stage.

Keywords: Cellulases; FPase; CMCase; β -glucosidase; Thermophilic fungi; Thermoascus aurantiacus; Mycothermus thermophilus.

Introduction

The excessive consumption of fossil fuels is the main reason for the current global energy problem, which are non-renewable resources and contribute significantly to greenhouse gas emissions and climate deterioration (Rempel *et al*., 2019). In order to meet future energy needs,

a sustainable and ecofriendly energy sources are required (Choudhary *et al*., 2017). Lignocellulose is considered a renewable carbon source which can be produced on a large-scale platform from a variety of materials, including marine algae, forestry wastes, and agricultural wastes of all kinds (Prasad and Chatterjee, 2019). The lignocellulosic materials are mainly composed of three polymers; cellulose, hemicellulose and lignin (Kim and Sunagawa, 2016). Because of the covalent and hydrogen connections that bind cellulose and hemicellulose to lignin, the lignin's structure is strengthened and more resistant to treatment (Ufodike, 2020).

Cellulose is D-glucose residues joined by β-1,4 glucosidic linkages. It is one of the most attractive and available raw materials for bioconversion into a wide range of products (Shajahan *et al*., 2017). Generally, fungi are among the thermophilic microorganisms that possess highly active enzymatic machinery that produce several types of enzymes including cellulase that is implicated in various industries (Singh, 2016). In addition, huge amounts of partially hydrolyzed or unused cellulosic resources are still building up in the environment and are improperly utilized as a result of the high costs of reuse (Lee *et al*., 2008).

Biodegradation of cellulose is an ecofriendly, pollution-free, inexpensive, and cost-effective bioprocesses, rather than physiochemical degradation (Ding *et al*., 2008). Exoglucanase (FPase), endoglucanase (CMCase), and β-glucosidase (βGL), are acting synergistically in the hydrolysis of cellulosic materials (Bhat, 2000; Zhang et al., 2015). In fact, cellulases are essential in bioconversion processing plants because they hydrolyze cellulose to provide fermentable sugars.

Bioprocesses have been effectively optimized by testing the influence of various variables and looking for the ideal circumstances using solid state fermentation (SSF) to manufacture the enzyme (Chen *et al.* 2005). Thus, despite some specific characteristics that make them more favorable at an industrial scale, the diversity and mode of action of thermophiles cellulases were nearly identical as they meet industrial needs by producing thermostable enzymes.

Thermophilic fungi are ubiquitous, widespread over 5,000 identified species that are isolated from various types of substrates and live in several environments such as aquatic sediments, waterlogged grasslands, dungs, industrial waste, mushroom compost, vegetable compost and municipal garbage (de Oliveira *et al*., 2015; Akpomie *et al*., 2021). The abundance of carbon rich microhabitats such as lignocellulosic wastes in temperate, dry, and tropical ecosystems, in daily temperature and moisture enhance thermophiles growth and dispersion (Maheshwari et al., 2000). Phylogenetic research of thermophilic fungi indicates a common origin to mesophiles based on the similarity of their enzymes and other structural features (Zeldes, 2015).

Thermophilic microorganisms are, thermophilic cell factories that produce thermostable enzymes more resistant to denaturing chemicals and can withstand high pressure than mesophilic enzymes. They are used in commercial applications for the manufacturing of value-added products and biofuels at higher temperatures providing an economic benefit. *Thermoascus aurantiacus* (Jain, 2015), *Thermomyces lanuginosus* (Kamra, 2004), *Scytalidium thermophilum* (Basotra, 2016), and *Melanocarpus albomyces* (Miettinen-Oinonen, 2004) are highly efficient thermophiles in cellulases productivity. Generally, at 45–50 °C, most thermophilic fungi produce large levels of cellulolytic enzymes (Singh, 2016) and over 50 °C, enzyme synthesis declined gradually (Kalogeris *et al*., 2003). *Thermoascus. aurantiacus*, and *Malbranchea cinnamomea*, are reported to be a potent cellulolytic species (Singh, 2016).

In this paper, thermophilic fungi were isolated and identified from various habitats in Egypt. The cellulolytic capability of the isolated species was recorded on Congo Red method and under solid state fermentation. Optimization of the condition governing the maximum cellulase production from *Thermoascus aurantiacus* MW559792 and *Mycothermus thermopilus* MZ723072 was were carried out using lignocellulosic waste substrates under solid state fermentation.

Materials and Methods

Collection of samples

Twelve samples of three different types (municipal refuge, dung soil and herbivore wastes) were collected in October 2018 from

various locations throughout Egypt's Damietta governorate. They were kept in clean polyethylene bags, taken straight to the lab, and kept there at 4 °C for future research.

Isolation and Identification of fungi

In 500 ml Erlenmeyer flask, 10 gm of each sample in 100 ml of sterile water were added, and mechanically shaken for 15 min at 120 rpm. Serial dilution was carried out and one ml of each dilution was transferred to CMC agar, that incubated at 50 °C for 10 days. For isolation of thermophiles from the herbivore wastes, collected materials were aseptically, spread over sterilized wet filter paper within a sterilized petri dish. Then, the growing fungi are transferred to sterile PDA agar plates. For further study, PDA agar slopes were made up of purified and roughly identified strains. Based on their morphological characteristics, using taxonomic manuals, appropriate literature and standard methods the purified species (on standard culture media) were identified (Raper and Fennel 1965; Ellis 1971, 1976).

Molecular Identification

Isolated species were sub-cultured on sterile PDA agar plates at 45 °C for 7 days under static conditions. Extraction of fungal genomic DNA was basically accomplished by applying ABT DNA mini extraction kit (Applied Biotech. Co. Ltd, Egypt), in accordance with the manufacturer's instructions. After that, the process of DNA electrophoresis was accomplished according to White *et al*. (1990). The partial sequences of the gene were obtained by PCR using the primers and the amplification program described by (O'Donnell *et al*., 1998). To make PCR, the reaction mixture contained forward ITS1 (5'- TCC GTA GGT GAA CCT GCG G -3') and reverse ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3) primers were employed. Following cycling parameters, DNA was quantified with gel electrophoresis (1% agarose in Tris acetate EDTA) and a low-DNA-mass ladder. For sequencing, the amplified products were submitted to South Korean Co. (Solgent Co Ltd). The BLAST from the NCBI website was used to examine the sequences retrieved. Phylogenetic analysis was performed using MegAlign (DNA Star) software version 5.05. The sequences were deposited in GenBank and the accession number of the fungal strain was received (Table 1). A phylogenetic tree was built up by comparisons of obtained sequences, with that from the database using blast tree and aligned using aligned sequences nucleotide BLAST.

Cellulolytic activity of thermophilic fungi by Congo red staining method

Cellulase production was examined by well diffusion method. The tested species were grown on PDA medium for 6 days. Each petri plate containing CMC agar was inoculated with an equal diameter (6 mm) disc of each isolate and incubated at 50 °C for 5 days, in three replicas. Then, each one was flooded with 1% Congo red dye for 30 min and washed with 1M NaCl for 20 min. The development of clear yellowish zones around the inoculated discs indicates the hydrolysis of cellulose. The fungi showing the largest zone of clearance were selected to be screened quantitatively by assaying cellulase activity (Ramesh *et al*. 2008).

Inoculum Preparation

Fungal PDA slants growing at 45 °C for 6 days scraped gently with a sterile wire loop after addition of 10 ml of sterile water containing Tween-80 (0.1% v/v) and then filtered. One ml of 10^5spores/ml was counted using a hemocytometer and used as primary inoculum for the fermentation process.

Cellulase production under solid state fermentation (SSF)

In this experiment 1.0 gm of dry grinding rice straw moistened with10 ml of basal salt solution (g/l): (KH_2PO_4, 15; $(NH_4)_2SO_4$, 5; $MgSO_4$. 7 H_2O, 0.6; $ZnSO_4$.7 H_2O, 0.14; $MnSO_4$.6 H_2O, 0.16; $CaCl_2 \cdot 6H_2O$, 0.37), were prepared with pH of 4.8 at 120 °C (15 psi) for 20 min. The mixture was then cooled, inoculated with 1.0 ml (10^5spore/ml) spore and incubated at 50 °C for 6 days. The flasks with fungal fermented rice straw (mycostraw) then were eluted with 0.05 M sodium citrate buffer (pH 4.8) solution (1:10 w/v) and shaken gently (200 rpm) for 30 min., centrifuge (5000 rpm) for 10 min and the supernatant was used for assaying the FPase, CMCase and β-glucosidase activities and their time course production.

Total cellulase Analysis

The activities of total cellulase (FPase, CMCase and β-glucosidase) were determined in accordance with the IUPAC procedures (**Ghose**, **1987)**. The activity of Exo-cellobiohydrolases (FPase) was assayed by measuring the released reducing sugars in a reaction mixture (1.0 ml of cultural filtrate; Whatman No.1 filter paper (1 x 6 cm; 50 mg); 1.0 ml of 0.05M sodium citrate buffer; pH 4.8) at 50 °C and after 60 min. The activity was expressed as the quantity of an enzyme needed to release reducing sugars equivalent to1µmol of glucose /min under test conditions. For assaying the activity of CMCase (Endoglucanase), the released reducing sugars after incubation of reaction mixture (1.0 ml of CMC; 0.5 ml of crude enzyme; aliquots of 0.05 mM sodium citrate buffer; pH 4.8) at 50 °C for 30 min. β-1,4-glucosidases (Cellobiase) activity was assayed in a reaction mixture (1.0 ml 0.1 % cellobiose; 0.05 M sodium citrate buffer; pH 4.8; aliquot diluted enzyme) that incubated for 30 min. at 50 °C. After that 3.0 ml of dinitrosalicylic acid reagent (DNS) was added to the reaction and boiled for 10 min. and cooled down to determine the O.D. at 575 nm for quantification of the released reducing sugars of the three enzymes (**Miller, 1959)**. The values obtained are compared with the glucose standard curve. The enzyme activity was expressed in (U/g) for solid state fermentation.

Optimization:

The optimization experiment was carried out following one factor at a time (OFAT) approach to find the best condition of the cellulase production by *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073. The effects of carbon sources (rice straw, wheat straw, rice hulls, maize straw and corn cob), nitrogen sources ($NaNO_3$, KNO_3, $NH4NO_3$, $(NH_4)_2SO_4$, Peptone, yeast extract & beef extract), initial pH (3.0–10.0), incubation temperature (30, 35, 40, 45, 50, 55, 60 and 65 °C) and incubation period (1-7 days) were tested in three replicas.

Results and discussion

Isolation and identification of thermophilic cellulolytic fungi

A total of 8 thermophilic cellulolytic species and 7 genera were isolated from various thermogenic habitats in Damietta governorate, Egypt, using Czapek's Dox agar medium with CMC in replacement of glucose (Table 1). The isolated species are identified morphologically to the genus level and classified into two major taxonomic phyla: ascomycota (orders Eurotiales, Sordariales and Onygenales) and Zygomycota (order Mucorales). These genera were identified to the species level based on their sequence variation present in the internal transcribing spacer (ITS) region, that aligned with NCBI reference sequences and based on 18 srRNA, as a perfect tool. The BLAST results revealed that, the isolated species exhibited high similarity (up to 100%) with the reference species in the GenBank (Table 1, Figure 1 & 2).

Phylogenetic Analysis

The phylogenetic analysis for taxa belonging to the phylum Ascomycota in figure 1 revealed 6 distinct genera and species namely; *Thermoascus aurantiacus*, *Thermomyces lanuginosus* strains (1&2), *Thermomyces dupontii* strains (1&2); *Mycothermus thermophilus* strains (1&2), *Melanocarpus albomyces* strains (1&2), and *Malbranchea cinnamomea*. Based on this sequence homology, sequence analysis indicated that *Thermoascus aurantiacus*, displayed maximum similarity of 99% with the *T. aurantiacus* on the same species in GenBank, and so for *Malbranchea cinnamomea*. The other species showed a similarity of 100% with their analogous species deposited in GenBank Table 1. The evolutionary relatedness deduced from the ITS region sequences revealed that *Thermoascus aurantiacus* (MW559792) clustered with the *Aspergillus lomoniformis. Thermomyces lanuginosus* st1&2, *Thermomyces dupontii* is in a single cluster Figure 1. *Rhizopus microsporus* phylogenetic tree was illustrated in Fig 2 belongs to the phylum Zygomycota revealed variable similarity with other recorded *R. microsporus* spp.

Table 1. List of isolated and identified thermophilic cellulolytic fungi showing source, classification, and similarities with reference species in GenBank and Accession number

Fungal species	Sample	Order	Similarity %	Accession number
***Thermoascus aurantiacus* Miehe**	Municipal waste	Eurotiales	99	MW559792
***Thermomyces lanuginosus* st1 Tsiklinsky**	Dung	Eurotiales	100	MW559790
***Thermomyces lanuginosus* st2**	Municipal waste	Eurotiales	100	MW559791
***Thermomyces. dupontii* st1 (Griffon et *Maublanc*) Houbraken and Samson,**	Municipal waste	Eurotiales	100	MW559788
***Thermomyces dupontii* st2**	Municipal waste	Eurotiales	100	MW559789
***Mycothermus thermophilus* st1 (Cooney et Emerson) Natvig, Taylor, Tsang. Hutchinson et Powell.**	Herbivores	Sordariales	100	MZ723072
***Mycothermus thermophilus* st2**	Dung	Sordariales	100	MZ723073
***Melanocarpus albomyces* st1 (Cooney & R. Emers.) Arx,**	Municipal waste	Sordariales	100	MW559793
***Melanocarpus albomyces* st2**	Municipal waste	Sordariales	100	MW559794
***Rhizopus microspores* Tiegh**	Dung	Mucorales	100	MZ723074
***Malbranchea cinnamomea* (Lib.) Oorschot & de Hoog 1984**	Herbivores	Onygenales	99	MW559795
Aspergillus fumigatus	Municipal wastes	Eurotiales	-	-

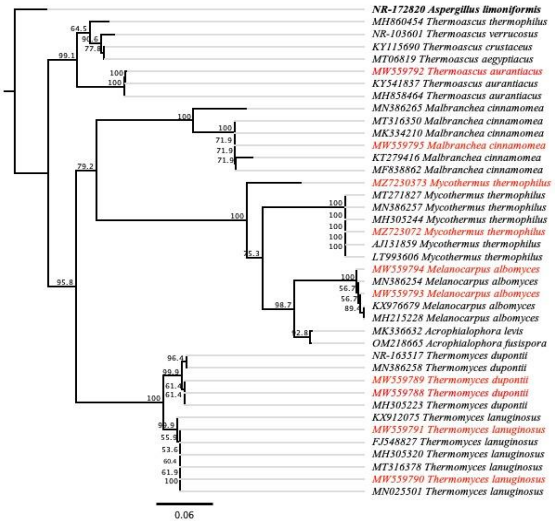

Figure 1. Phylogenetic tree generated with the ITS-rDNA sequences of Ascomycetes members including thermophiles species in orders Eurotiales and Sordiales and allied genera from gene bank. Phylogenetic tree analysis based on 18S rDNA sequence alignment with some other related species, which possessed the highest similarity. The neighbor-joining was performed using the maximum composite likelihood methods.

Cellulolytic activity

Table 2 illustrated that all the isolated species could produce cellulases in various degrees and based on the diameter of the clearing zone, 5 species (*T. aurantiacus* MW559792 and *My. thermophilus* MZ723072, *Melanocarpus albomyces* MW559793, *Rhizopus microsporus* MZ723074 and *My. thermophilus* MZ723073) have the highest activity and therefore they were selected for next experiment.

Table (2): Screening of isolated species for their cellulolytic activity on CMC agar plates flooded with Congo red dye**.**

Fungal species	Accession NO.	Clear zone diameter(mm)
***Mycothermus thermophilus* st2**	MZ723072	80.0 ± 1.15
Thermoascus aurantiacus	MW559792	68.66 ± 1.45
***Melanocarpus albomyces* st1**	MW559793	55.0 ± 2.08
Rhizopus microsporus	MZ723074	48.33 ± 0.33
***My. thermophilus* st1**	MZ723073	48.0± 1.52
***Thermomyces lanuginosus* st2**	MW559791	43.0 ± 1.73
***Melanocarpus albomyces* st2**	MW559794	42.33 ± 0.33
Malbranchea cinnamomea	MW559795	42.0 ± 1.08
***Thermomyces dupontii* st1**	MW559788	40.66 ± 0.66
***Thermomyces dupontii* st2**	MW559789	35.66 ± 1.85
***Thermomyces lanuginosus* st1**	MW559790	22.0 ± 1.15
Aspergillus fumigatus	-	20.0 ± 1.33

Cellulase production under solid state fermentation

The activity of cellulolytic enzymes (FPase, CMCase and β-glucosidase) of the five selected species that grew on rice straw- basal salt mixture at 50°C and pH 4.8 for five days were assayed under SSF condition. Table 3 and

Figure 3 illustrate that *Thermoascus aurantiacus* MW559792 was the maximum producer of FPase (1.75 U/g), CMCase (6.35 U/g) and β-glucosidase (25.12 U/g), followed by *Mycothermus thermophilus* MZ723073, FPase(1.56 U/g), CMCase(5.12 U/g) β-glucosidase(22.82 U/g). Thus, *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 were selected for the next experiments to determine the optimal conditions for their cellulase production.

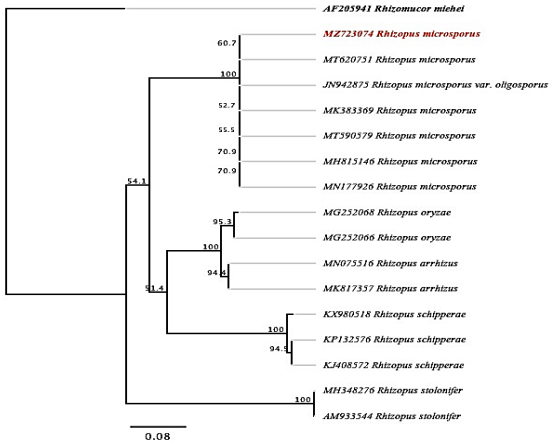

Figure 2. Phylogenetic tree generated with the ITS-rDNA sequences of thermophilic *Rhizopus microsporus* (red color). Phylogenetic tree analysis based on 18S rDNA sequence alignment with some other related species, which possessed the highest similarity. The neighbour-joining was performed using the maximum composite likelihood methods.

Table (3): Assaying cellulase activity of selected species on solid-state fermentation

Fungal species	**Cellulase activity U /g**		
	FPase	CMCase	β-Glucosidase
***T. aurantiacus* MW559792**	1.75 ± 0.05	6.35 ±0.20	25.12 ±0.38
***My. thermophilus* MZ723073**	1.56 ± 0.1	5.12 ±0.37	22.82 ±0.81
***My. thermophilus* MZ723072**	1.36 ± 0.12	4.70 ±0.36	20.51 ± 0.71
***Rhizopus microspores* MZ723074**	1.02 ± 0.10	4.42 ±0.14	18.46 ±0.46
***Melanocarpus albomyces* MW559793**	1.48 ± 0.05	4.46 ±0.12	19.05 ±0.43

Effect of nutritional parameters on cellulase production

Carbon sources

Carbon sources are very critical in the metabolism and synthesis of the enzyme. In fact, fungi produce large amounts of extracellular cellulolytic enzymes (Dashtban et al., 2009). Five lignocellulosic wastes were used as alternative substrates to carbon sources for enhancing cellulase production by *T. aurantiacus* MW559792 and *My. Thermophilus* MZ723073. Table 4 illustrates that rice straw was the most favorable carbon source for maximum production of FPase (1.78, 1.45 U/g), CMCase (6.18, 4.09 U/g) and β-glucosidase (31.93, 16.23 U/g) from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 respectively. The suitable substrate for maximum production of (FPase, CMCase andβ-glucosidase) activities were in the following order: rice straw> maize straw > wheat straw>corn cob> rice hulls in case of *T. aurantiacus* MW559792 and rice straw>wheat straw > maize straw >corn cob> rice hulls in the case of *My. thermophilus* MZ723073. Thus, rice hulls were not promising for cellulase production by selected species. The inappropriateness of some lignocellulosic substrates for enhancing the production of cellulases might be due to unsuitable physical properties of the substrate (Krishna, 2005). Ultimately, the cellulolytic activity of *T. aurantiacus* and *My. thermophilus* on the bases of the high lignocellulose degrading efficiency so it has been suggested for bioconversion processes especially in the saccharification stage of plant wastes **(**Gomes et al.,2000; Basotra et al., 2016; Schuerg et al., 2017).

Nitrogen sources

Nitrogen is very important in protein synthesis and so for cellulase activity (Mandels, 1975). Table 5 illustrates that cellulase production by *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 have been affected by the nature of nitrogen sources (organic or inorganic). The result reveals that the effect of nitrogen source on cellulolytic activity of *T. aurantiacus* MW559792 and *My. thermophilus*

Table (4): Influence of different types of carbon sources on cellulases activities by *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

Fungal species	Cellulases (U/g)	Carbon Source				
		Wheat Straw	Rice Straw	Rice Hulls	Maize Straw	Corn Cob
***T. aurantiacus* MW559792**	Fpase	1.56± 0.08	1.78 ± 0.06	0.85 ± 0.03	1.66 ± 0.06	1.37 ± 0.03
	CMCase	5.17 ± 0.13	6.18 ± 0.15	3.08 ± 0.09	5.51 ± 0.06	4.63 ± 0.23
	β-glucosidase	28.66 ± 0.35	31.93 ± 0.26	17.44 ± 0.09	28.67 ± 0.46	25.4 ± 0.36
***My.thermopilus* MZ723072**	Fpase	1.26 ± 0.01	1.45 ± 0.11	0.64 ± 0.02	1.14 ± 0.01	1.04 ± 0.03
	CMCase	3.80 ± 0.19	4.09 ± 0.12	1.96 ± 0.05	3.51 ± 0.19	3.07 ± 0.10
	β-glucosidase	13.68 ± 0.29	16.23 ± 0.30	8.68 ± 0.21	12.33 ± 0.51	11.82 ± 0.16

MZ723073 are in the following order: Peptone > Yeast Extract>$(NH_4)_2SO_4$> Beef Extract> $NH4NO_3$> $NaNO_3$> KNO_3. In fact, peptone was the most suitable source for maximum FPase (1.88, 1.46 U/g), CMCase (7.02, 4.89 U/g) and β-glucosidase (28.60, 19.91U/g) production by *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 respectively. It is followed by yeast extract. However, beef extract and ammonium sulfate produced almost the same quantity and the rest of nitrogen sources exhibited low enzyme activities. In general, organic nitrogen sources exhibit significant increases in cellulase production than inorganic nitrogen sources. Alike it is stated that organic sources caused maximum cellulase production whereas inorganic sources did not affect significantly the enzyme activity (Daroit et al., 2007; Deswal et al., 2011). Conversely, it is reported that inorganic nitrogen increases cellulolytic activity more than organic nitrogen sources (Sasi et al., 2012; Kalogeris et al., 2003).

Table (5): Influence of organic and inorganic Nitrogen sources on cellulases activities from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

Species	Cellulases (U/g)	Nitrogen source						
		$NaNO_3$	KNO_3	$NH4NO_3$	$(NH_4)_2SO_4$	Peptone	Yeast Extract	Beef Extract
T. aurantiacus MW559792	Fpase	1.09 ± .05	0.86±0.03	1.26±0.12	1.43±0.18	1.88±0.06	1.64±0.08	1.49±0.05
	CMCase	4.55±0.09	2.39±0.28	5.16±0.10	5.94±0.36	7.01±0.43	6.19±0.46	5.91±0.36
	β-glucosidase	22.24±0.28	19.40±0.37	23.92±0.57	26.14±0.40	28.60±0.86	26.79±0.66	25.43±0.35
My. thermopilus MZ723072	Fpase	1.01±0.01	0.86±0.03	1.20±0.25	1.30±0.26	1.46±0.27	1.37±0.17	1.24±0.03
	CMCase	4.05±0.11	3.46±0.13	4.21±0.38	4.53±0.10	4.89±0.58	4.77±0.37	4.29±0.14
	β-glucosidase	14.17±0.23	12.06±0.27	15.06±0.46	17.2±0.58	19.91±0.88	18.88±0.68	16.55±0.43

Effect of physiological parameters

a. Effect of incubation time

The incubation time is associated directly with the growth, production of enzymes and other physiological functions up to a certain extent. The activities of FPaseCMCase and β-glucosidase were assayed daily up to 7 days of incubation on SSF. Figures (3, 4 and 5) illustrate that *T. aurantiacus* MW559792, showed the maximum activity of FPase (1.92 U/g) was obtained at the 5th day while CMCase (6.47 U /g) and β-glucosidase (26.54 U/g) reached their maximum yields at the 4th day of incubation. Incidentally, *My. thermophilus* MZ723073, confer maximum FPase (1.40 U/g) and CMCase (5.18 U/g) on the 5th dayof incubation. β-glucosidase retained its maximum activity (17.87 U/g) on the 6th day. Alike, it is stated that 96 and 120 hrs were reported as the optimal time for cellulase productivity from *Aspergillus niger* and *Trichoderma viride* (Mrudula and Murugammal *et al*., 2011; Mai *et al*., 2018). The peak cellulase activity of *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 was achieved during the incubation period from the 4th to the 6th day which was commercially suitable time. The decrease in activities after that was attributed to the nutrients depletion, that stressed the physiology of the fungal cell resulting in the deactivation of the enzyme machinery (Nochur *et al*., 1993).

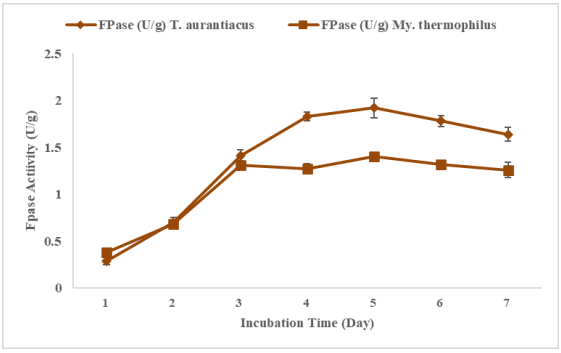

Figure (3): Effect of incubation period on Fpase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

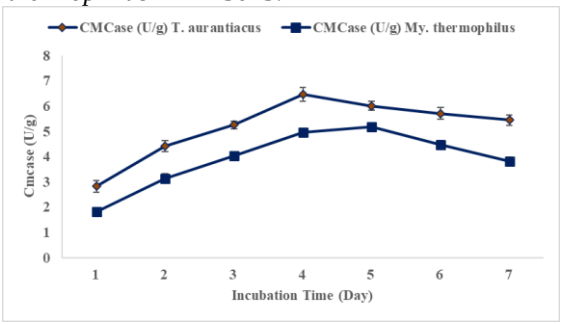

Figure (4): Effect of incubation period on CMCase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

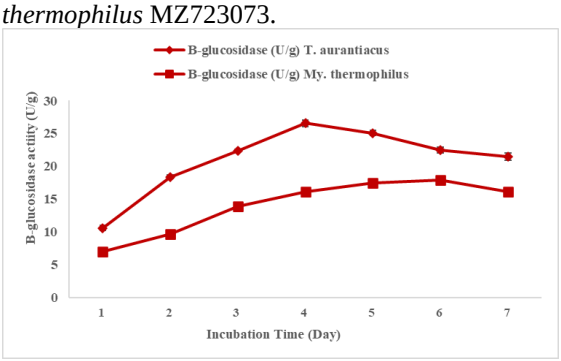

Figure (5): Effect of incubation period on β-glucosidase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

Temperature

Temperature is an essential parameter that is significantly influences cellulases productivity (Krishna, 2005). Its optimization from 30 to 65 °C for cellulases production by *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 was evaluated. The results revealed that *T. aurantiacus* MW559792 reached its maximum activity of FPase (1.82 U/g), CMCase (6.83 U/g) and β-glucosidase (32.63 U/g) at 50 °C (Figure 6, 7 and 8). It is also found that the enzyme activity decreased with the increase of temperature up to 65°C. Alike, Kawamori *et al*. (1987), stated that *T. aurantiacus* A-131 was thermostable and did not inactivate even at 60°C. Similar results have been reported for *T. aurantiacus* growth under SSF by Grajek *et al*. (1986) and Kalogeris *et al*. (2003). For *My. thermophilus* MZ723073, the enzyme activity is increased progressively from 30°C until reaches its maxima at 45 °C for FPase(1.65 U/g), CMCase(5.52 U/g) and β-glucosidase(22.26 U/g). After that the enzyme activity of the three enzymes decline up to 65°C (Figure 12, 13 and 14). This could be owing to the suppression of substrates transport via the fungal cells at low temperature (Aiba *et al*. 1973). The optimum temperature for cellulase production by *M. thermophilus (*MZ723073) was 45 °C. Likewise, Sriyamjalla *et al*. (2016) reported that 45°C was the optimum temperature for both endo and exoglucanase activities from *Scytalidium thermophilum* SKESMBKU02. Ultimately, above optimal temperature, a successive decline in cellulases activity was recorded and this was attributed to the enzyme denaturation (Khare & Upadhyay 2011; Olajuyigbe *et al*. 2016).

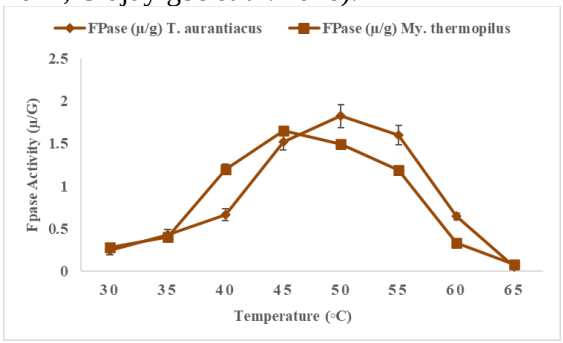

Figure (6): Effect of temperature on Fpase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

Effect of initial pH

pH is an important environmental parameter affecting the fungal growth, active transport and various metabolic activities in any fermentation process **(**Krishna, 2005). The optimum pH is required to retain the three-D structure of the enzyme active site (Mmango- Kaseke *et al*., 2016). The effect of pH on cellulases activities of *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073 was examined at pH from 3.0 to 10.0. The results in Figs. (9, 10 and 11) revealed that the highest FPase (1.80 U/g), CMCase (6.39 U/g) and β-glucosidase (31.31 U/g) activities were obtained at pH 5 by *T. aurantiacus* MW559792. On the contrary Kalogeris *et al*. (2003), reported that pH 4 was the optimum for cellulase production by *T.*

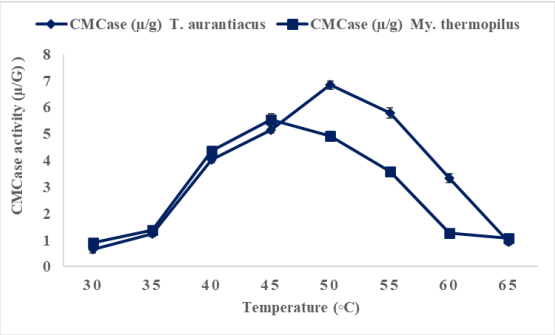

Figure (7): Effect of temperature on CMCase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

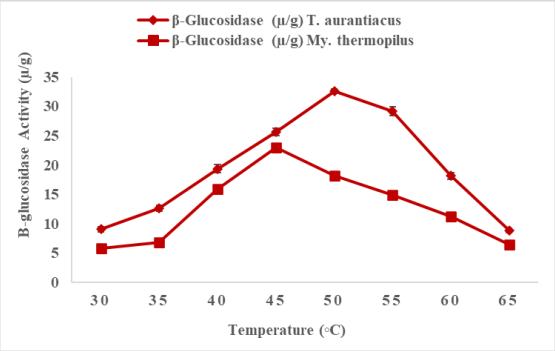

Figure (8): Effect of temperature on β-glucosidase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

aurantiacus. In general, filamentous fungi prefer acidic environments with optimal growth at pH (3.8 – 5) (Prior *et al*., 1992). On the other hand, the maximum activities of Fpase (1.69 U/gm), CMCase (5.24 U/gm) and β-glucosidase (22.80 U/g) of *My. thermophilus* MZ723073 was recorded at pH 5 Figure (15, 16 and 17. Likewise, analogous results were obtained from *Scytalidium thermophilum* (Sriyamjalla *et al*., 2016) and thermophilic *Humicola sp*. (Kumar *et al*., 2014). In fact, both fungi have high activities at pH 5. Then, the enzymes activity slightly and consistently decreases with increasing pH up to 10.0. It was noted that, above or below the optimum pH (5) the activity decreases, therefore, highly acidic and alkaline pH has negative impacts on enzyme activity, where fungi prefer weak acidic culture conditions for their growth. Likewise, it was stated that increasing the pH of the medium above the optimum weak acidic condition to 10.0 exhibited a significant reduction in the production of cellulases (El-Katatny *et al*., 2000; Kalogeris *et al*., 2003; Deswal *et al*., 2011; Gorems and Alemu, 2014).

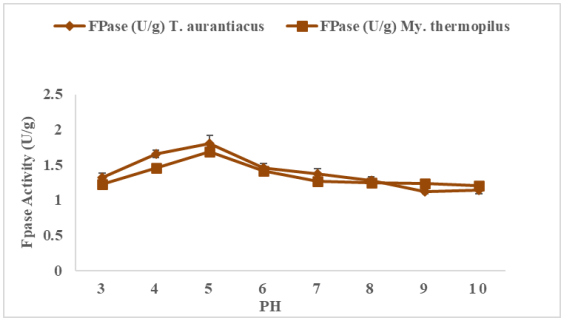

Figure (9): Effect of pH on Fpase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

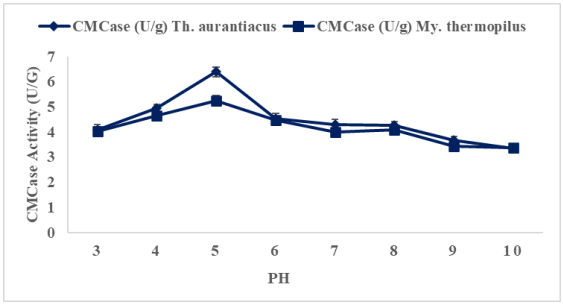

Figure (10): Effect of pH on CMCase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

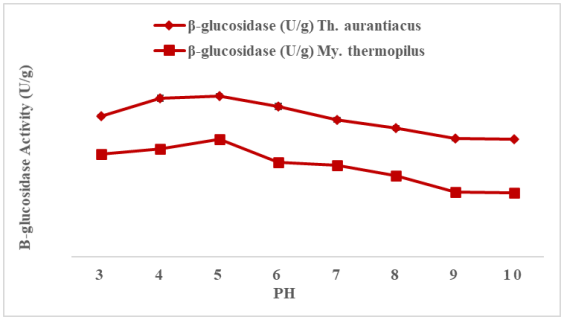

Figure (11): Effect of pH on β-glucosidase activity from *T. aurantiacus* MW559792 and *My. thermophilus* MZ723073.

Conclusion

Thermoascus aurantiacus MW559792 and *Mycothermus thermophilus* MZ723073 were active cellulolytic fungi. The cellulolytic activity of these fungi optimized on solid-state fermentation at various culture condition parameters. Utilization of various lignocellulosic wastes by SSF is an economical technique for enzyme production. Their cellulolytic machinery gave them the potentiality for using industrially in bioconversion processes of lignocellulosic material especially in the saccharification processes. Ultimately, fungal enzymes can be produced on large scale using agriculture

residues and used effectively in the mass production of cellulolytic enzymes.

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الملخص العربي

عنوان البحث: الأنشطة المحللة للسليلوز للفطرين المحبين للحرارة ثيرموأسكس أورانتياكس وميكوثيرمس ثيرموفيللس

السيد محمد المرسي[١]، محمد مجدي المتولي[١]، سالي أحمد شبارة[١]، مروة توكل محيسن[١]
[١] قسم النبات والميكروبيولوجي- كليه العلوم - جامعة دمياط - دمياط الجديدة - مصر

تم عزل سبعة أنواع محللة للسليلوز ومحبة للحرارة من بيئات حرارية مختلفة على وسط أجار المعدلة مع كربوكسي ميثيل سليلوز لتحل محل الجلوكوز. Czapek's Dox حيث تم التعرف على الفطريات المعزولة بناءً على خصائصها المورفولوجية والتماثل المتسلسل لتسلسلات جينات أر إن إيه الريبوسي الخاصة بها. و تم استخدام اختبار الكونغو الأحمر لعرض نشاطها السليلوزي, (نشاط التحلل السليلوزي لخمس أنواع مختارة تم اختباره على البيئة الصلبة FPase – CMCase – β -glucosidase). كم تم إختيار الفطرين ثيرموأسكس أورانتياكس وميكوثيرمس ثيرموفيللس لتجربة التحسين بناءً على إنتاجيتهم القصوى من السليوليز. كما تم تحسين النمو على البيئة الصلبة, من بين خمسة من مخلفات ليجنوسليلوزية استخدمت كمصادر للكربون ، كان قش الأرز هو الأكثر ملاءمة لإنتاج أقصى قدر من FPase (1.78, 1.45 U/g), CMCase (6.18, 4.09 U/g) and β-glucosidase (31.93, 16.23 U/g) من الثيرموأسكس أورانتياكس والميكوثيرمس ثيرموفيللس على الترتيب. المواد الليجنوسليولوزية لفطرة ثيرمواسكس اورانتياكس حسب الترتيب التالي: قش الأرز> قش الذرة> قش القمح> كوز الذرة> قشر الأرز،ولفطرة ميكوثيرمس ثيرموفيللس:قش الارز>قش القمح>قش الذرة>كوز الذرة>قشر الارز.. كما كان البيتون هو المصدر النيتروجيني المناسب لأعلى نشاط FPase (1.88, 1.46 U/g), CMCase (7.02, 4.89 U/g) and β-glucosidase (28.60, 19.91 U/g) من الثيرموأسكس أورانتياكس والميكوثيرمس ثيرموفيللس على الترتيب. و كانت ملائمة المصادر النيتروجينية على الترتيب التالي البيتون > مستخلص الخميرة > كبريتات الأمونيوم> مستخلص اللحم>نترات الأمونيوم> نترات الصوديوم> نترات البوتاسيوم. كما كان أقصى نشاط سليلوزي لكلا الفطرين الثيرموأسكس أورانتياكس والميكوثيرمس ثيرموفيللس عند الرقم الهيدروجيني ٥, درجة حرارة ٥٠ و ٤٥, وبعد ٤ إلى ٦ أيام من التحضين على الترتيب. وعلى هذا الأساس تم اقتراح استخدام الثيرموأسكس أورانتياكس والميكوثيرمس ثيرموفيلس صناعيا للتحويل الحيوي للمخلفات السليوليزية في مرحلة التسكير.