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Impact of Thermotolerance Response on the Physiological and Genomic Variation of *Fusarium oxysporum*

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FUSARIUM oxysporum strain F2 was isolated from the soil. It was morphologically and molecularly identified. The isolate showed promising thermotolerance characteristics. This was shown through the production of permanent proteins after 120min of pre-thermal exposure at 40°C, as detected by SDS-PAGE. On the other hand, the RAPD marker revealed higher genetic variations produced by *Fusarium oxysporum* than the ISSR marker. Thirteen ISSR primers yielded 137 total bands, of which 81 were polymorphic bands with a percentage of 59.12%. While 18 RAPD primers yielded 223 total bands, 141 of which were polymorphic bands with a percentage of 63.23%. Notably, elevated temperatures triggered the isolate to enhance the production of hydrolytic enzymes. *Fusarium oxysporum* strain F2 produced more cellulolytic and pectinolytic enzymes until 120min pre-thermal exposure at 40°C, displaying 3.262 and 1.797 (IU/mL), with corresponding percentages of activation 82.031% and 73.288%, respectively. This promising yield of cellulolytic activity could be employed in biofuel production.

Keywords: Cell viability, *Fusarium oxysporium,* Heat shocked Proteins (Hsps), Hydrolytic enzymes, ISSR and RAPD markers, SDS- gel electrophoresis.

Introduction

Fusarium oxysporum is a well-known species of soil-borne fungi capable of causing significant losses to horticultural and ornamental crops throughout temperate and subtropical climatic regions. The *Fusarium oxysporum* isolates are responsible for wilting vascular plant tissues and damping-off of many annual vegetables along with perennial weeds. Control and disease management of *Fusarium* is complicated due to the high frequency of the fungus as a soil saprophytic organism (Chen et al., 2019).

To resist stress, organisms exhibit molecular chaperones modulated as heat-shock proteins (Hsps) with specific biological functions. They are classified into six main families according to their molecular weights: (i) Hsp20, (ii) Hsp40, (iii) Hsp60, (iv) Hsp70, (v) Hsp90 and (vi) Hsp100 (Ahmad et al., 2015). In most organisms, HSPs are induced by abnormal physiological conditions. However, their expression can suddenly increase in response to abiotic physical stimuli, including radiation, heat shock, or chemicals. It may be expressed in response to biotic stress, such as microbial interactions with pathogenic viruses, bacteria, fungi, and parasite stimuli. These proteins employ a variety of elicitors responsible for cellular signaling pathways to simultaneously protect cells against biotic and abiotic stresses (Gong et al., 2017).

The thermal shock proteins are thought to play a functional role during the repair of heat-injured cells and are involved as molecular chaperones

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in the re-folding of denatured proteins. Examples of such heat shock-associated chaperones are DnaK, GroEL, and GroES (Rosen & Ron, 2002). Other heat shock proteins, such as CplC and CplP, have ATP-dependent protease activity, and their induction is thought to ensure stress tolerance and degradation of heat-damaged proteins (Krüger et al., 2001).

Different molecular markers, Random Amplified Polymorphic Deoxyribonucleic acid (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Inter Simple Sequence Repeats (ISSRs) helped focus on genetic diversity and to identify markers associated with stresses in microorganisms (Dubey & Singh, 2008; Dubey et al., 2010; Noman et al., 2022). The major advantage of markers such as RAPD and ISSR is that they do not need any pre-sequence information. ISSR has been employed to develop Sequence Characterized Amplified Region (SCAR) markers for studying genetic diversity, phylogeny, gene tagging, genome mapping, and/or evolutionary biology within *Fusarium oxysporum* (Singh & Kapoor, 2018).

Fusarium is a cellulase source used as a hydrolytic compound in the food, cosmetic, drug, paper, textile, and detergent industries. Cellulase is also used to produce orange vinegar and clarify citrus juice. Like the pectinase enzyme, cellulase can be used in the wine industry (Soares et al., 2012). Pectinase enzymes have shown considerable influence in the fruit and textile industries. The role of acidic pectinases in decreasing the cloudiness and bitterness of fruit juices. At the same time, alkaline pectinases in the textile industry are used for retting and degumming fiber crops, producing good quality paper, fermentation of coffee and tea, oil extractions and treatment of pectic wastewater recorded (Kashyap et al., 2001).

Cellulases occupy about 20% of the enzyme market (Singhania et al., 2010). They are the third most commonly consumed enzymes in the applied industry. With the growing demand for bioenergy and bio-based materials, the future of commercial cellulase production is looking bright (Yoon et al., 2014). One of the biggest hindrances to employing cellulose in bio-refinery is the cost of large-scale production, especially since it is difficult to increase the over-enzyme production using cheap substrates or optimize enzymes with higher stability and specific activity on synthetic fermentative media (Zhang et al., 2006). Based on estimates, nearly 25% of every dollar used to produce bioethanol commercially is spent during enzyme production (Huberman et al., 2016), with around 100 grams of cellulase being used per gallon of ethanol (Zhu et al., 2009).

Improving the fungus's productive capacity at the hydrolytic enzymatic activity level is one of the positive factors that can be industrially exploited to produce biofuels. Waste management and producing clean and low-cost energy are two major challenges facing our societies. Food waste (FW) can be used as a source for ethanol production due to its rich composition of cellulose, hemicellulose, and starch, but the cost is an obstacle. Production of bioethanol from biomass is one way to minimize the consumption of crude oil and environmental pollution. According to Prasoulas et al. (2020), *Fusarium oxysporum* was used to hydrolyse FW and the subsequent ethanol production. A combination of *F. oxysporum* and *S. cerevisiae* cultures also boosts ethanol production.

The current work's objective is to evaluate heat shock's effect on some physiological and molecular characteristics of *Fusarium oxysporum* following heat adaptation at different elevated temperatures. This will improve the productivity of cellulase.

Materials and Methods

Rhizospheric isolation of Fusarium oxysporum to obtain an axenic culture

Fusarium oxysporum (Fo) was isolated from the rhizospheric region of infected tomatoes. One gram of soil sample was added to 10mL of diluent. One mL of the previous sample was serially diluted (ten-fold dilution) by transferring aliquots to the diluent. The diluent is (0.85gm/100mL NaCl and 0.1gm/100mL peptone). One mL from each aliquot was plated onto Czapek-Dox agar plates and incubated at 25°C for five days.

Molecular identification of Fusarium sp.

DNA extraction was carried out using DNeasy® Plant Mini Kit (Cat. No. 69104, QIAGEN, USA) following the manufacturer's directions. Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′- GCTGCGTTCTTCATCGATGC-3′) were used to amplify the ITS1 region of the rRNA genes (White et al., 1990). The amplified

PCR products were sequenced in Macrogen, Inc. (Seoul, Republic of Korea) using an ABI 3730xl DNA sequencer. BLAST search analysis was performed against the nucleotide sequences deposited in GenBank. The obtained ITS sequence was aligned using the ClustalW algorithm (https://www.genome.jp/tools-bin/clustalw).

Heat-shock treatment

Freshly prepared fungal cells were inoculated into 50 ml sterile Czapek-Dox broth flasks (parent *Fusarium oxysporum*; PFo) and were kept at the optimum temperature (25°C) for five days. To track Hsps; the parent culture was then heated to a selective heat shock temperature (40°C; Sterflinger et al., 2012) for different periods; 0.5, 1, 1.5, and 2h; (thermally treated *Fusarium oxysporum*; TFo). The latter was recovered by incubating at the optimum temperature for five days (recovered *Fusarium oxysporum*; RFo).

Fusarium oxysporum response against different elevated temperatures:

Aliquots of 50mL Czapek-Dox broth medium, inoculated with 0.5 mm disc of thermal preheated two hours RFo cells, were incubated at different temperatures (25, 35, 40, 45, and 50ºC) for five days (Stressed *Fusarium oxysporum*; SFo).

The protein content of (TFo, RFo, and SFo) cells was assayed compared to PFo cells using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, genetic differences were determined using ISSR and RAPD markers.

The percentage of viability was determined as a relative percentage ratio compared with the control as follows:

Survival rate (%) = (A / T) x 100

where A is the (CFU) of the survival colonies after thermal treatment and T is the (CFU) of the control (untreated) colonies (Aly et al., 2016).

Protein extraction and identification

After heat shock and thermal stress treatments, the dry fungal mats were recovered for protein and molecular assays.

Fungal mycelia were harvested using Whatman filter paper and washed carefully with distilled water. The harvested dry mycelia were weighed, mixed with liquid nitrogen, and ground to powder. The obtained culture-free supernatant (CFS) was used for SDS PAGE analysis (12% acrylamide gel), according to Laemmli (1970). Gels were stained with Coomasie brilliant blue R-250 before being photographed and scanned. Data were documented through Alphatec 2200 software.

DNA extraction

The genomic DNA was extracted from all fungal cells using DNeasy® Plant Mini Kit (Cat. No. 69104, QIAGEN, USA), according to the manufacturer's directions.

ISSR and RAPD analysis

Out of 30 ISSR (Eurofins, Germany) and 30 RAPD (Operon, Germany) primers initially tested, 14 ISSR primers and 19 RAPD primers were further selected to screen genomic variation in fungal cells. The reactions were applied in 25µL including 200µM of dNTPs, 2.5mM of $MgCl_2$ in 1X GoTaq® Flexi buffer, 20pM of each. The authors did not design the primers. Primers were designed and provided by Eurofins Genomics, Germany. The primer, 30ng of template DNA, and 1 U of GoTaq® Flexi DNA (Promega Corporation; USA). The amplification process was done in a Gene Amplification® PCR 9700 System thermal cycler. The oriented program applied for the ISSR reaction was; primary denature at 94°C for 5min (1 cycle), followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 48°C for 50sec, extension at 72°C for 60sec; and a final extension at 72°C for 7min (1 cycle). The parameters applied for the RAPD reaction were; primary denature at 95°C for 5min (1 cycle), followed by denature at 95°C for 45sec, annealing at 37°C for 60 sec, extension at 72°C for 120sec for 40 cycles; and a final extension at 72°C for 7min (1 cycle) and was stored at 4°C. Amplification products were visualized on 1.2% agarose gels stained with ethidium bromide (Mulè et al., 2004).

Determination of fungal biomass and viability

According to Wu et al. (2009) and El Hassni et al. (2021), the fungal biomass was recovered by filtration, dried at 60°C for 24h, and weighed. In contrast, fungal viability was estimated as a conidial forming unit (CFU). Each Erlenmeyer flask was amended with 0.1mL inoculum from 7 days old stock culture, shacked well, and then serially diluted. 0.1 from dilution (10^4) was inoculated on potato dextrose agar (PDA) by spread plate method. Three triplicate dishes from

each thermal treatment were incubated at 25°C for five days, after which the colonies were counted.

Physiological assays

Cellulase and pectinase assay

A total of 0.1mL from pre-cultivated five days old stock culture of PFo cells was grown in Czapek broth medium supplemented with 1% carboxymethylcellulose (CMC) or 1% citrus pectin as a unique carbon source instead of sucrose to estimate the enzymatic activity of cellulase and pectinase respectively. Cultures were pre-heated at 40°C for 0, 30, 60, 90, 120, and 150min. All treatments were assayed in triplicates, using 0.3mL of fungal filtrate as a source for the enzyme. The reaction mixture also included 1.7mL phosphate citrate buffer (pH 6), and 1mL 0.25% of CMC. The reaction was incubated at 55°C for 15min. and then terminated using 1mL 3,5-dinitro salicylic acid (DNS) solution and heating the mixture in a 100°C water bath for 10min, then cooled down to room temperature. Shortly afterwards, the absorbance was measured at 546nm, and a calibration curve was prepared using an aqueous D-glucose solution. Cellulase activity was estimated according to Kumar et al. (2012) using CMC as the substrate, while Pectinase activity followed the method of Roboz et al. (1952). Enzyme activation percentage was estimated according to this equation:

% Enzyme activation= (activity of treated (TFo)-activity of control (PFo))/ activity of control (PFo)

Statistical analysis

Statistical analysis was performed through the standard SPSS program (version 20, SPSS Inc., Chicago, IL, USA) to measure standard deviation.

Results

Molecular identification for the selected microorganism

The selected isolate (Fo) was subjected to ITS sequence analysis. BLAST analysis of the nucleotide sequence of the region amplified from Fo showed a similarity of 99.6% with *Fusarium oxysporum* species and was recorded with accession number MW722788.

Detection of heat shock temperature

Fusarium oxysporum revealed higher permanent HSPs protein production after 120min prethermal exposure at 40°C. This analysis identified seven novel protein bands at molecular weights of 147, 88, 73, 57, 39, 22, and 12kDa. On the other hand, three temporarily expressed protein bands were recorded at molecular weights 37, 27, and 18 that disappeared after a recovered-incubation for five days (Fig. 1).

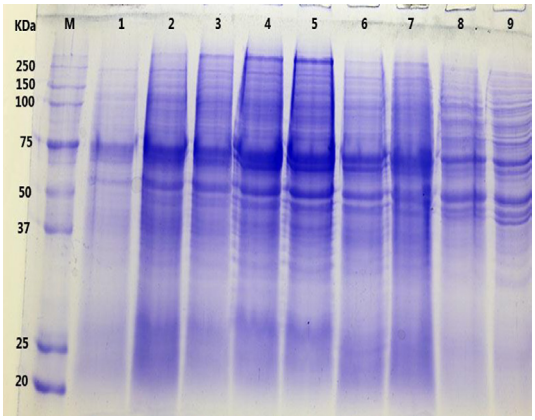

Fig. 1. SDS-PAGE gel image shows Protein bands of *F. oxysporum* (Fo) included lane 1, Marker; lane 2, Control (PFO); lane 3, Fo treated thermally for 30min; lane 4, Fo treated thermally for 60min; lane 5, Fo treated thermally for 90min; lane 6, Fo treated thermally for 120min; lane 7, Fo recovered after 30min; lane 8, Fo recovered after 60min; lane 9, Fo recovered after 90min; lane 10, Fo recovered after 120min [Note: The thermal treated Fo is subjected directly to heat shock in their original media but the recovered treatment is reinoculated to newly fresh prepared media without further heat shock]

Protein pattern of the HSPs

Heat treatment of PFo at 40°C induced a set of new heat shock proteins which belong to different families of heat shock proteins, four of which (12, 39, 57, and 147kDa) were common for all thermal treatments and were permanently stable throughout the remaining treatments. Notably, the number of unique bands expressed at 88kDa protein occurred when cells were subjected to prethermal exposure for 30min and at 73kDa through pretreated heat shock for 90min. A much higher rate of protein synthesis was observed at 120min, which induced three newly formed proteins at 22, 73, and 88kDa in addition to the previously mentioned four common heat shock proteins. A 120min heat shock-induced treatment was further chosen as the treated control for other stresses due to the high expression of new numbers of heat shock proteins by Fo (Table 1).

The abundant existence of heat shock proteins was monitored during different thermal exposure times to detect the permanent stability of these proteins.

Effect of heat shock at the molecular level for Fusarium oxysporum

To detect the effect of heat shock on the PFo, TFo, and RFo cells at the molecular level, an initial experiment was conducted that used 30 ISSR primers, out of which only three (ISSR UBC-836, ISSR UBC-841, and ISSR UBC-842) generated recordable results (Fig. 2), with a total polymorphism of 50 % (Table 2).

TABLE 1. Analysis of protein bands of PFo, TFo, and RFo cells

Molecular weight (kDa)	Control PFo cells	Treatment (40°C) TFo cells and RFo cells							
	$Fo_{0\ min}$	$Fo_{30\ min}$		$Fo_{60\ min}$		$Fo_{90\ min}$		$Fo_{120\ min}$	
		T	R	T	R	T	R	T	R
147.250	-	+	+	+	+	+	+	+	+
123.382	+	+	+	+	+	+	+	+	+
107.143	+	+	+	+	+	+	+	+	+
96.620	+	+	+	+	+	+	+	+	+
90.142	-	-	-	-	-	-	+	-	-
88.313	-	+	+	+	-	+	-	+	+
80.625	+	+	+	+	+	+	+	+	+
73.038	-	+	-	+	-	+	+	+	+
67.589	+	+	+	+	+	+	+	+	+
57.874	-	+	+	+	+	+	+	+	+
45.258	+	+	+	+	+	+	+	+	+
39.942	-	+	+	+	+	+	+	+	+
37.477	-	-	-	-	-	-	+	+	-
35.309	+	+	+	+	+	+	+	+	+
32.682	+	-	+	+	+	-	+	+	+
28.884	+	-	-	+	+	-	-	+	-
27.536	-	-	-	-	-	-	+	+	-
25.104	+	+	+	+	+	+	+	+	+
22.454	-	+	-	-	-	+	-	+	+
19.956	-	-	-	-	+	-	-	-	-
18.679	-	-	-	-	+	+	-	+	-
15.026	+	+	+	+	+	+	+	+	+
12.042	-	+	+	+	+	+	+	+	+
11.459	+	-	-	-	-	-	-	-	-

T: is thermally treated Fo cells while R: is recovered Fo cells

TABLE 2. Effect of heat shock on Fo as revealed by ISSR primers

Primer name	Primer sequence (5'→3')	Total bands	Polymorphic bands	Polymorphic bands (%)	Unique positive bands(bp)	Unique negative bands(bp)	Total unique bands
ISSR UBC-836	$(AG)_8YA$	8	3	37.5	-	2200(RFo120)	1
ISSR UBC-841	$(GA)_8YC$	9	7	77.8	-	1300(TFo60)	1
ISSR UBC-842	$(GA)_8YG$	11	4	36.4	-	3000(RFo90), 2500(RFo90), 2200(RFo90)	3
TOTAL		28	14	50	-	5	5

A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C or T), H: (A or C or T), V: (A or C or G), R: (A or G)

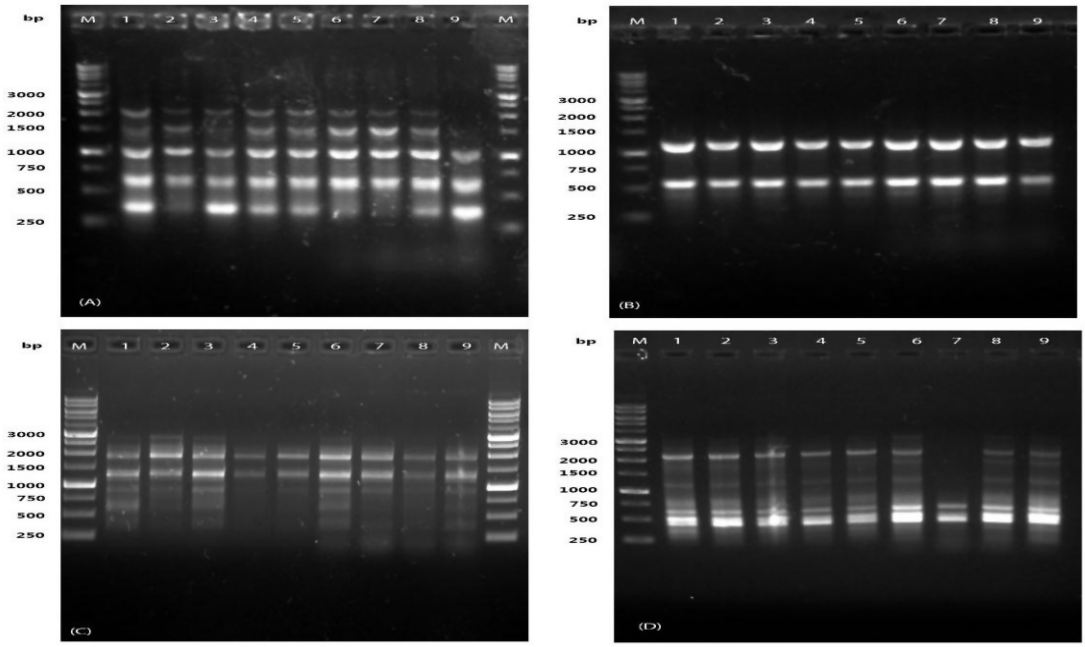

Fig. 2. A representative ISSR profile of the *Fusarium oxysporum* cells under Heat Shock with primers UBC-836 (A), UBC-840, (monomorphic) (B), UBC-841 (C), and UBC-842 (D) [M 1kb DNA Ladder]

Effect of elevated temperature stress on Fusarium oxysporum proteins as revealed by SDS-PAGE profile

Raising the temperature above the optimum produced new temporary proteins (140.9 and 31.7kDa) in cells treated at 40°C. Higher temperatures decayed most of the small heat shock protein family. Otherwise, the most permanent proteins produced from heat shock (39.9, 57, 73.7, and 88.8kDA) were considered common for thermal stress. In contrast, the small heat shock protein family (12.4 and 22.6kDa) could not tolerate higher temperatures (Fig. 3, Table 3).

Effect of temperature stress on Fusarium oxysporum DNA as revealed by ISSR and RAPD

Remarkably, 4 ISSR primers (Fig. 4) yielded 47 total bands; 36 were polymorphic with an average percentage of 76.6%. The ISSR UBC836 primer produced 11 full bands with nine unique bands (all found at control 25°C), seven distinct positive bands, and two negative bands). The ISSR UBC840 primer provided 15 full bands, including 8 unique bands (all at control 25°C); 5 were the only positive bands, while the remaining 3 were unique negative ones. Furthermore, the ISSR UBC841 primer generated thirteen bands without any unique bands. The distinct negative bands at 450bp and 600bp were observed with ISSR UBC842 primer at 40°C SFo (Table 4I).

Interestingly, 6 RAPD primers (Fig. 5) gave a polymorphic band percentage of 54.7% with 53 total bands. The highest polymorphic percentage was achieved by OPC16 primer (88.9%) with the highest total unique bands (7 unique bands; 3 unique positive bands and 4 unique negative bands), while the lowest polymorphic percentage was recorded by OPA04 primer (22.2%) with 2 unique positive bands (150 and 350) (Table 4II).

Viability test of PFo, TFo, and SFo cells

Data revealed no significant difference between the growth rate of all RFo mats with time and heat shock treatments (Fig. 6A). RFo cells, on the other hand, grew significantly faster than PFo and TFo cells. Notably, at 120 min., the total number of permanent heat shock proteins was at its highest (Table 1). Thus, RFo cells at 120 min were further chosen to represent the parent for the subsequent elevated temperature stress. In this scenario, thermal exposed mycelial mats of Fo showed variable growth patterns between PFo cells and TFo cells. Increasing temperature led to growth suppression, but TFo cells showed significant growth rates at 45°C while PFo cells did not (Fig. 6B).

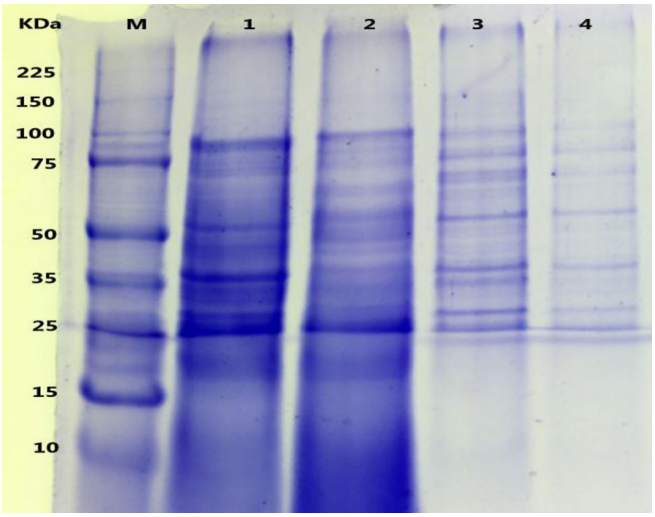

Fig. 3. SDS-PAGE gel image of Protein bands of *Fusarium oxysporum* under elevated temperatures as heat stress: lane 1, Marker; lane 2, *F. oxysporum* pre-thermally exposed to 25°C lane 4, *F. oxysporum* pre-thermally exposed to 35°C; lane 5 , *F. oxysporum* pre-thermally exposed to 40°C; lane 6, *F. oxysporum* pre-thermally exposed to 45°C

TABLE 3. Effect of different temperatures on Fo as revealed by SDS-PAGE

Molecular weight (kDa)	Treatment			
	Temperature stress on Fo			
	25 °C	35 °C	40 °C	45 °C
166.501	+	+	+	-
140.927	-	-	+	+
123.147	+	+	+	-
107.498	+	+	+	-
88.853	+	+	+	+
73.673	+	+	+	+
67.534	+	+	-	-
57.024	+	+	+	+
50.008	+	+	+	-
45.417	+	-	-	-
39.879	+	+	+	+
37.784	+	-	-	-
31.754	-	-	+	-
28.579	+	+	+	+
25.404	+	-	-	-
22.588	+	+	+	-
20.235	+	+	+	+
12.394	+	+	-	-

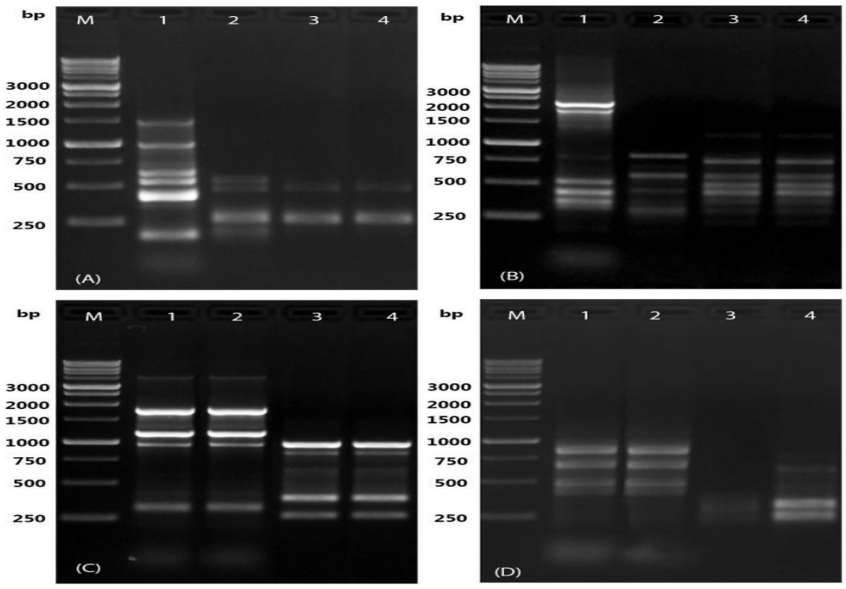

Fig. 4. ISSR profile of *Fusarium oxysporum* under temperature stress with primers ISSR 836 (A), ISSR UBC840 (B), ISSR UBC841 (C), and ISSR UBC842 (D) [M 1kb DNA Ladder]

TABLE 4. Effect of temperature stress on Fo as revealed by ISSR primers and RAPD primers

I) ISSR primers							
Primer Name	**Primer sequence (5'→3')**	**Total Bands**	**Polymorphic bands**	**Polymorphic Bands (%)**	**Unique positive bands(bp)**	**Unique negative bands(bp)**	**Total Unique bands**
ISSR UBC-836	$(AG)_8$ YA	11	10	90.9	1450, 1000, 700, 400, 350, 200, 150	450,250	9
ISSR UBC-840	$(GA)_8$ YT	15	10	66.7	2600, 2400, 1400, 800, 150	850, 350, 250	8
ISSR UBC-841	$(GA)_8$ YC	13	10	76.9	-	-	-
ISSR UBC-842	$(GA)_8$ YG	8	6	75.0	-	600, 450	2
TOTAL		47	36	76.6	12	7	19
II) RAPD primers							
OPA 02	TGCCGAGCTG	11	5	45.5	1600(25°C),750(25°C), 600(45°C),	300(25°C)	4
OPA 04	AATCGGGCTG	9	2	22.2	800(25°C)	150(25°C)	2
OPB 04	GGACTGGAGT	12	9	75.0	350, 150(both at 40°C)	-	2
OPC 11	AAAGCTGCGG	7	3	42.9	-	-	-
OPC 16	CACACTCCAG	9	8	88.9	1400(25°C), 500(25°C),350(35°C)	900(35°C), 700(35°C), 300(25°C), 150(25°C)	6
OPD 08	GTGTGCCCCA	5	2	40.0	750, 650(both at 25°C)	-	2
TOTAL		53	29	54.7	11	6	16

A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C or T), H: (A or C or T), V:(A or C or G), R: (A or G)

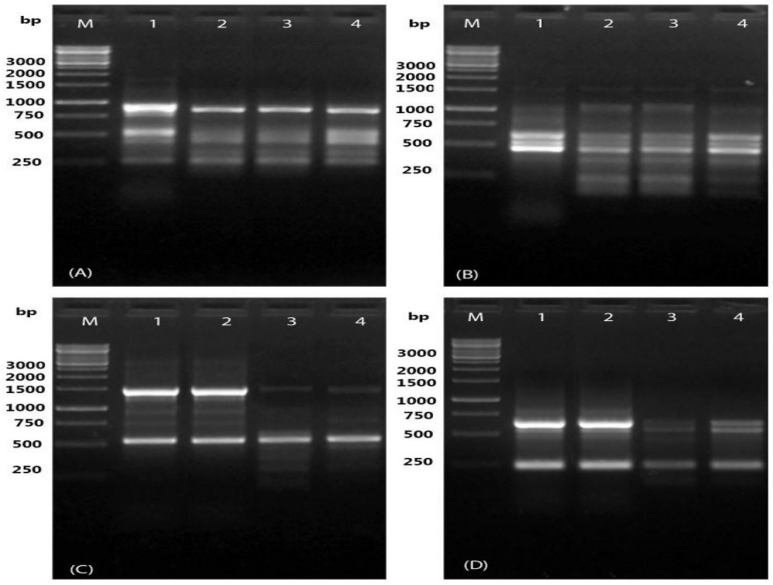

Fig. 5. RAPD profile of *Fusarium oxysporum* under temperature stress with primers OPA02 (A), OPA04 (B), OPB04 (C), and OPC11 (D) [M 1kb DNA Ladder]

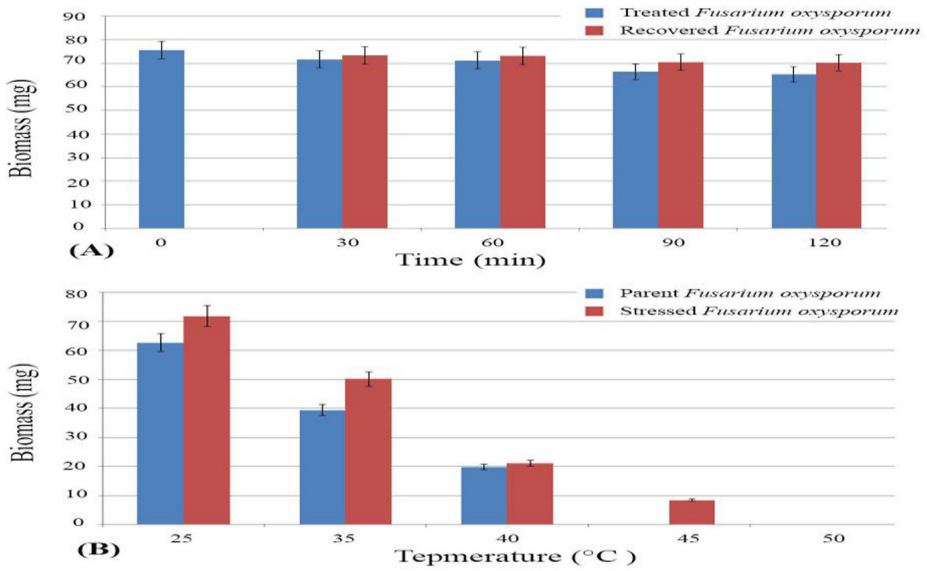

Fig. 6. The effect of heat shock (A) and temperature (B) on the TFo and RFo cells' growth compared with the PFo cells

Effect of different preheated application times on cellulolytic and pectinolytic activities of Fusarium oxysporum Strain F2

The cellulolytic and pectinolytic activities are presented in Table 5 after five incubation days (expressed as one IU/mL reducing sugar/ one mL medium). Data indicated a substantial up-regulation of the cellulolytic activity (3.262IU/mL) of Fo till 120min with a corresponding 82.031% per cent of activation over the control (PFo). Similarly, a significant increase in the pectinolytic activity (1.797IU/mL) of Fo till 120min with a corresponding 73.288% per cent of activation. There was no significant reduction in cellulolytic and pectinolytic activity as the exposure time increased from 120 to 150min. Heat shock-induced a marked-significant increase with time from 30 to 120min. The cellulolytic and pectinolytic per cent of activation varied from 6.194 to 82.031% and 10.125% to 73.288%, respectively (Table 5).

TABLE 5. Effect of different preheated application times on cellulolytic and pectinolytic activity of Fo after five incubation days (expressed as one IU/mL reducing sugar/ one mL medium)

Treatment / Time of heat pre-exposure	Cellulolytic activity (IU/mL)	% of activation regarding control treatment	Pectinolytic activity (IU/mL)	% of activation regarding control treatment
Control (0 time)	1.792	-	1.037	-
30	1.903	6.194	1.142	10.125
60	2.287	27.623	1.272	22.662
90	2.683	49.720	1.483	43.009
120	3.262	82.031	1.797	73.288
150	3.218	79.576	1.762	69.913

The micromoles (µ mol/min) of reducing sugars released by one ml enzyme in one min is regarded as one IU/mL.

Discussion

Heat shock response is a common cellular reaction that protects cells and organisms against elevated temperatures. This reaction is associated with the biosynthesis of heat shock proteins (HSPs), whose synthesis is also induced by other stressors, such as osmotic pressure and pH variation (Wang et al., 2004).

The detected viability of the tested microorganisms showed significant variation in growth after heat shock treatments as their growth decreased by increasing the time of exposure to heat. This may be attributed to thermal stress responses in microorganisms that influence the life cycle and cellular processes. In this trend, the optimum growing temperature for most fungi is around 25°C. The sudden increase in temperature and time of exposure generally causes virulence attenuation and eventually results in the death of the tested organism (Lamoth et al., 2012).

In contrast, Hsps production represents a rapid and potent cellular response to different stresses. During cell stress, Hsps usually interact with denatured protein subunits and inhibit the biosynthesis of cytotoxic protein molecules; hence they maintain the stability of cellular metabolic proteins (Sharma et al., 2009). The heat shock response induction improves cell survival during thermal drying (Corcoran et al., 2008).

Heat stress activates the production of various reactive oxygen species (ROS) (Abrashev et al., 2008). Various ROS-digesting proteins can be enhanced due to heat stress pre-exposure, such as superoxide dismutase, catalase, glutaredoxin–glutathione reductases (Zhang et al., 2017) thioredoxin reductases, and several protein families, in addition to cellulose, pectinase, protease, lipase, and chitinase enzymes along with Hsps are involved in heat stress response (HSR).

In the study conducted by Ruiz-Roldán et al. (2008), it was demonstrated that the sti35 gene of *Fusarium oxysporum* was induced under different stress conditions. The expression of the sti**35** gene is repressed by nucleotide base-thiamine and activated at high temperatures. Heterologous gene expression of sti**35** by *Saccharomyces cerevisiae* occurred in the thi**4** mutant and enhanced UV tolerance in a mutant uvr (-) of *Escherichia coli.* Deltasti35 mutants of *F. oxysporum* showed tolerance to menadione (superoxide-generating agent), indicating that Sti35 has a bifold action in thiamine biosynthesis and during the oxidative stress response. Our results showed that most of the permanent proteins produced by heat shock (39.9, 57, 73.7 and 88.8kDA) were commonly induced proteins for all our thermal treatments, corresponding to the over-expression of sti35 in *Fusarium oxysporum* during high temperatures.

Singh et al. (2019) reported that ISSR and RAPD markers effectively determined genetic variability among *Fusarium* isolates. Out of **90** RAPD and ISSR primers screened, **34** primers provided a comprehensive representation of genetic diversity. The results agree with abundant reports wherein ISSR and RAPD markers have been applied and were useful in analyzing the genetic biodiversity of members belonging to *F. oxysporum* (Dubey & Singh, 2008; Thangavelu et al., 2012).

Although a sounding level of polymorphism was attained using both markers, ISSR showed a higher level of polymorphism than MI and PIC. ISSR markers exhibit higher polymorphism when they amplify conserved repeated regions in-between microsatellite sequences, whereas RAPD markers act randomly through the entire genomic sequences. (Zietkiewicz et al., 1994).

RAPD and ISSR techniques proved to be potent in identifying resistant genotypes and effective in marker-assisted selection if applied to variable genetic backgrounds (Maisuria et al., 2017). Several researchers used RAPD and ISSR markers as identifier markers related to *Fusarium* wilt resistance, especially in chickpea cultivars (Ratnaparkhe et al., 1998 a, b) and in wheat and rice (Prabhu et al., 2009). The differences in polymorphism between RAPD and ISSR may be referred to as the amount of genetic variation between the samples under the study, as indicated by Poerba & Ahmad (2010).

Akbar et al. (2018) used ISSR to assess the genetic diversity between members of *Fusarium* species using two ISSR primers, (GA)9C and (GA)9T. ISSR analyses showed substantial genetic variability between all the *Fusarium* populations as a causative organism for tomatoes. These primers exhibited **303** bands corresponding to **400**–**4500**bp. However, the minimum and the maximum number of polymorphic ISSR fragments› limits fluctuate from three to seven per isolate.

Fusarium is a source of cellulase enzyme, which is used as a hydrolytic compound for different purposes. In the present study, it seemed beneficial to study the *in vitro* activities of hydrolytic enzymes, including cellulase and pectinase of *Fusarium oxysporum*, in response to different heat pre-exposure times (from 30 to 150min). The results clarified that there was progressive activation in the cellulolytic and pectinolytic activities of the test pathogen till 120 min pre-heat exposure, followed by a non-significant decrease from (90 to 150 preheat exposure). The maximum statistically significant increment was achieved at 120 min preheat exposure. Cellulolytic or pectinolytic activity was measured at 3.262 and 1.797, with associated activation per cent recorded at 82.031 and 73.288, respectively. Abrashev et al. (2008) and Zhang et al. (2017) also reported heat shock-induced fungi. They may involve the overexpression of catalytic enzymes of heat-shocked fungi to promote the ability to tolerate further stressors (Nantapong et al., 2019). However, the overproduction of cellulase due to thermal stress may be advantageous in producing bioethanol using cellulose as the sole carbon source utilized by *Fusarium oxysporum*.

Our future work will focus on optimizing cellulase in biofuel production using the costly large-scale overproduction of preheated stressed microorganisms and for a better understanding of the over-induction mechanisms of hyper cellulolytic and pectinolytic activities during different abiotic stresses.

Conclusion

Thermal shock positively affected fusarium enzymatic productivity and enhanced its stress-tolerance ability. Thus, it would be highly interesting to develop *Fusarium oxysporium* (FO) as a starter strain with an improved potential to temperature stress for a basic manufacturing application restricted to bioethanol production.

Competing interests: The authors report no conflicts of interest regarding this work.

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تأثير التحمل الحراري المستحث على الخصائص الفسيولوجية و التباين الجينومي لفطرة الفيوزاريوم اوكسيسبورم

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تم عزل فطرة الفيوزاريوم اكسيسبورم، وقد اظهرت عزلة من عزلات فطرة الفيوزاريوم اوكسيسبورم استجابة واعدة لدرجات الحرارة المرتفعة عن طريق إنتاج بروتينات دائمة وذلك بعد 120 دقيقة من التعافي من الاجهاد الحراري عند 40 درجة مئوية، كما تم اكتشافه بواسطة الفصل الكهربائي للبروتينات. وعلى الجانب الاخر، باستخدام المقارنة بين الدليل الجيني (رابيد) والدليل الجيني (اي اس اس ار) وجدت اختلافات جينية أعلى فى الدليل الاول عن نظيره الاخر بالنسبه لفطرة الفيوزاريم اكسيسبورم. باستخدام ثلاثة عشر بادئة من الدليل الجيني (اي اس اس ار) أنتجت 137 نطاق جيني مرجعي ،منها 81 نطاق متعدد الأشكال بنسبة ٪59.12. بينما باستخدام ثمانية عشر بادئة من الدليل الجيني (رابيد) أنتجت 233 نطاق جيني مرجعى، منها 141 متعددة الأشكال بنسبة ٪63.23 . يليها عند التعرض لدرجات حرارة مرتفعة أدت إلى استحثاث بروتينات قابلة للذوبان (إنزيمات تحلل مائي). ومن الجدير بالذكر ان إنتاج السليوليز لفطره فيوزاريوم اوكسيسبورم قد ارتفع كميًا بعد 120 دقيقة من التعرض الحراري المسبق للعزلة عند 40 درجة مئوية والتي سجلت 3.262 ميكرو مول لكل دقيقه نشاطًا لتحلل النسيج السيليولوزي مع نسبة تنشيط موازية و التي سجلت ٪82.031. وبالمثل و قد اتبع انزيم البكتينيز نفس نظيره السليوليزي مع قيم منخفضة نسبيًا عنها من القيم السيلوليزيه. يمكن استخدام هذه الزيادة المحتملة الواعدة في الانتاجية للإنزيم في إنتاج الوقود الحيوي.