Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Tracking of thermal microbial shock proteins and genomic variation of *Bacillus cereus* due to some abiotic stresses

Rastreamento de proteínas de choque microbiano térmico e variação genômica de *Bacillus cereus* devido a alguns estresses abióticos

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Abstract

Bacillus cereus is considered the most potent bacterial strain in terms of the increment in induced proteins during thermal treatment at 52 °C for 90 min. Protein production in food-born microorganism (*Bacillus cereus*) recovered from contaminated food was investigated in response to heat shock treatment. Bacterial tolerance towards pH, salinity, and temperature at various levels was also investigated. Heat-shock proteins (HSPs) produced when exposed to 52 °C for up to 60 minutes led to significant differences (30%) above the untreated control (37 °C), and the maximum difference was recorded at 52 °C at 90 minutes. ISSR detected a higher number of bands/primer than RAPD (13.7 vs. 12.7, respectively), and more polymorphic bands (10.7 vs. 8.4 bands/primer, respectively). The untreated bacterial strain did not grow at pH levels lower than 3, whereas the thermally treated strain grew significantly at pH two. A consistent increase in HSPs was observed, with a gradual increase in salinity of less than 16%. Surprisingly, the gradual increase in temperature did not induce tolerance against higher temperatures. However, a significant growth rate was noticed in response to heat-shocked treatments. The untreated *Bacillus cereus* demonstrated antibiotic resistance to gentamycin and clindamycin (1.54 and 1.65 cm, respectively), much lower than the corresponding inhibition areas with preheat-treated test pathogen which were recorded (2.37 and 2.49 cm, respectively).

Keywords: *Bacillus cereus*, HSPs proteins, SDS gel electrophoresis, ISSR and RAPD markers, genomic variations, hydrolytic enzymes, bacterial sensitivity, gentamycin and clindamycin.

Resumo

Bacillus cereus é considerada a cepa bacteriana mais potente em termos de incremento de proteínas induzidas durante o tratamento térmico a 52 °C por 90 min. A produção de proteínas em microorganismos de origem alimentar (*Bacillus cereus*) recuperados de alimentos contaminados foi investigada em resposta ao tratamento de choque térmico. A tolerância bacteriana ao pH, salinidade e temperatura em vários níveis também foram investigadas. Proteínas de choque térmico (HSPs) produzidas quando expostas a 52 °C por até 60 minutos levaram a diferenças significativas (30%) acima do controle não tratado (37 °C), e a diferença máxima foi registrada a 52 °C em 90 minutos . O ISSR detectou um maior número de bandas/iniciador do que o RAPD (13,7 vs. 12,7, respectivamente) e mais bandas polimórficas (10,7 vs. 8,4 bandas/iniciador, respectivamente). A cepa bacteriana não tratada não cresceu em níveis de pH abaixo de 3, enquanto a cepa tratada termicamente cresceu significativamente em pH dois. Observou-se aumento consistente de HSPs, com aumento gradual da salinidade inferior a 16%. Surpreendentemente, o aumento gradual da temperatura não induziu tolerância a temperaturas mais altas. No entanto, uma taxa de crescimento significativa foi observada em resposta aos tratamentos de choque térmico. O *Bacillus cereus* não tratado demonstrou resistência antibiótica à gentamicina e clindamicina (1,54 e 1,65 cm, respectivamente), muito menor do que as áreas de inibição correspondentes com patógeno de teste pré-tratado que foram registradas (2,37 e 2,49 cm, respectivamente).

Palavras-chave: *Bacillus cereus*, proteínas HSPs, eletroforese em gel SDS, marcadores ISSR e RAPD, variações genômicas, enzimas hidrolíticas, sensibilidade bacteriana, gentamicina e clindamicina.

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Received: October 10, 2022 – Accepted: February 9, 2023

1. Introduction

Microbial cells undergo stresses, including suboptimal growth variations and adverse environmental conditions that can impair cell viability or fitness (De Nadal et al., 2011). Stresses are either biological or non-biological; these include thermal (hot or cold) and non-thermal stress such as pH, salinity, and pressure (Mafart et al., 2001). A wide range of microorganisms, including thermophiles and psychrophiles, have adapted their life cycles to severe conditions. Other microorganisms develop temporary survival mechanisms until conditions change. To survive in favorable conditions, cells undergo physiological changes. These changes vary depending on the environment, stress, and type of organism.

HSPs are fast-hardening chaperons formed when cells are exposed to pre-lethal thermal temperatures for a short period of time. They protect cells and organisms from heat damage, restoring regular physiological cell activities and eventually leading to a higher thermal tolerance level. In addition, in some cases, small HSPs have been proven to play an essential role in the pathogenesis of microbes (Feder and Hofmann, 1999). Furthermore, HSPs significantly contribute to regulating protein quality by renaturing proteins denatured through heat shock (Zhong et al., 2013). Nevertheless, molecular chaperones are abundant within cellular environments, serving as a defensive mechanism against unfavorable conditions (Kim et al., 2013; Kumar et al., 2016). They play a vital role in protein-protein interactions, such as folding, forming the proper protein composition, and limiting the unwanted protein to be synthesized.

In addition, HSPs exist in non-stressful environments, controlling the cell's proteins. They transmit old proteins to proteasomes while assisting newly synthesized proteins in folding properly (Sharma, 2010). The size of molecular chaperones ranges from 10 to over 100 KDa, depending on the size, specific sites, and physiological principles of these chaperones (Jee, 2016).

In vitro HSPs protect altered proteins from irreversible aggregation by forming several soluble oligomeric complexes with non-native proteins (Haslbeck and Buchner, 2002). In addition, HSPs contribute to stabilizing biological substrates, assembling large molecules, the degradation of polypeptides, organizing transcription mechanisms (Bukau et al., 2006), and competitiveness among rhizobial isolates (Yap and Lim, 1983). While the possibility of survival of *Bacillus* spp. under heat shock is partially understood, the mechanism of cellular protection is not fully resolved (Noor et al., 2019).

Bacillus cereus is a positively gram-staining bacterium, a facultative anaerobe in terms of oxygen requirement, and a rod-shaped endospore-forming microorganism that inhabits soil to complete its saprophytic life cycle (Vilain et al., 2006; Hariram and Labbé, 2016). *B. cereus* lives in water, vegetables, and various other food resources due to its saprophytic soil life cycle. Because Bacillus has been shown to adapt to various stresses, it is crucial to understand the molecular basis of the response to stresses imposed on Bacillus (e.g., heat, salt, acid, and ethanol) (Browne and Dowds, 2001). Physiological studies demonstrated the effect of pH, ethanol (Casadei et al., 2001), and salt (Mahakarnchanakul and Beuchat, 1999) on heat tolerance in *B. cereus*. Unlike other PCR techniques, such as Real-time PCR (Zhao et al., 2018) and PCR-RFLP (Abdel-Rahman and Ahmed, 2007) no prior knowledge of the DNA sequence of the studied genome is required as primers bind to the genomic sequence at random.

The current study aims to investigate the effect of heat shock on the responses, biochemical changes, and physiological activities of *Bacillus cereus* isolate in terms of temperature, pH, and salinity, as well as to examine the microbial responses to such stresses by determining the HSPs novel protein synthesized and monitoring the variation at DNA molecular level using two different molecular markers; ISSR and RAPD.

2. Material and Methods

2.1. Isolation and purification of Bacillus cereus

Bacillus isolate was obtained from one gram of contaminated food samples by suspending it in 10 ml of osmotic diluent (0.85 gm/100 ml NaCl and 0.1gm/100 ml peptone) and allowing them to stand for 30 min. One ml from the diluted solution was seeded into 100 ml of King's medium B Base (Protease peptone 20 g/l, Dipotassium hydrogen phosphate 1.5 g/l, Magnesium sulfate heptahydrate 1.5 g/l, Agar 20 g/l; pH 7.2). Serial dilutions were performed using sterile distilled water. Then, one ml from each dilution was transferred into nutrient agar media (0.5% NaCl; 0.5% Peptone, 0.3% Beef extract powder, 20 g/l Agar; pH: 7.2) and was incubated at 37 °C. *Bacillus* is a spore former and heat-resistant microorganism. Under pasteurization 72 °C for 15 Sec, we get rid of all competitive microorganisms that form vegetative cells only during isolation. *Bacillus* can germinate from their spores after the finalization of thermal treatment. Isolation followed by purification was performed twice; one typical colony of each strain was suspended in 100 ml sterile distilled water and kept overnight at 37 °C with shaking. The suspension was then distributed over LB agar media (0.5% NaCl; 0.5% Peptone, 0.3% Beef extract powder; pH: 7.2). Afterward, one standard colony from the nutrient agar plate was selected for further purification using 100 ml of King B media for *Bacillus isolate*.

The bacterial strain was identified according to staining procedures, microscopic examination, and cultural characteristics of fresh samples used by a combination of API system 20E and API 50 CHB strips and some additional biochemical tests described by (Borsodi et al., 2007). Moreover, the isolate was molecularly identified using 16S ribosomal RNA sequence analysis using 27F primer (5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991).

2.2. Inoculum preparation

Ten-fold dilution of bacterial inoculum using LB as a diluent was performed. The culture was incubated in a rotary shaking incubator at 200 rpm and 37 °C until the quantitative growth optical density at 600 nm was recorded.

2.3. Heat-shock treatment for bacterial isolate

As a preliminary heat shock treatment, a stressful temperature of 52 °C was used. Then, 1 ml of freshly prepared bacterial cells were inoculated into a 10 ml sterile LB broth medium, and the cultured media was incubated at 37 °C. A set of LB cultured broth media was held at the normal growth temperature (control) for *Bacillus* isolate, whereas other sets of LB broth media were heated to maintain the heat shock temperature for an isolate. Each tube was subjected to the tested heat shock temperature for different periods (30, 60, 90, and 120 min). The recovered filtrate was centrifuged at 6000 rpm for 10 min, and the precipitate was collected and used on sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) analysis.

2.4. Recovery of the thermally tolerant bacterial cells

As previously mentioned, a suspension of freshly prepared bacterial cells was heated at 52 °C for (30, 60, 90, and 120 min) and then was incubated at 37 °C. At the same time, a suspension of thermally untreated mid-exponential-phase bacterial cells was used as a control.

2.5. Abiotic stresses

pH, salinity, and thermal stresses were applied to the *Bacillus* isolate. The recovered Bacillus cells from each stress were assayed against the control by using both protein SDS-PAGE and ISSR and RAPD analysis.

2.6. Determination of the lethal point for pH, salt, and temperature

Bacillus isolate grown in LB at 37 °C for 4 h was diluted 1/10-fold and was grown to reach an OD of 0.5 at 600 nm to determine the lethal and non-killing (adaptive) levels of stresses. 1 ml from the tested bacteria was subjected to various pHs (2-12) and NaCl concentrations as an osmoticum with varying concentrations (2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, and 20%). For thermal stress, cells were diluted 1/10-fold using an LB broth medium preheated to the required temperatures (40, 45, 50, 55, 60, and 65 °C). Treated samples were serially diluted onto LB broth medium and were grown in a set of triplicates overnight at 37 °C. The viability percentage was determined compared to the untreated control, which had a 100% survival rate. The mean and standard deviation of colony counts were calculated using various dilutions from the same treated culture.

2.7. Protein extraction and identification

Cells were incubated and pre-thermally treated using an LB broth medium. Heat shock-treated and untreated cells were collected from each flask by filtration, washed with 0.9% NaCl, weighed, and re-suspended in ten-folded Tris buffer (62.5 mM Tris, 20% Glycerol, 2% SDS, and 5% β-mercaptoethanol), pH was adjusted to 8.0. Cells were lysed (10% sucrose; 50 mM Tris-HCl (pH 7.6); 1 mM dithiothreitol; lysozyme (600 µg/mL) and EDTA (2 mM)) and the extracted proteins were separated by one-dimensional SDS-PAGE, carried out for whole-cell protein extracts using 15% gel and Tris-glycine buffer (pH 8) at 125 V for 90 minutes. The gel was stained with Coomassie brilliant blue R-250, photographed, scanned, and data was documented using Alphatec 2200 software for protein analysis.

2.8. DNA extraction

Total genomic DNA was extracted from the parents (control) and the treated *Bacillus* cells using DNeasy Genomic Extraction Kit (Qiagen, CA, USA) according to the manufacturer's instructions.

2.9. ISSR and RAPD analysis

30 ISSR (Eurofins, Germany) and 25 RAPD (Operon, Germany) primers were used to screen the genomic variation in *Bacillus* isolate pre-subjected to thermal shock. The PCR reaction was applied in a final volume of 25 µl containing 500 µM of dNTPs, 2.5 mM of $MgCl_2$ in 1X GoTaq® Flexi buffer, 20 pM of each primer, 30 ng of template DNA, and 1 U of GoTaq® Flexi DNA (Promega, Promega corporation; USA). The amplification process was carried out in a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems), and the program applied for ISSR reaction was: 94 °C /5 min (1 cycle); [94 °C /0.45 sec, 48 °C/0.50 sec, 72 °C/1 min] (35 cycles); 72 °C /7 min (1 cycle) and was stored at 4 °C while the program applied for RAPD reaction was: 95 °C /5 min (1 cycle); [95 °C /0.45 sec, 37 °C/1 min, 72 °C/2 min] (40 cycles); 72 °C /7 min (1 cycle) and was stored at 4°C. The PCR products were separated on 1.5 % agarose gel in 0.1 M Tris Acetic Acid (TEA) buffer by electrophoresis at 100 V for 1h and were visualized by Bio-Rad™ Molecular Imager® Gel Doc™ XR+ System.

2.10. Determination of Bacillus isolate sensitivity to gentamycin and clindamycin after a heat stress challenge

A culture of *Bacillus isolate* was subcultured in LB broth at 37 °C for one day. Heat shock at 52 °C for 90 min was applied using two sets of sigma tubes, one represented as control and the other mentioned as a preheated isolate (treatment). 100 µl of each bacterial suspension was spread evenly over the surface of the LB agar plate. Before amending the antibiotic-containing diffusion discs, the inoculated plates were lifted and dried. The susceptibility of the tested isolate was assayed against two types of antibiotics using the disc diffusion method (Bauer et al., 1966). Gentamycin (10 µg) and clindamycin (2 µg) were centrally placed on the surface of the agar plates. All plates were then incubated at 37 °C for 24 h. Finally, the plates were examined, the diameter of the inhibition zone was measured, and the data were tabulated.

2.11. Physiological assays

1. Cellulase assay

Cellulase activity was evaluated (Bernfield, 1955; Areekijseree et al., 2004; Kumar et al., 2012) using 1% carboxymethylcellulose (CMC) as the substrate. A preheated (52 °C) reaction mixture containing 0.3 ml of enzyme

extract, 1.7 ml of phosphate citrate buffer pH 6, and 1 ml of 0.25% CMC in a final volume of 2 ml was utilized. The reaction was allowed to continue for 15 minutes and then terminated using 1 ml of 3,5- dinitro salicylic acid (DNS) solution. The mixture was placed in a 100 °C water bath for 10 minutes before being cooled to room temperature, and the optical density was measured at 546 nm. The results were expressed in (IU reducing sugars/ ml).

2. Pectinase assay

The method for producing pectinolytic enzymes was similar to that of cellulose except for adding 1 % citrus pectin as the sole carbon source replacing CMC in the liquid medium.

2.12. Statistical analysis

Statistical analysis was conducted using the SPSS program (version 20, SPSS Inc., Chicago, IL, USA). Spearman's rank correlation test was used to determine the correlation between powder type and colony count.

3. Results and Discussion

Bacillus isolate was identified using a combination of API system 20E and API CH strips (Borsodi et al., 2007). Furthermore, the isolate was identified using 16S rDNA-based primers and it was submitted to the Gene Bank under accession number (MW721258).

The detected viability of the tested microorganisms showed significant variation in growth rate after heat shock treatments, with their growth decreasing as heat exposure time increased (Figure 1A).

3.1. Detection of the induced HSPs

In the preliminary experiments, extracted total proteins were prepared from treated cells pre-incubated at 37 °C before adjusting the temperature to 52 °C for time intervals of 30, 60, 90, and 120 min and separated by SDS-PAGE. Nevertheless, Periago et al. (2002) determined that 30 °C was the optimal growth temperature for the *Bacillus cereus* ATCC 14579. Furthermore, they exposed *B. cereus* cells to a significant temperature increase from 30 to 42 °C. Differences in optimum temperatures in our results (37 °C) and theirs (30 °C) could be attributed to the differences in strains used. Also, Lv et al. (2018) suggested that the formation of spores can be a way to resist stressors since vegetative cells of *B. cereus* could not endure heat stress.

While a 30-minute heat shock treatment induced only one protein (26 kDa), a 60-minute heat shock treatment increased the expression of the 9, 16, 26, and 40 kDa proteins. At 90 minutes, a more elevated synthesis rate was observed which was maintained in recovery samples at 9, 16, 26, 31, 40, and 44 kDa before suddenly declining in the treated isolate into 26, 31 kDa proteins at an exposure time of 120 min. Due to the high expression of new HSPs produced by *B. cereus* isolates, a 90-minute heat shock-promoted treatment was chosen as the control for other stresses. Furthermore, no further induction was evident after prolonged stress exposure (30 ± 120 min). Periago et al. (2002) successfully identified 250 different

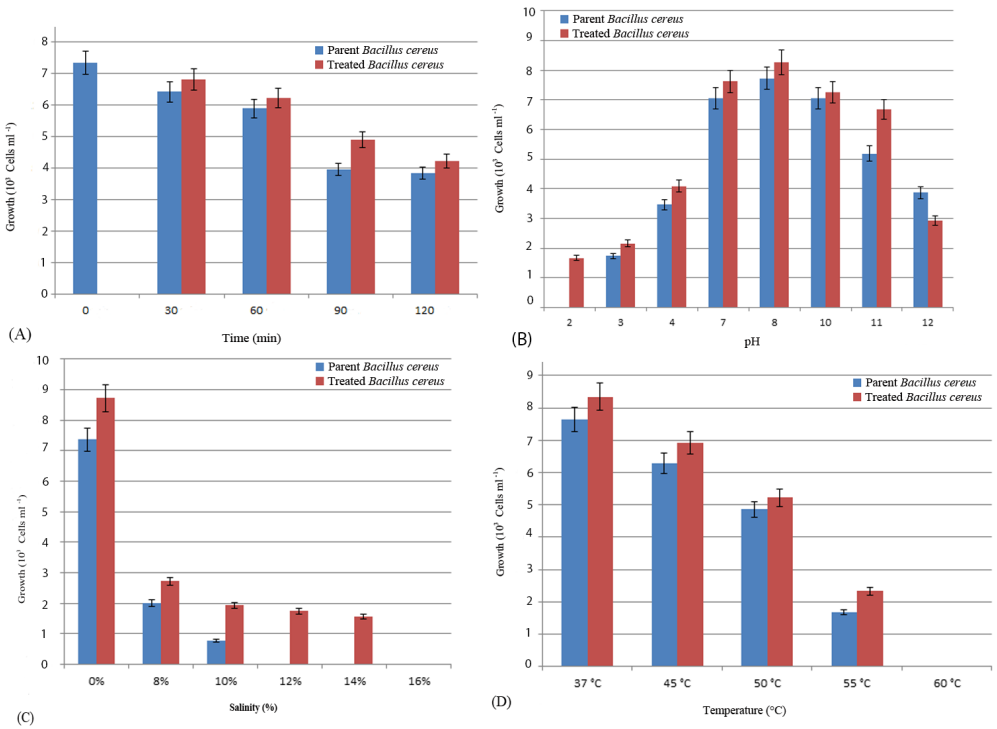

Figure 1. The effect of heat shock (A), pH (B), salinity (C), and temperature (D) on the mutant *Bacillus cereus* growth compared with the parent.

proteins induced in heat-shocked *B. cereus* cells and categorized them based on the time points of maximal production. However, the difference in the number of proteins induced in our study would be related to their usage of 2 D- Gel Electrophoresis.

3.2. Effect of heat shock at the molecular level of the Bacillus cereus

Out of 30 ISSR primers tested, seven primers successfully generated a total of 90 bands, 72 of which were polymorphic, with band sizes ranging from 150 to 2150 bp (Figure 2). The seven primers successfully characterized heat-shocked *Bacillus cereus* isolate by revealing 17 unique bands; 14 were unique positive, while the other three were unique negative.

The highest number of bands resulting from heat shock (17) was amplified by primer (ISSR 03), whereas the lowest one (6) was that revealed by primer (ISSR 11), as presented in (Table 1). The ISSR marker yielded polymorphism ranging from 50 (for ISSR 11) to 83.3% (for ISSR 06 and ISSR 12), with an average polymorphism of 80% (Table 1).

Verma et al. (2018) used ISSR to assess the genetic diversity among three *Bacillus* spp using seven primers. They obtained a polymorphism percentage of 100% with two primers with several amplicons ranging from 7 to 13 with a size range of 250-2250 bp. However, the difference in the number of amplicons would be attributed to the difference in the species studied and the treatment used.

3.3. Effect of pH stress on the protein level

After exposure to initial heat shock, treated *Bacillus cereus* control cells were selectively exposed to three types of stresses following their optimum growth conditions; pH, thermal, and salinity.

When *Bacillus cereus* isolate was pH-stressed, the untreated bacterial strains fail to grow at a pH lower than 3, whereas the treated *Bacillus cereus* grows significantly at pH 2 after thermal treatment (Figure 1B).

While the control pH (8) induced 9, 16, 26, 31, 40, 44, and 107 kDa proteins, 9 kDa protein was detected from a pH range from 7 to 12. However, 10 kDa protein was only detected at a pH lower than 7. On the contrary, the 12-kDa protein was detected at pH 3, 4, 11, and 12. Surprisingly, all treatments had three common proteins at 16, 26, 40, and 44 kDa. However, the second one disappeared at pH 2, and the latter at pH 12. Only one 24, 34, 60, and 117 kDa protein band appeared at pH, 3, 2, and 4, respectively. In contrast, two bands of 30 kDa proteins appeared at pH 3 and 4. Four bands of 107 kDa appeared at pH 4, 7, 8, and 12.

The development of acid resistance after the pre-incubation of bacterial cells at sublethal pH has been reported previously for many bacterial species, including *Lactococcus lactis* (Sanders et al., 1999) and *Lactobacillus* (De Angelis and Gobbetti, 2004). Nevertheless, our results demonstrated that *Bacillus cereus* isolate increased its acid resistance after exposure to heat shock. However, the level of tolerance depends on the shock duration. Bore et al. (2007) suggested that cross-resistance response

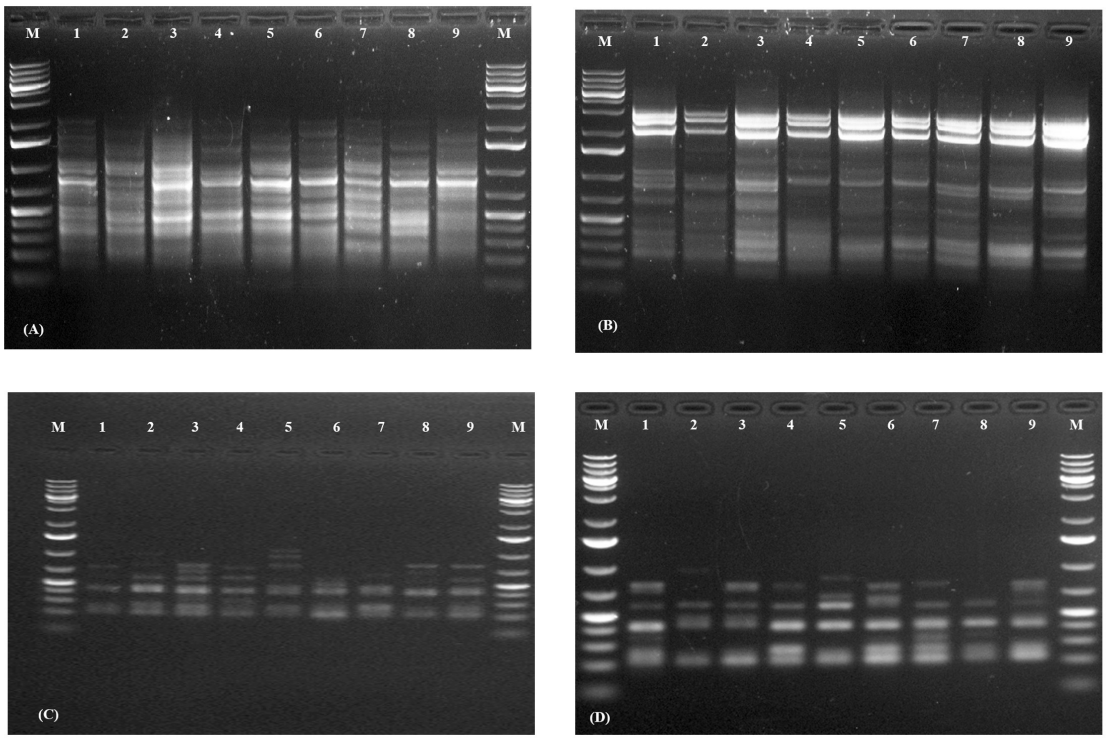

Figure 2. Representative ISSR profile of *Bacillus cereus* isolate under heat shock stress with primers 3 (A), 4 (B), 5 (C), and 6 (D). Lane (1) represents the control. M 1kb DNA Ladder.

Table 1. ISSR primers that produced polymorphic fingerprinting as a result of exposing *Bacillus cereus* to heat shock, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Primer Name	Primer sequence (5'→3')	Total Bands	Polymorphic bands	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
ISSR 03	ACACACACACACACACY*T	17	14	82.4	-	-	-
ISSR 04	ACACACACACACACACYG	15	10	66.7	700	150	2
ISSR 06	CGCGATA GATAGATAGATA	12	10	83.3	2150, 2000, 1950	-	3
ISSR 07	ACGATAGATAGATAGATA	12	9	75	1000, 850, 650, 600	250	5
ISSR 09	GATAGATAGATAGATAGC	16	16	100	1700, 1500, 1100, 800, 700, 250	-	6
ISSR 11	ACACACACACACACACYA	6	3	50	-	-	-
ISSR 12	ACACACACACACACACYC	12	10	83.3	-	680	1
TOTAL		90	72	80	14	3	17

between different stresses allows cellular resistance against other agents.

3.4. Effect of pH salinity, and temperature stress on the molecular level

Because of the lack of polymorphism, out of the 30 ISSR and 25 RAPD primers initially tested, 11 RAPD and 16 ISSR primers were further used to screen the genomic variation in the *Bacillus cereus* isolate pre-subjected to thermal shock, and the tested organism exposed to different stresses (pH, salinity, and temperature).

3.5. Effect of pH stress on the molecular level

Thirteen primers, eight ISSR, and five RAPD successfully produced polymorphic patterns due to pH stress on *Bacillus cereus*. Moreover, a total of 99 bands were produced by the eight ISSR primers, and 50 bands resulted from the five RAPD primers; 78 and 40 were polymorphic, respectively. Band sizes varied from 250 bp (ISSR 06) to 1600 (ISSR 03) bp and from 600 bp (OP-G19) to 2500 bp (OP-D20). The total number of unique bands resulting from pH stress was 21 for ISSR and 12 for RAPD; 17 and 3 were unique positive, while the remaining (4 and 9) were unique negative, respectively (Table 2 and 3).

The highest number of bands recorded by the ISSR marker for pH stress (15) was amplified by primer ISSR 01 and ISSR 03, whereas the lowest one (8) was that presented by ISSR 06. In contrast, the highest number of bands obtained by the RAPD marker was that recorded by primer OP-B17 (13), while the least number (5) was that revealed by primer (OP-C03). The ISSR marker yielded polymorphism ranging from 57.1 (for ISSR 08) to 100% (for ISSR 09), with an average of 78.8%. The level of polymorphism obtained by the RAPD marker, on the contrary, ranged from 54.5 (for OP-D20) to 100% (for OP-B17 and OP-G19), with an average of 80%.

3.6. Effect of thermal stress on the protein level

Thermal stress-induced permanent proteins can be classified under heat shock for *B. cereus*. A control heat stress promoted treatment-induced 9, 16, 26, 31, 40, 44, and 107 kDa proteins. Interestingly, new proteins were recorded by gradually increasing the temperature to 45°C, where a 48 kDa protein was recorded. Furthermore, at 50 °C, 20, 48, and 51 kDa proteins were recorded. However, increasing the temperature to 60 °C caused the 20 kDa protein to disappear. Consequently, while heat stress affects all protein groups, other stresses including salt stress are thought to interact with the novel synthesized ones (Hantke et al., 2019).

According to Periago et al. (2002), the optimum temperature for the growth of *Bacillus cereus* is 30°C, and the lethal temperature is 50°C. They also reported that pre-exposing the thermal dose of *Bacillus cereus* cells to 42 °C for 1/4 h led to a more than 100-fold increased survival rate. Moreover, longer heat pre-exposures of 1/2 h to 1 h at 42 °C resulted in a significant increase in *B. cereus* thermo-tolerance compared to untreated cells (3- to 4-log increased survival rate after 20 min. However, Lv et al. (2018) indicated that heat-induced inactivation might primarily cause severe damage in the protein rather than the original cell.

3.7. Effect of thermal stress on the DNA level

With temperature-stressed *Bacillus cereus*, nine ISSR primers and ten RAPD primers demonstrated reproducible results. However, only eleven primers (4 ISSR, 7 RAPD) revealed polymorphic results (Tables 4 and 5). The four ISSR primers produced a total of 30 bands, and 48 bands resulted from the seven RAPD primers; 25 and 24 were polymorphic, respectively. Interestingly, the amplified products ranged from 150 to 1200 bp for the same primer (ISSR 13). Conversely, the amplified products by RAPD markers ranged from 200 bp (OP-A02) to 3500 bp

Table 2. ISSR primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to pH stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Name	Primer sequence (5'→3')	Total Bands	Polymorphic bands	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
ISSR 01	AGAGAGAGAGAGAGAGY*C	15	13	86.7	900, 400	800	3
ISSR 03	ACACACACACACACACY*T	15	13	86.7	1600, 1400, 850, 700, 350	-	5
ISSR 04	ACACACACACACACACYG	10	8	80	-	-	-
ISSR 05	GTGTGTGTGTGTGTGTYG	12	8	66.7	850, 650	800	3
ISSR 06	CGCGATA GATAGATAGATA	8	6	75	-	800, 250	2
ISSR 07	ACGATAGATAGATAGATA	12	9	75	1100, 900, 650	-	3
ISSR 08	ACA CAC ACA CAC ACA CG	14	8	57.1	-	-	-
ISSR 09	GATAGATAGATAGATAGC	13	13	100	1000, 900, 750, 700, 600	-	5
TOTAL		99	78	78.8	17	4	21

Table 3. RAPD primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to pH stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Primer Name	Primer sequence (5'→3')	Total Bands	Polymorphic Bands (bp)	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
OP-B17	AGGGAACGAG	13	13	100	800	1900	2
OP-C03	GGGGGTCTTT	5	4	80	-	-	-
OP-C04	CCGCATCTAC	12	8	66.7	1700	2500, 650	3
OP-D20	ACCCGGTCAC	11	6	54.5	2400	2500, 1700, 1600, 1500	5
OP-G19	GTCAGGGCAA	9	9	100	-	700, 600	2
TOTAL		50	40	80	3	9	12

Table 4. ISSR primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to temperature stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Primer Name	Primer sequence (5'→3')	Total Bands	Polymorphic Bands (bp)	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
ISSR 13	AGAGAGAGAGAGAGAGYT	8	7	87.5	1200, 600, 400, 150	750, 300	6
ISSR 14	CTCCTCCTCCTCCTCTT	10	10	100	700, 600, 400, 350, 300, 280	500	7
ISSR 16	TCTCTCTCTCTCTCT CA	4	4	100	750, 600, 350	200	4
ISSR 19	TGCTGCTGCTGCTGCTGC	8	4	50	900, 500, 350	300	4
TOTAL		30	25	83.3	16	5	21

Table 5. RAPD primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to temperature stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Name	Primer sequence Primer Sequence (5'→3')	Total Bands	Polymorphic bands	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
OP-A02	TGCCGAGCTG	7	5	71.4	2700	1600, 650, 450, 200	5
OP-B03	CATCCCCCTG	7	3	42.9	1400, 750	-	2
OP-C04	CCGCATCTAC	7	4	57.1	3500, 350	-	2
OP-C11	AAAGCTGCGG	8	3	37.5	-	400	1
OP-C16	CACACTCCAG	7	5	71.4	650, 600	550	3
OP-D20	ACCCGGTCAC	3	1	33.3	-	1800	1
OP-G19	GTCAGGGCAA	9	3	33.3	-	-	-
TOTAL		48	24	50	7	7	14

(OP-C04). The total number of unique bands resulting from temperature stress was 21 for ISSR and 14 for RAPD; 16 and 7 were unique positive bands, while the other (5 and 7) were unique negative bands.

The ISSR marker amplified the most bands (10) when amplified by primer (ISSR 14), while the primer (ISSR 16) produced the fewest. In contrast, the highest number of bands (9) recorded by RAPD was that of primer OP-G19, while primer OP-D20 revealed the fewest. Furthermore, the level of polymorphism obtained by the ISSR marker ranged from 50 (for ISSR 19) to 100% (for ISSR 14 and ISSR 16) with an average of 83.3%, while that obtained by the RAPD marker ranged from 33.3 for OP-D20 and OP-G19 to 71.4% for OP-A02 and OP-C16 with an average of 50%.

The findings indicate the level of damage caused by the thermal treatment. However, DNA damage observed during this step might be the indirect cause consequence of the preheat shock (Wesche et al., 2009).

3.8. Effect of salinity stress on the protein level

A regular increase in growth with a continuous increase in salinity tolerance up to 14% was recorded compared to the control cells, which could tolerate salinity concentrations up to only 10%. Bacterial response to osmotic changes affects cellular processes, whereas synthesizing osmo-protective compounds facilitates the restoration of cellular functions after changing osmotic conditions (Hoffmann and Bremer, 2017; Rojas and Huang, 2018). However, heat stress-induced proteins differ from that produced due to salt stress, whereas the latter can be incited by the fast cell size reduction that is accompanied by changes in turgor pressure due to water efflux (Stadmiller et al., 2017; Hantke et al., 2019).

Control salinity stress-induced proteins of 9, 16, 26, 31, 40, 44, and 107 kDa. Furthermore, after treatment with a 12% NaCl concentration, inductions of proteins of 77, 112, and 123 kDa were observed. Nevertheless, increasing salinity concentration to a minimum of four (16, 31, 77, and 107 KDa) after treatment with 14% salt concentration resulted in a reduction in the synthesized protein (16, 31, 77, and 107 KDa) (Figure 1C).

3.9. Effect of salinity stress on the molecular level

Twelve primers (five ISSR primers and seven RAPD primers) effectively characterized the effect of salinity on the *Bacillus cereus* isolate genome. While the five ISSR primers showed a polymorphic trend, only three RAPD markers demonstrated the same trend (Tables 6 and 7). Salinity stress showed a total of 64 bands; 45 were polymorphic (25 ISSR and 20 RAPD bands). Band sizes ranged from 150 bp (ISSR 14) to 2400 bp (ISSR 08) and from 150 bp (OP-C16) to 850 bp (OP-B04). The total number of unique bands that characterized salinity stress was 17 for the ISSR marker and 9 for the RAPD marker, 11 and 8 of them were unique positive, while the others (6 and 1) were unique negative, respectively.

The primer ISSR 14 produced the most bands recorded by ISSR markers due to salinity stress (11 bands), whereas the primer ISSR 16 produced the lowest. On the contrary, primer OP-C16 amplified the most bands (12 bands) revealed by the RAPD marker, while primer OP-B17 produced the fewest. The ISSR marker yielded polymorphism ranging from 42.9 for ISSR 19 to 100% for ISSR 16 with an average of 71.4%, and the RAPD marker yielded polymorphism ranging from 16.7 for OP-B17 to 83.3% for OP-C16 with an average of 69%.

Sen et al. (2015) characterized seventeen *Bacillus subtilis* genotypes using PCR-based molecular markers. Furthermore, based on genetic diversity, high genotypic and phenotypic heterogeneity in *Bacillus* isolates was observed (Cottyn et al., 2011) using ISSR markers for gram-positive bacterium *Clavibacter michiganensis* (Baeyen et al., 2013).

3.10. Determination of Bacillus cereus isolate sensitivity to gentamycin and clindamycin after a heat stress challenge

To elucidate the effect of heat-shocked bacteria (*Bacillus cereus*) on their antibiotic susceptibility, the exposure of *B. cereus* strains to heat stress may alter their antibiotic

Table 6. ISSR primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to salinity stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Name	Primer sequence (5'→3')	Total Bands	Polymorphic bands	Polymorphic Bands (%)	Unique positive bands (bp)	Unique negative bands (bp)	Total Unique bands
ISSR 08	ACA CAC ACA CAC ACA CG	5	3	60	-	2400, 1800, 1200	3
ISSR 13	AGAGAGAGAGAGAGAGYT	8	6	75	600, 400	350	3
ISSR 14	CTCCTCCTCCTCCTCTT	11	9	81.8	1050, 850, 750, 350, 250, 150	-	6
ISSR 16	TCTCTCTCTCTCTCT CA	4	4	100	450	400	2
ISSR 19	TGCTGCTGCTGCTGCTGC	7	3	42.9	400, 250	200	3
TOTAL		35	25	71.4	11	6	17

Table 7. RAPD primers that produced polymorphic fingerprinting after exposing *Bacillus cereus* isolate to salinity stress, their corresponding sequences, the total number of bands, number of polymorphic bands, percentage of polymorphism, number of unique positive, number of unique negative bands as well as the total number of unique bands obtained.

Primer Name	Primer sequence (5'→3')	Total Bands	Polymorphic bands	Polymorphic Bands (%)	Unique positive bands	Unique negative bands	Total Unique bands
OP-B04	GGACTGGAGT	11	9	81.8	850, 800, 350	-	3
OP-B17	AGGGAACGAG	6	1	16.7	-	300	1
OP-C16	CACACTCCAG	12	10	83.3	600, 500, 400, 200, 150	-	5
TOTAL		29	20	69	8	1	9

susceptibility against antibiotics is dependent on the selective potentiality of each strains, formation of either temporary or permanent heat stress proteins and type of antibiotics. In the current study (Figure 3), the untreated *Bacillus cereus* demonstrated antibiotic resistance to gentamycin and clindamycin (1.54 and 1.65 cm respectively), much lower than the corresponding values with preheat treated test pathogen which recorded (2.37 and 2.49) (Figure 4). Using the same concentrations, Heugens et al. (2001) and Sokolova and Lannig (2008) illustrated that the organisms could increase susceptibility to chemicals and reduce thermal tolerance when exposed to elevated temperatures.

Zhang et al. (2017) evaluated the effect of sodium chloride on heat resistance and antibiotic susceptibility. NCCP10812 and *Salmonella enteritidis* NCCP12243 were subjected to 0, 2, and 4% NaCl as well as a gradual increase of salt concentrations from 0 up to 4% for 24 h at 35 °C. The isolates were then investigated regarding heat resistance at 60 °C and antibiotic susceptibility to eight antibiotics. *S. Typhimurium* NCCP10812 demonstrated increased heat resistance after being treated with a single NaCl concentration. A gradual increase in NaCl concentration suppresses the antibiotic sensitivities of *S. Typhimurium* NCCP10812 to chloramphenicol, gentamicin,

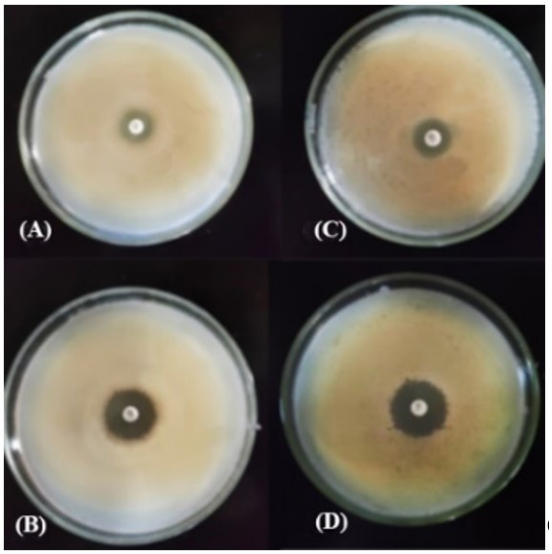

Figure 3. Antibiotic susceptibility test performed by using the Kirby-Bauer disk diffusion method, gentamicin (10 µg) with parent *Bacillus cereus* (A) and heat-shocked *Bacillus cereus* (B), and clindamycin (2 µg) with parent *Bacillus cereus* (C) and heat-shocked *Bacillus cereus* (D).

and oxytetracycline. These results indicate that NaCl in food may cause increased thermal resistance, cell invasion efficiency, and antibiotic resistance of *Salmonella*.

The general stress response regulator σs (RpoS); is the common stress response regulator (Hengge-Aronis, 2002) that was upregulated when bacteria were subjected to osmotic stress. In this regard, Huang et al. (2009) stated that RpoS regulates the antibiotic resistance of *Escherichia coli* against ampicillin, chloramphenicol, and rifampicin. Therefore, it can be hypothesized that NaCl may control RpoS expression and consequently affect the antibiotic sensitivity of *Salmonella* NCCP10812 and NCCP12243.

3.11. The effect of different preheated application times on cellulolytic and pectinolytic activity of Bacillus cereus

Data indicated a significant reduction in the cellulolytic and pectinolytic activity of *Bacillus cereus* isolate with relatively lower values of pectinase activities than the corresponding ones of cellulose until 90 min, with 0.269 cellulolytic activity with a corresponding percent of activation of 40.104%. Nevertheless, pectinolytic activity reached 0.241 with a corresponding activation percentage of 48.765 (Table 8). There was no significant decline in cellulolytic or pectinolytic activity as the exposure time increased from (90 to 120 min). Heat shock induced a substantial increase in time (from 30 to 90), with cellulolytic percent of activation varying from (10.938% to 40.104%). Moreover, pectinolytic activation percentages range from (14.198% to 48.765%).

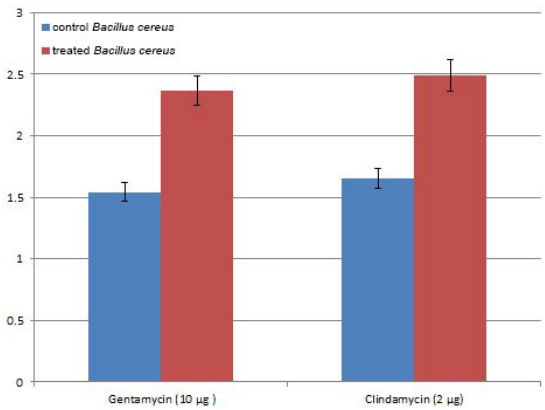

Figure 4. Antibiotic resistance pattern to tested antibiotics in heat-shocked *Bacillus cereus* with gentamycin (10 µg) and clindamycin (2 µg) on LB agar plate at 37 °C for 24 hrs.

B. cereus strains were found to produce extracellular enzymes, gelatinase 83%, DNase 83%, lecithinase 83%, protease on skim milk agar 100%, protease on milk agar 100%, xylanase 100%, protease on casein agar 83%, amylase 83%, cellulose, 41%, and pectinolytic activity, 4%. *B. cereus* also produces several toxins, including cytotoxin K (CytK) proteins, the primary virulence factors in *B. cereus* (Lund et al., 2000).

According to Namasivayam et al. (2011), the optimal temperature for maximum pectinase activity from *B. cereus* is 37°C. Similarly, 35 °C was reported for pectinase from *Penicillin chrysogenum* (Banu et al., 2010; Phutela et al., 2005) reported that 60 °C was an optimal temperature for *Aspergillus fumigatus* pectinase. Other thermophilic fungi have been reported to optimally produce enzymes at 50 °C (Rubinder et al., 2002).

Abrashev *et al* ., 2014 reported that heat stress resulted in the production of reactive oxygen species (ROS). Nevertheless, various ROS-scavenging proteins can respond to heat stress, such as catalase (CAT), superoxide dismutase (SOD), thioredoxin reductases (TrxRs), and glutaredoxin–glutathione reductases (GRX-GRs) (Zhang et al., 2017). In addition to HSPs, several protein families are involved in heat stress response (HSR), including protease, lecithinase, amylase, cellulase, and pectinase.

It is significant to investigate the *in vitro* hydrolytic enzyme activity, including cellulase and pectinase of *Bacillus cereus*, in response to different times of heat pre-exposure (from 30 to 120 min). The results demonstrated progressive activation in cellulolytic and pectinolytic activities of the test pathogen until 90 min preheat exposure, followed by a non-significant decrease from (90 to 120 preheat exposure). The maximum statistically significant increase was achieved upon exposure to preheating for 90 min, in which the activity of either cellular activity or pectin hydrolysis was measured at 0.269 and 0.241, with the associated activation ratio recorded at 40.104 and 48.765, respectively. The enzymatic activation of heat stress microorganisms was also reported by (Abrashev et al., 2014; Zhang et al., 2017), and it can

Table 8. Effect of different preheated application times on cellulolytic and pectinolytic activity of *Bacillus cereus* isolate after two incubation days (expressed as 1IU/ ml reducing sugar/1 ml medium).

Treatment / Time of Heat pre-exposure	Cellulase enzymatic activity (IU/ml)	% of activation regarding control treatment	pectinase enzymatic activity (IU/ml)	% of activation regarding control treatment
Control (0 time)	0.192	0	0.162	0
30	0.213	10.938	0.185	14.198
60	0.233	21.354	0.207	27.778
90	0.269	40.104	0.241	48.765
120	0.262	36.458	0.238	46.914
150	0.260	35.417	0.236	45.679

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

be attributed to the overexpression of gene of catalytic enzymes of heat-shocked microorganisms to enhance their ability of thermotolerance to tolerate further continues stressing, aligning with Nantapong et al. (2019).

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