The Mutating Effect of Microwave Irradiation on Spores and Crystal Protein Formation of Iraqi *Bacillus thuringiensis* kurstaki KS3

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Abstract

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Bacillus thuringiensis is the most important biological control agent that is used in fields and stores against insect pests of agricultural importance. This research was conducted to study the effect of microwave radiation on sporogenesis and crystal protein production by the Iraqi bacterium *B. thuringiensis* KS3 strain. The bacterium was enriched by Lauria bertani broth (LB) pH 7.0 for spore formation. Spore suspension was microwave irradiated at 1000 Watt for 5, 10 and 15 seconds. Spore inactivation rate for each time period of microwave treatment was calculated and the macroscopic differences were examined. Crystal protein and spore production were estimated for irradiated and non-irradiated bacterial cultures. Viable spores in the control treatment was 3×10^8 viable spores/ml, and decreased after 5, 10, and 15 s of microwave treatment to 3×10^7, 2×10^6 and 2×10^4 viable spores/ml, respectively. The reduction of spore viability reached to 49.263% 15 seconds after treatment. The appearance of colonies on the top of nutrient agar (NA) were almost similar with the control, with no differences in color, margin and surface of treated colonies following the three time periods treatment. Colonies with dense color, smaller in size and with straight margin appeared on congo red culture for all treatments in contrast to the control. Microscopic examination showed that treated bacilli were similar regarding their shape, diameter and arrangement, however, smaller bacterial cell size following 15 s irradiation treatment was observed. Control culture after 72 h started to form spores, whereas after 24 h, irradiated spores for 5, 10 and 15 s formed 75, 90 and 90% viable spores, respectively. Crystal protein reached the highest concentration after 72 h in control culture, whereas it varied based on treatment period. The production of viable spores from microwave irradiated isolates increased about one logarithmic cycle compared with the control culture of *B. thuringiensis* KS3.The peak of the UV spectrum of Crystal protein extracts was recognized at 255-280 nm and the peak of the curve indicated the protein concentration at a given wavelength. Differences were recognized in the UV light wavelength range of 220-235 nm.

Keywords: Sporogenesis, Physical effect, Delta toxin.

Introduction

The rapidly increasing world population reached 7.8 billion in August 2020 according to the most recent United Nation estimate, which imposed increasing pressure on the agricultural community (Han-Ming *et al*., 2019; Long *et al*., 2015; World Population Prospects, 2019) urging the development of highly efficient pest control strategies to minimize crop losses worldwide to overcome the need for food (Bravo *et al*., 2007).

Agricultural products like cereal grains, legumes, vegetables, fruits, dates and nuts have to go through different treatments before and after harvesting until they reach consumers. Around 10-15% of post-harvested food grain is lost due to un-proper storage conditions or attack by different deleterious biological agents.

Integrated pest management is used to manage infestation of insects in the fields, after harvest and during marketing of food products. Biological and physical strategies are more favorable for cleaning the environment and food products as well as for overcoming the development of resistant insects to chemicals following repeated chemical pesticides treatment (Subramanyam & Hagstrum, 2000).

The biological agent the bacterium *Bacillus thuringiensis* is widely applied in the field to control the important agricultural pests which belong to the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera as well as nematodes (Bravo *et al*., 2007; Palma *et al*., 2015). The major advantage of this bacterium is the strict specificity of delta-endotoxins (Crystal proteins) to certain insect species, but not harmful to humans, birds and other farm animals.

Microwaves are electromagnetic fields that oscillate in the 300 MHz–300 GHz frequency range. The thermal effect of microwaves (microwave dielectric heating) is largely employed for the heating and processing of food and in production of industrial materials. The effect of the electromagnetic field induced by radio frequency has been attracting attention of scientists due to its deleterious effect on living organisms (including humans) if the field intensity reaches beyond a certain level.

Many investigators have studied the inactivation of microorganisms by microwave irradiation since the mid-twentieth century. Many researchers have studied the inactivation of microorganisms by microwave irradiation. Early studies suggested that the inactivation was primarily due to heat induction by irradiation (Das & Shah, 2013; Fung & Cunningham, 1980). Later studies (Fujikawa *et al*., 1992; Wang *et al*., 2003) clarified further that there was no

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significant difference between the microbial thermal inactivation patterns produced by microwave irradiation. However, there were several studies that demonstrated non-thermal effects of microwave irradiation on microbial inactivation and metabolic activity; these effects include electroporation, cell membrane rupture and magnetic field coupling (Kang & Kato, 2014; Kozempel *et al*., 1998; Shamis *et al*., 2011).

This research aimed to predict the mutating effect of microwave radiation on the spores of Iraqi isolate of *B. thuringiensis* KS3 and its impact on the production of spores and crystal protein production under artificial medium conditions in the laboratory.

Material and Methods

Spores/crystal mixture formation by *Bacillus thuringiensis* kurstaki KS3

The Iraqi bacterial strain of *B. thuringiensis* KS3 kurstaki was isolated from soil and identified by (Al-Khafaji *et al*., 2017), and it was used in this work. Lauria bertani broth (LB) pH 7.0 was prepared and the bacteria were cultured and incubated for 72 h at 30±2ºC in a shaker incubator at 120 rpm/min for spore and crystal protein formation. Cultures were heat treated at 80ºC for 30 min, and then spores were counted based on a serial dilution technique.

Microwave treatment for *Bacillus thuringiensis* kurstaki KS3

Spore suspension 3×10^8 (control trial) was divided into equal volumes of 5 ml using screw- capped vials. Each vial was separately positioned in the middle of the working chamber in the microwave instrument. Microwave irradiation was applied at 1000 Watt for 5, 10 and 15 seconds with three replicates for each time period. Temperature was monitored in an open system for each treatment to adjust for time with heating in the operator. Three additional 5 ml of spore suspension were immersed in water paths pre-equipped at 50º, 80º and 100ºC for 5, 10 and 15 s as control treatments. The control, heated spores suspension, and irradiated samples were serially diluted using distilled water, and 0.1 ml of each diluent was streaked on the nutrient agar (NA) plate. Plates were incubated at 30±2°C and the growth of cultures was monitored for 72h. The colony count was estimated as colony forming units per milliliter (CFU/ml) and transformed to log10 that was expressed as the average number of surviving spores (Celandroni *et al*., 2004; Wang *et al*., 2003).

The percentage of spore inactivation for each time interval of microwave treatment was calculated using the following equation (Skowron *et al*., 2016):

$$I\% = (A–B)/A\times100$$

where A: number of viable spores germinated in a control culture, B: number of viable spores which can germinate after treatment period.

All experiments were performed in triplicate and 100 random colonies were screened and selected isolates were subjected to further experimentation.

Characterization of microwave irradiated bacteria

Microwave irradiated bacterial cells were streaked over nutrient agar and incubated for 24, 48 and 72 h. The appearance of bacterial growth as colony shape, color, margin and surface were all screened on NA. Microscopic examination was used to determine the differences between control culture and treated bacterial growth. The spore formation period and cell shape were detected in the gram-stained slide. Biofilm formation was evaluated for control culture of *B. thuringiensis* KS3 and microwave irradiated cultures as described earlier (Gundogan & Ataol, 2013). Briefly, Congo red stain 0.8% was incorporated in the brain heart infusion agar containing 1% sucrose. Bacterial cultures, controlled and treated, were serially diluted and streaked before being incubated at 30±2ºC for 72 h. Colonies with a dry crystalline consistency were observed for biofilm formation. Three irradiated isolates were chosen for further experiments; *B. thuringiensis* KS3.1, *B. thuringiensis* KS3.2 and *B. thuringiensis* KS3.3.

Bioassay efficiency of microwave irradiated bacteria:

Control bacteria and microwave-irradiated isolates (BtKS1.1, BtKS2.1, and BtKS3.1) were inoculated into LB broth pH 7.0 and incubated for 72 hours at 30ºC in a shaker incubator at 120 rpm/min. Spores were counted by serial dilution method and Crystal protein was estimated as described earlier (Somerville & Hazel, 1975) with minor modifications. Briefly, one ml of culture was centrifuged at 4000 rpm/min for spores sedimentation, and the supernatant was centrifuged at 9700 rpm/min for 10 min. The supernatant was discarded and the pellet was suspended in 1 ml of 0.5 M of sodium hydroxide. Crystal protein concentration was estimated by the formula: OD_{280}= 1 is equivalent to 1 mg of protein using Thermo scientific, nanodrop 2000.

Bioactivity of spores/crystal mixture was determined against the 2nd instar of *Ephestia cautella* (walker) larvae (Kothari *et al*., 2014) maintained in the laboratory. Insect larvae were reared on a medium composed of crushed whole wheat 81%, glycerin 12%, date syrup (dibis) 6% and Saccharomyces yeast 1%. Larvae were grown at ambient temperature 28±2°C and humidity 18%. Spores/crystal protein mixtures were collected from 4 ml bacterial culture as mentioned above, mixed well with only 5 g of larval medium then 10 larvae of each replicate of 2nd instar larvae were placed on the medium surface, incubated for 72 h, and mortalities were counted for each treatment.

Statistical analysis

The data was statistically analyzed using the second edition of the Genestat program, one-way and two-way ANOVA were used for analysis. LSD was used to compare treatment rates at P= 0.05.

Results and Discussion

Results obtained showed that the temperature of 5 ml spore suspensions increased from 20º to 100ºC within 15 s in an open vial in a microwave oven. Microwave irradiation for only 5 s increased heat to 50ºC, however, irradiation for 10s and 15s increased the temperature of irradiated spore suspension to 80º and 100ºC, respectively, which is in agreement with what has been reported earlier (Ryynanen &

Ohlsson, 1996; Yadav *et al*., 2014). Celandroni *et al*. (2004) reported that exceeding output power of microwave oven would decrease the time required to reach boiling point in contrast with low output power.

Serial dilutions revealed that viable spores decreased with time. The concentration of 3×10^8 viable spores/ml in the control decreased to 3×10^7, 2×10^6 and 2×10^4 viable spores/ml after 5, 10 and 15 s, respectively, of microwave irradiation (Figure 1). The decrease in viable spores count after microwave treatment may result from thermal and non-thermal factors. Microwave radiation lies between radio frequencies and infrared radiation in an electromagnetic field (E-field). That might influence the chemistry of biological molecules and the assembly of structural cell components independently of the thermal effect generated by waves.

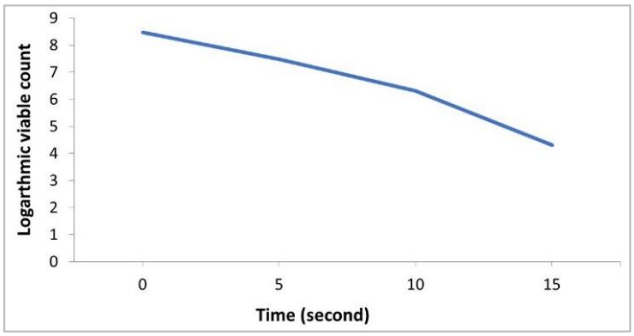

Figure1. Effect of microwave irradiation period on viability of *Bacillus thuringiensis* kurstaki KS3 spores.

Microwave heating depends on the transformation of alternating electromagnetic field energy into thermal energy by affecting polar molecules of biological systems such as water, protein, some amino acids and fat which rotate with a magnetic field to adjust to the alternating electric field induced by the microwave beam. On the other hand, microwave heating is most efficient on liquid water and much less on fatty and sugar containing molecules (Sutar & Prasad, 2008). Conventional heat treatment revealed that there were no observed differences in viable spore count after 50, 80 and 100ºC treatment for 5, 10 and 15 s, respectively (Table 1).

Table 1. The *Bacillus thuringiensis* kurstaki KS3 spores viability after conventional heat treatment at different exposure periods.

Time (seconds)	Logarithmic viable count at different temperatures (ºC)			
	50	80	100	$LSD_{0.05}$
5	8.477	8.477	8.477	1.235
10	8.45	8.44	8.40	1.235
15	8.45	8.45	8.43	2.135
$LSD_{0.05}$	1.244	1.244	2.488	

Spore suspension exhibited a 1-log reduction after 5 s of microwave irradiation while the reduction reached 2.176-log and 4.176-log after 10s and 15 s respectively (Table 2). Statistical analysis revealed that significant decrease in spore viability occurred after exposure to microwave radiation for 10 and 15 sec. The efficiency of spore destruction depends on factors such as microwave power, time of exposure and the humidity of samples. Results presented here agreed with previous work (Kim *et al*., 2009) which indicated that time of exposure and heat resulted from microwave power would interfere with the destruction of *B. lichniformis* spores.

Table 2. The viability of spores from *Bacillus thuringiensis* kurstaki KS3 after microwave irradiation

Time (seconds)	Log (CFU/ml)	Reduction in Log Number	Spores viability (%)	Reduction in viability (%)
0	8.477	0.00	100.00	0.00
5	7.477	1.00	88.203	11.79
10	6.301	2.17	74.330	25.67
15	4.301	4.10	50.737	49.263

LSD= 1.24 at P=0.05.

The results indicated that bacterial spores displayed a higher resistance to microwave radiation within a short exposure time. Unlike vegetative bacterial cells, spores coat contains dipicolonic acid rendering them more resistant to harsh conditions (Park *et al*., 2006). However, ultra-structural alteration of microwave irradiated spores released molecular components and proteins outside the spores coat for *B. subtilis*, *B. cereus* and *B. licheniformis*, *Clostridium sporogenes*, in contrast to the differences attributed to conventional boiling water bath (Cao *et al*., 2018; Celandroni *et al*., 2004; Kim *et al*., 2009; Skowron *et al*., 2016).

Results also showed that the appearance of colonies on the top of the NA plate was almost similar with control culture. No differences in the color, margin and surface of treated colonies for the three-time exposures. However, biofilm formation ability differed between control cultures and microwave treated cultures. In contrast to control culture (Figure 2), colonies with dense color, smaller size, and straight colony margins appeared on the Congo Red Plate and for all durations of microwave irradiation, in contrast to the control culture.

Figure 2. Biofilm formation on congo red medium of *Bacillus thuringiensis* kurstaki KS3 after microwave irradiation (A= Microwave treated culture, B= Control culture).

Also microscopic examination showed that treated bacilli were similar in their shape, size and arrangement, however, smaller bacterial cell size were recognized in 15 s

irradiation treatment. The most apparent difference was the time required for spore formation. The control culture began to form spores after 72 hours, whereas only 75, 90 and 90% of the cultures formed spores after only 24 hours for 5, 10 and 15 s microwave treatments, respectively (Figure 3).

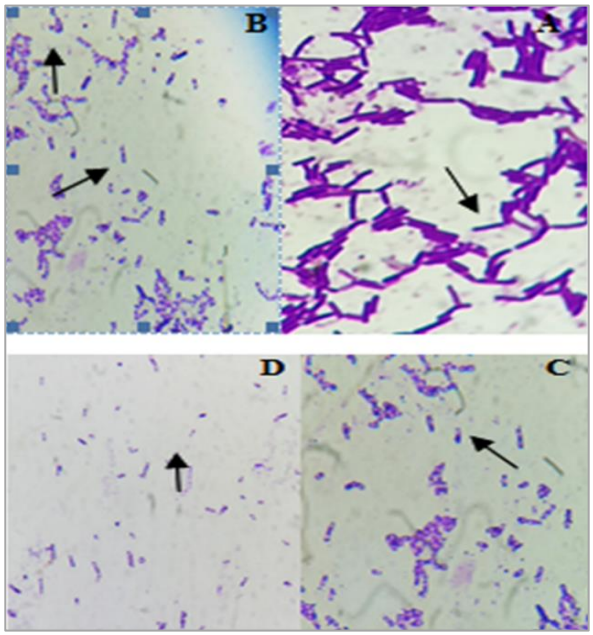

Figure 3. Microscopic examination of *Bacillus thuringiensis* kurstaki KS3 survival cells on nutrient agar plate after microwave irradiation (culture examined after 24 h incubation at 30±2ºC). A= control culture, B= culture after 5 s microwave treatment, C= culture after 10s microwave treatment, D= culture after 15s microwave treatment.

The analysis of crystal protein production in LB broth differed with incubation time. Crystal protein concentration produced by the control culture increased with time to reach its highest concentration after 72 h, whereas concentration of crystal protein varied with exposure time for each treatment (Table 3). On the other hand, the production of viable spores from microwave irradiated isolates would increase by about one logarithmic cycle compared to the viable spores/ml in the control culture of *B. thuringiensis* KS3 (Figure 4).

Table 3. The production of crystal protein (mg/ml) from microwave irradiated mutations of *Bacillus thuringiensis* KS3 following different exposure periods.

Bacterial isolate	Incubation period (hour)			Mean
	24	48	72	
Control Bt KS3	0.1303	2.5417	3.3117	1.9946
Bt KS3.1	0.7287	0.7063	0.4740	0.6363
Bt KS3.2	0.5440	0.5247	0.6350	0.5679
Bt KS3.3	0.4850	0.4620	0.2313	0.3928
Mean	0.4720	1.0587	1.1630	

$LSD_{0.05}$ for treatments = 0.01035, for time period= 0.00896, for treat × time = 0.01793

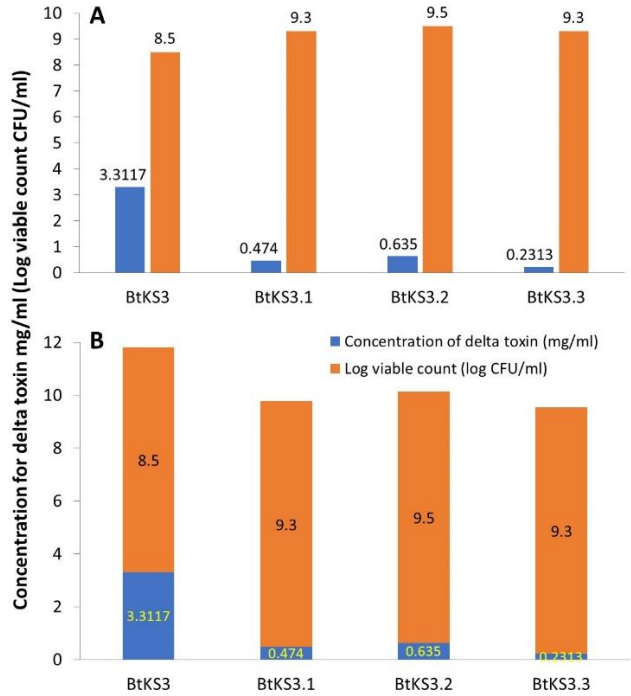

Figure 4. The production of crystal protein and viable spores from microwave irradiated Iraqi *Bacillus thuringiensis* kurstaki KS3. A= Separated crystal protein concentration and viable spores; B= Protein/spores mixture.

The difference in crystal protein production in microwave treated isolates might result from the mutagenic effect of microwave, which requires more work to identify genetic alterations (Jangid *et al*., 2008; Gosai *et al*., 2014; Kothari *et al*., 2014). These workers reported that microwave non-thermal effects are capable of inducing mutations and altering gene expression in different bacterial species.

The decrease in crystal protein concentration in this study may be attributed to expression of genes responsible for crystal protein production, or to the complete sporogenesis in which delta toxin is produced and secreted after cell lysis. On the other hand, protease enzymes might have affected the structure of crystal protein in the medium during production.

Our results demonstrated that the maximum absorption features of the crystal protein extracts spectra for control and other treatment, were recognized at around 255-280 nm wave length for all extracts. This may indicate the presence of chromophores in the crystal protein structure. However, the peak of the curve indicates the difference in protein concentration at a given wavelength. The UV spectrum of crystal protein extracted from the control culture after 72 hours of incubation differed significantly from the spectrum of crystal protein extracted from microwave treated culture. All three irradiated survival bacterial cultures Bt KS1.1, Bt KS2.1 and Bt KS3.1 at the three periods of incubation showed UV spectrums similar to those of the control culture incubated for 48 h. The recognized difference was in the range of 220-235 nm and this might be due to the excretion of some uncharged amino acids or peptides devoted to sulfur bond or chromatophore amino acids as suggested previously

(Prasad *et al*., 2017). Furthermore, polysaccharides produced by microwave treated isolates as a co-form with spore formation by bacterial culture might absorb UV light in the range of 190- 230 nm, and this is in argument with the finding of this research that microwave irradiated isolates Bt KS3.1, Bt KS3.2and Bt KS3.3 were early producers of spores inside the mother cell as well as their ability to accumulate of congo red stain on their colonies. In addition, other researchers determined polysaccharide production using the same UV-wavelength range such as Cyanobacteria (Jiang *et al*., 2018; Trabelsi *et al*., 2009).

The insecticidal activity of spores/crystal mixture of irradiated isolates under study was conducted with 2nd instar larvae of *E. cautella* and revealed that despite reduction in crystal protein concentration in the extract of Bt KS3.1, Bt KS3.2 and Bt KS3.3 isolates, elevated spore count would furnish the mixture bioactivity with no visualized difference in the mode of action of spore/crystal mixture between control culture and mutant isolates under study. Black dead larvae appeared in both the control and microwave-irradiated mutants, which is related to the action of *B. thuringiensis* delta toxin on insects. This is in agreement with other findings (Pederson *et al*., 1995) that referred to the action of spore count associated with the bioactivity of *B. thuringiensis*.

الملخص

الخفاجي، خلود عبد الإله، فلاح حنش نهار، سميرة عودة خليوي، محمد عبد الرحيم عبد الله، أحمد جعفر فياض، أميرة علوان مزبان وصابرين عبد الهادي صالح. 2023. التأثير المطفِّر للتشعيع بالموجات الميكْرويَّة على تكوين الأبواغ والبروتين البلوري في العزلة العراقية kurstaki KS3 للبكتيريا *Bacillus thuringiensis*. مجلة وقاية النبات العربية، 41(3): 285−291. https://doi.org/10.22268/AJPP-041.3.285291

تعدّ البكتيريا *Bacillus thuringiensis* kurstaki KS3 أحد أهم عوامل المكافحة الحيوية المستعملة لمكافحة الحشرات ذات الأهمية الزراعية في الحقل والمخازن. هدف هذا البحث الى دراسة تأثير الإشعاع الميكْرَوي على تكوين الأبواغ والبروتين البلوري المنتج من العزلة العراقية KS3 للبكتيريا *B. thuringiensis*. استعمل وسط لوريا بوتاني السائل ذي الرقم الهيدروجيني 7.0 لتغذية البكتيريا وإنتاج الأبواغ. عُرِّض محلول الأبواغ الى الأشعة الميكْرَوية وبقدرة 1000 واط لفترات 5، 10 و15 ثانية. تمّ حساب تثبيط الأبواغ لكل فترة زمنية، كما فحصت الاختلافات المجهرية للخلايا المشعة لاستقصاء حدوث الاختلافات المظهرية للمستعمرات وتركيز البروتين البلوري وعدد الأبواغ المنتجة وتقييمها مقارنةً مع الشاهد غير المعامل. قُدِّر عدد الأبواغ في مزروع المقارنة 3×10^{8} بوغة/مل ولوحظ انخفاض عددها إلى 3×10^{7}، 2×10^{6} و 2×10^{4} بوغة/مل بعد فترات تعريض 5، 10 و 15 ثانية، على التوالي، وبنسبة انخفاض قدرها 49.263% بعد 15 ثانية. لوحظ أن شكل مستعمرات البكتيريا كان متشابهاً للفترات الثلاث المستخدمة، كما ظهرت مستعمرات ذات حجم أصغر وحافات ملساء على وسط الكونغو الأحمر في المعاملات الثلاث بالمقارنة مع معاملة الشاهد. أوضح الفحص المجهري أن المزروع المُعامَل متشابه مع المزروع الأصل من حيث شكل الخلايا وقطرها وترتيبها مع ظهور خلايا أصغر حجماً عند المعاملة لمدّة 15 ثانية، كما أُنتجت أبواغ للمزروع غير المعامل بعد 72 ساعة من التنمية مقارنة مع 75، 90 و 90% من إنتاج الأبواغ في الخلايا المعاملة بعد 24 ساعة فقط وذلك لفترات التعريض 5، 10 و 15 ثانية، على التوالي. بلغ إنتاج البروتين البلوري ذروته بعد 72 ساعة في المزروع غير المعامل، وتذبذب إنتاجه في العزلات المشعة تبعاً لمدّة التعريض.

كلمات مفتاحية: تكوين الأبواغ، تأثير فيزيائي، سم دلتا.

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