EVALUATION OF DIFFERENT AGRO- BASED MEDIA ON THE PRODUCTION OF CRYSTAL TOXIN FROM THE INSECTICIDAL BACTERIUM *Bacillus thuringiensis*KS3

Kh. A. A. Alkhafaji **S. O. Khleoy**
S. A. Alrahem **S. A. Alhadi**

ABSTRACT

The aim of this research work was evaluation of some agro-based material and by product in the production of spore/crystal mixture from a local isolate of *Bacillus thuringienisis* KS3(BtKS3) in semi solid fermentation. Growth, spore formation and production of crystal protein toxin from (BtKS3) were determined on conventional and nonconventional media in shake flasks. Lauria Bertani broth was the medium of choice, it gave the highest crystal protein concentration reached to 0.925 mg ml^{-1} compared with semi synthetic broth (0.536 mg ml^{-1}),rich medium (0.219mg ml^{-1}) and nutrient broth (0.831 mg ml^{-1}). Powder of wheat, corn, sorghum, faba straw, millet, chickiling, mung bean and cheese whey were used each alone for preparation of a medium. All components except faba straw and cheese whey were supported bacterial growth. Spore count was higher at media composed of sorghum, millet and chickiling. The best agro- based media was composed of sorghum and millet that both supported crystal protein synthesis which reached to 1.616 mg ml $^{-1}$ and 1.519 mg ml $^{-1}$ respectively, no significant difference was recognized between the two components. No significant differences observed between Sorghum concentration at 2.5 and 5% wt/ vol without any additive which gave superiority result compared with Laurea bertani broth which reached to1.666 $mgml^{-1}$ and 1.774 $mgml^{-1}$respectively, while, reduction of crystal protein synthesis was detected at medium contained 10% sorghum. Considering all three factors, relative yield, productivity and cost, the use of the mono-component medium was proved much more economical for an industrial production of crystal protein toxin than the references broths.

INTRODUCTION

Bio insecticides based on the bacterium *Bacillus thuringiensis* (*Bt*) are the most broadly used microbial biological agents in the control of most harmful insects (4,11).They represent natural, biodegradable and safe pest control means. *B. thuringiensis* is a ubiquitous, gram positive, spore forming bacterium. Its insecticidal activity resides mainly in the production of a proteinaceous parasporal crystal called δ-endotoxin, which is formed during sporulation. Despite the increasing need for environment friendly, chemical residue free and safe control agents such as *Bt* based biopesticides (10),The production and use of bio-insecticides remains very limited compared to chemical insecticides for two main reasons: the production cost and the low stability of the product in the environment over time leading to reoccurrence of applications. Growth and synthesis of toxin need many

Ministry of Sci. and Tech., Baghdad, Iraq.

requirement include a carbon (energy source) like glucose, nitrogen sources such as peptone or yeast extract and ammonium sulfate, and mineral salts (6,13).Metal ions such as Ca_2+Mg_2+and Mn_2+were found essential for *Bt* growth and potassium ions were showed necessary for the production of *Bt* crystals (9,20).Synthetic laboratory mediums with all these elements are generally used for small and large scale production. However, the synthetic and expensive substrates are usually replaced by cheap agro materials or agro-industrial by-products to reduce the cost of mass production of *Bt*. This approach allows also to process waste into valuable products and help dispose of these byproducts in an ecologically safe manner. Different agro-industrial residues and byproducts were used in *Bacillus thuringiensis* production. Soybean meal, corn steep liquor, groundnut seed meal extract, fish meal, gruel fish, cheese whey, *Bombyx mori* pupae, powders of edible leguminous seeds and fodder yeast were used as sources of proteins (1,7,8,14,16,21) but some time carbohydrates (glucose, starch or molasses) and/or mineral sources may be necessary to add. Usually *Bt* strains isolated locally are more effective than imported strains due to higher specificity on target host, greater field persistence due to higher adaptation to the natural environment and toxicity at a higher temperature range (3). To infer the benefit from the *Bt* based biopesticides, there is a need for studying influence of raw material on growth, spore formation and crystal protein production by local *Bt* strain that would be used as biopesticides for the indigenous crops. Thus this work was conducted to achieve the reproducibility of many agro material and by product for B.t crystal toxin protein production.

MATERIALS AND METHODS

Bacterial cultures and Media preparation

Soil bacterial isolate designated as KS3 identified as *B. thuringiensis* by al-Khafaji in the laboratory of Agriculture Research Directorate was used for toxin production the whole of study, using agro based materials by semi solid fermentation. The bacterial spores were inoculated in the nutrient broth and incubated at 30°C for 18 h to use as mother inoculums.

Three liquid media were used as reference cultures, briefly, Laurea Bertani broth (LB) (tryptone1%, yeast extract 0.5% and NaCl 1% pH7.8). semi synthetic broth consisted of (g L^{-1}): soybean flour 15; glucose 5; starch 5; K_2HPO_4 1; KH_2PO_4 1; $MgSO_4.7H_2O$ 0.3; $FeSO_4.7H_2O$ 0.02; $ZnSO_4.7H_2O$ 0.02; $CaCO_3$ 1.0 (2). The third medium was proposed by Anderson and designated as complete rich medium (g L^{-1}); composed of Glucose 9.77; Yeast extract 4.62; Bacto peptone 4.62; $(NH_4)_2SO_4$ 1; KH_2PO_4 3.4; K_2HPO_4 4.15; $MgSO_4.7H_2O$ 0.3; $CaCl_2.2\ H_2O$ 0.106; Fe-Citrate 0.075; $ZnSO_4.7H_2O$ 0.0075; $CuSO_4$ 0.0045; $MnSO_4.7H_2O$ 0.05 (12).

Many different agro material and by products were used in the semi solid fermentation media for the production of crystal toxin from the insecticidal bacterium Bt KS3. Raw material includes cereals (wheat flour), legumes (faba straw, millet, chickiling) corn, sorghum and cheese whey.Seeds and straw were grinding with the aid of electric grinder while, whey was used as liquid material.

The FDA information data was used to assess the protein content, sugars, minerals and vitamins for each agro materials used in the present study.

Semi solid mono- component medium was prepared by incorporated the powder of raw material at 2.5% to distilled water. All pHs were adjusted to 7.8 with NaOH and sterilized by autoclave for 15 min at 1.5 bar. All laboratory synthetic and semi solid media were prepared in 100 ml conical flasks containing 20 ml of each media.

All media were inoculated with $1*10^{8}$ cfu ml^{-1} of starter culture of *B.thuringiensis* KS3, incubated for 72h at 30C with constant agitation using shaker incubator at 150 rpm.

Recovery and estimation of spore/crystal protein mixture

Spores were counted by dilution method for all treatment, briefly, one ml of culture was diluted with nine ml of distilled water to obtain 10^{-1} dilution, then heat treated at 80 C for 10 min to inactivate vegetative bacterial cell and dilution continued to 10^{-7}.Only 0.1 ml of 10^{-5} to 10^{-7} was spread over nutrient agar, incubated at 30 °C for 24 hr and colonies number were recorded in plates with 30 to 300 colonies to enumerate spores in the culture.

For crystal protein estimation, a procedure modified from (15,16,17) was applied, briefly, samples were withdrawn, centrifuged at 4000 rpm for 10 min to remove spores and the cell debris, second centrifugation was applied at 10000 rpm for 10 min to precipitate crystal protein which dissolved with 0.5 N NaOH. Protein concentration was quantified using Nano drop (Thermo scientific, nanodrop2000) for protein determination in which 1OD at 280 nm equivalent to 1 mg ml^{-1} protein.

The sorghum medium was chosen as agro- based medium for the production of crystal toxin, MgSO4 at 0.01% was added and spore forming and crystal protein production was estimated in new medium (2). Sorghum based medium prepared with 2.5% and 5% concentration, also with or without MgSO4 at 0.01%

Statistical analysis

All experiments were done with triplicate and statistical analysis was applied using ANOVA statistical program where the confidence level was set at 95%.Any significance difference ($p \leq 0.05$) was analyzed using LSD test.

RESULTS AND DISCUSSIONS

The production of crystal protein/ spores mixture differed according to laboratory synthetic media composition and the data showed that LB was the best media for crystal toxin protein production from a local isolate B. thuringiensis SK3 (0.925mg ml^{-1}) with significant differences as presented in figure (1).

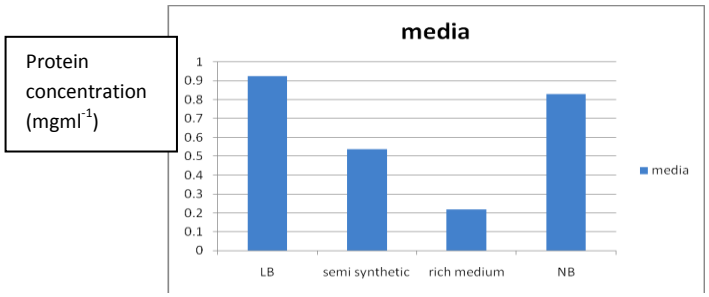

Figure 1: production of crystal protein from *B. thuringiensis* KS3 in laboratory media.

Since crystal toxin of *B. thuringiensis* mainly constituted of protein thus media components play a very important role in determining the yield and insecticidal activity of the spore crystal complex. Also, different strains belong to the same species may have different growth requirements, spore formation and crystal toxin production this in accordance with Boulenouar *et al.* (5) who observed that the different strains of the same bacteria may show different growth and toxic activities, which may be due to the differences in growth requirements of different strains.

First experiment showed that raw agro material supported the growth of a local isolate BtKS3. Different bacterial growth rate and spore formation observed in a comparison with LB. Millet, Chickeling and sorghum based media showed good spore formation of bacteria while, low sporulation observed with media based on corn and wheat, while no sporulation on faba straw and whey based media.

Crystal protein content varied according to media composition, sorghum and millet gave best crystal concentration which reached to 1.616 and 1.519mg ml-1 respectively with no significant differences among them and LB broth. The second best production raw material was corn and wheat flour;crystal protein concentration reached to 1.26 and 0.887 respectively with appearance significant differences followed by chickiling (0.835mg ml^{-1}) and mug bean (0.833 mg ml^{-1}). Bacterial growth and crystal protein reduced drastically with media contained faba straw and why, this may due to the high phenolic compound content in faba straw and the reduction of whey pH through fermentation and overall nutrition factors of whey may had a negative effects on the toxin production. Also, Low spore formation may due to many factors include substrate utilization, final pH and incubation time of semi solid fermentation in such media.

Many enzymes activity, spore formation, nutrition and secondary by product of bacteria require specific metal ions such as magnesium, manganese, calcium, zinc and ferrous. A lot of minerals are normally present in the sorghum seed used here in fermentation and there may be no need to add these minerals in the fermentation media as presented by data sheet of FDA.

Table 1:Crystal protein production by BtKS3 using agro- based media

Agro- based material	Means crystal protein concentration(mg ml^{-1})	Spore formation CFU ml^{-1}
wheat	0.887^{B}	1×10^{2}
corn	1.26^{A}	1×10^{3}
sorghum	1.616^{A}	1×10^{8}
Faba straw	0.680^{B}	nil
millet	1.519^{B}	1×10^{8}
chikeling	$.833^{B}$	1×10^{7}
whey	$.630^{B}$	nil
Mung bean	$.835^{B}$	1×10^{7}
LB	1.451^{A}	1×10^{7}
LSD= 0.114 at 95% difference		

LSD for crystal protein concentration; A,B =significant differences between A and B;A= no significant difference among A

Study results showed that no significant difference between using 2.5% and 5% of sorghum flour while a reduction in protein production was observed

at 10% of sorghum (table 2). This may due to the reduced agitation resulted from high viscosity of medium or high concentration of many minerals that exist in sorghum seed.

The survey of FDA data revealed that Sorghum contains high protein concentration, different sugars, vitamins and minerals which all supported the growth, spore formation and crystal protein formation. Many researchers used raw material as it is as Anyika (1) and Devidas (8), they used plant material including corn, soya, wheat and legumes. While, A lack of research dealing with using sorghum seed in fermentation for spore/crystal toxin production from *B. thuringiensis* to compare with.

Table2:Crystal protein production by BtKS3 at different concentration of sorghum

Sorghum concentration gm %	Means of crystal protein concentration mg ml^{-1}	Spore count cfu ml^{-1}
2.5%	1.666	1×10^{8}
5%	1.774	1×10^{8}
10%	1.353	1×10^{7}
LB broth	1.493	1×10^{7}
LSD=0.1565		

The fermentation of the different isolates of *B.t.*, regardless of subspecies, have some general characteristics in common. They all use sugar (usually glucose, molasses, or starch), producing acid during the fermentation. In general, they have similar requirements for proteins or protein hydrolysates. However, the individual isolates are unique entities, and a particular medium that may support good growth or toxin production by one isolate may be less favorable for another(17,18,19,21).

In conclusion: Some agro- based media selected in the present work could represent an economical benefit for biopesticide production, because they allowed, spore formation, and ultimately higher toxin production levels at lower cost, compared to conventional nutrient source. Each separated preparation of agro- based media proved adequate for cultivation of a local isolate of B. thuringiensis KS3 and spore/ crystal toxin mixture formation, at least on a laboratory scale. Medium based on sorghum or milt, which provided the best choose fermentation medium for the growth and spore formation of the local isolate BtSK3 in this study, can be considered for further development. If these low- cost Bt preparations are as successful in field trials as they are in the laboratory, they could be an important tool for use in an integrated pest control programmed.

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تقويم اوساط ذات أصل زراعي في إنتاج البلورات السامة من البكتريا *Bacillus thuringiensis* KS3

خلود عبد الاله الخفاجي سميرة عودة خليوي
صفاء عبد الرحيم صابرين عبد الهادي

الملخص

الهدف من البحث هو تقويم قابيله عدد من الأوساط المصنعة من المواد الزراعية أو مخلفاتها في إنتاج مزيج الذيفان البلوري/السبورات للعزلة المحلية ***Bacillus thuringienisis*** **KS3(BtKS3)** باستخدام تخمرات شبه الصلبة. تم التحري عن قابلية العزلة المحلية على النمو وإنتاج السبورات إضافة الى إنتاج البروتين البلوري في الأوساط التقليدية وغير التقليدية باستخدام الدوارق والهزاز. تم اختيار وسط لوريا برتاني في الإنتاج والمقارنة، إذ اعطى اعلى انتاجية من تركيز البلورات البروتينية التي وصلت الى **0.925** ملغم مل$^{-1}$ مقارنة مع الأوساط نصف المصنعة **0.536** ملغم مل$^{-1}$ والوسط الإغنائي **0.219**ملغم مل$^{-1}$ ووسط المرق المغذي**0.831**.ملغم مل$^{-1}$. أظهر استعمال أوساط احتوت على مسحوق الطحين والذرة الصفراء والذرة البيضاء ومخلفات الباقلاء والدخن والهرطمان والماش وشرش الأجبان ان الأوساط جميعها تدعم النمو البكتيري وتكوين السبورات عدا وسطي مسحوق مخلفات الباقلاء والشرش. كان تقدير العدد الكلي للسبورات أعلى في اوساط الذرة البيضاء والدخن والهرطمان، وكان افضل الأوساط تلك التي احتوت على الذرة البيضاء او الدخن، إذ دعمت إنتاج البروتين البلوري وبدون فروق معنوية ليصل تركيزه الى كل من **1.616** ملغم مل$^{-1}$و**1.519**ملغم مل$^{-1}$ على التوالي. لم تظهر فروق معنوية في تركيز البروتين البلوري عند استخدام تركيزي **2.5**% و**5**% من مسحوق الذرة البيضاء وبدون أية إضافات أخرى بينما أدى إضافة تركيز **10**% منها الى انخفاض في تركيز البروتين البلوري.تفوق وسط الذرة البيضاء على وسط السيطرة (لوريا برتاني)، فوصل التركيزين الى **1.666**ملغم مل$^{-1}$ و**1.774** ملغم مل$^{-1}$ عند تركيزي **2.5**% و**5**% من الذرة البيضاء على التوالي. وباعتماد العوامل الثلاثة التي شملت الناتج النهائي، الإنتاجية والكلفة يكون استخدام الوسط أحادي التركيب أكثر اقتصاديا من أوساط السيطرة المختبرية للإنتاج الصناعي للبلورات البروتينية.

وزارة العلوم والتكنولوجيا، بغداد، العراق.