The possibility of manufacturing a biocidal of *Bacillus* spp. and their growth on different fermented media

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Abstract:

The study was conducted in the Molecular Biology Laboratory - Department of Plant Protection, Faculty of Agriculture, University of Kufa during 2020-2021 to evaluate the possibility of producing a biocide using *Bacillus* spp. *B. firmus*, *B. cereus*. and *B. simplex*. The results of manufacturing bionematicide from these bacteria showed that the three species did not differ in their growth on the same compost medium (palm fronds, rice residues, wheat residues), while the growth of each *Bacillus* spp. differed from other species on different culture media. The palm frond medium, regardless of the type of bacteria, was the least favorable and did not support many bacterial populations, and allowed no more than $83*10^8$ CFU. Whereas the bacterial colonies ranged from $219*10^8$ CFU to $254*10^8$ CFU on fermented wheat residues, with a slight difference from rice residues, which led to bacterial colonies of $198*10^8$ CFU to $214*10^8$ CFU. In the case of carriers used for the biocide, preparation using sand was always better than with calcium sulfate. The effect of packing materials (plastic bags, paper bags, or plastic containers) on the efficiency and vitality of bacteria in the biocide content was also evaluated. It is noted that the highest bacterial colonies, which ranged from 267 to 253 $*10^8$ CFU, in the case of using plastic containers significantly differed from storing in paper bags ($233-230*10^8$ CFU) and plastic bags ($235 - 216 *10^8$ CFU), after 15 days of storage in laboratory conditions. However, the viability and effectiveness of the prepared biocide decreased with the increase of storage periods to 30, 60, and 90 days, regardless of the type of bacteria.

Keywords: Biocides, *Bacillus*, Fermentation media, Carrier.

Introduction:

The biopesticide industry is the processes and additives that help in the survival and effectiveness of the biological control agent active and play an important role in delivering it to the target organisms or pathogens. The use of biopesticides to control plant pests and diseases provides an effective and environmentally friendly solution (15), as they are less threatening to the environment and human health compared to chemical preparations. The most common biocides are pathogens of economically important pests. These include bio-insecticides such as *Bacillus thuringiensis*, and *B. sphaericus* (1).For many decades, soil, seeds, roots and more have been treated with bacteria to improve plant growth and development. The objective of adding bacteria is to improve plant growth through the beneficial relationship of nitrogen fixation, improve plant resistance to toxic compounds, and biological control of plant pathogenic microorganisms (14).

The genera *Acinetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Frankia*, and *Pantoea* (16 and 3) constitute most of the genera that are used in biological control. In general, biological control agents are multiplied and maintained using appropriate, available, low-cost media that contain the elements and compounds necessary to maintain the viability of the biological agent vaccine. Also, the biological factor under production should not pose risks to humans and the environment and is rapidly degraded in the environment, which means less pollution. The main requirements for the preservation of the organism (6) provide a more favorable microenvironment for microbial strains, along with physical or chemical protection over a long period to avoid the rapid deterioration of cells. viability during storage, (ii) to support competition between more adapted native soil strains and plants, and (iii) to reduce losses from plundering microorganisms after introduction into the soil. All of these functions are intended to provide a reliable source of living cells available to interact with plants (10 and 11).

Carriers in the concept of pesticides are materials used to provide appropriate volume in the physiological state. They generally must have positive or no negative effect on the prepared biocide, be available with low cost, ease of use, packability and mixability, and have good storage capacity. The carrier must also have a high water holding capacity, high content of organic matter, and high ability to sterilize (17). Calcium carbonate was used in previous studies as an effective carrier for biocides preparations, including bacterial ones. It was found that calcium carbonate was the most efficient carrier, compared to talc, for Pseudomonas fluorescence(5). For carriers that are used to coat the seeds, they must have good seed adhesion, soil biodegradability, and non-polluting compounds (18). Mixtures used for carriers can be dry materials such as clay, coal, peat, vermiculite, perlite, rock phosphate, and calcium sulfate, or inert materials (bentonite, kaolin, silicate, vermiculite), and organic ones such as sawdust, wheat bran, soybean flour and rice husks as carriers. The carriers must be suitable for bacteria survival and activity,

non-toxic, easily sterilized, adjustable pH, heat, humidity, and packaging (7,8). Packaging is one of the three main obstacles affecting the use of biocides. Therefore the study aim is to test the suitability of three fermented media (palm fronds, rice residues, wheat residues), two carriers (sand and calcium phosphate), and three types of packaging (plastic bag, paper bag, or plastic container) for synthesizing a bionematicide from *Bacillus* spp.

Materials and Methods:

Evaluating bacteria growth and determining inoculum concentration:

Nutrient broth medium (N.B.) was prepared in 250 ml glass flasks and previously purified (*Bacillus* spp.) on N.A medium was added and incubated at 28 ± 2 °C for 48 hours. Then, the number of bacteria colonies for each *Bacillus* species of counted. The bacteria cultures were subjected to a dilution method of up to 10^9 to determine the best concentration. Then 1ml of each dilution was added to a sterile Petri dish with 3 replications and the culture medium N.A was poured and incubated at 28 ± 2 °C for 48 hours and the number of colonies resulting from each dilution was counted. And the dilution (10^8) was chosen, which was adopted to be used in the subsequent experiments.

Synthesis of a biocidal using *acillus* spp. under study:

The efficiency of three plant residues as growth media for Bacillus species under study

Some fermented media, palm fronds, rice plant residues, and wheat residues were tested as growth media for the Bacillus species under study. The materials were finely ground and a fine powder was obtained for each medium. The powdered materials were distributed in glass bottles of 250 gm, sterilized with an autoclave, and re-sterilized after 24 hours. The media were inoculated with bacterioplankton of different species prepared in advance on N.B liquid media at 10 days old using 50 ml of the bacterial suspension for each 250 g of powder. Then incubated at 28 + 2 C for 10 days and the bottles were shaken every two days. To ensure the distribution of bacterial growth on all parts of the development medium, after the end of the incubation period, the numerical density of the different bacterial species in the dilutions up to 10-8 dilutions growing on the N.A culture medium was calculated after 48 hours and the number of colonies resulting from each dilution was calculated (4).

Loading *Bacillus* spp. inoculum on carrier materials:

Two types of the carrier (sand and calcium sulfate) were sterilized and prepared to which the *Bacillus* spp. inoculums were loaded. Bacterial (inoculum on fermented material) was loaded onto the carrier in a ratio of 5:1 and dried at 35°C until the weight is stable. Then the processed mixture (Bacillus sp + fermented plant residue +carrier) was subjected to three types of packing which are plastic bags, paper bags, and plastic containers with three replicates, and stored for 15, 30, 60, and 90 days in the laboratory conditions at 4°C. The experiment was set up according to the completely randomized design (C.R.D) with three replications.

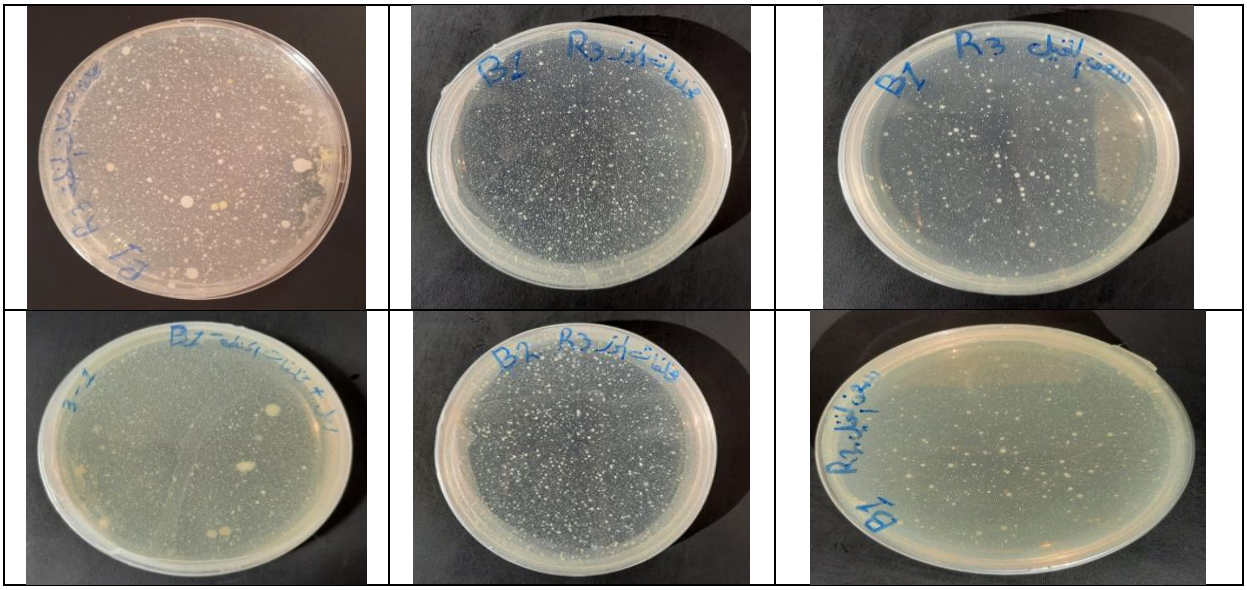
Statistical Analysis System (SAS, 2011) was used for data analysis. Means were compared among treatments using the least significant difference L.S.D. ($P \leq 0.05$).

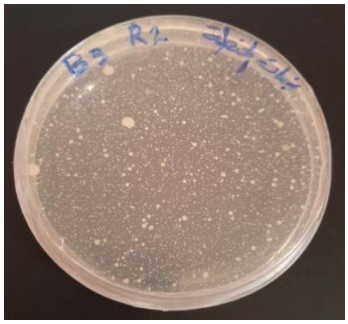
Results and Discussion:

Manufacture of a biocide from *Bacillus* spp.

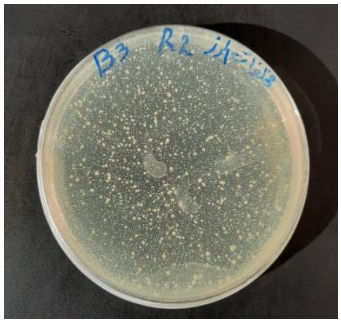
The efficiency of three plant residues as growth media for Bacillus species under study

The results showed that three bacteria species *Bacillus firmus*, *B. cereus*, and *B. simplex* did not differ among themselves in the growth of the same medium. While the different media (palm fronds, rice residues, wheat residues) differed significantly from each other in influencing the growth of bacterial colonies (Figure2,3). Palm fronds medium (regardless of the type of bacteria) did not allow to increase in the number of biological units to higher than $83*10^8$ CFU compared to the highest growth of bacterial colonies ranging from $219*10^8$ CFU to $254*10^8$ CFU on wheat residues medium, which slightly differed from the medium of rice residues that of $198*10^8$ CFU to $214*10^8$ CFU. These results agreed with previous results that the corn residues were the best medium among the four residues including alfalfa hay, ceratophyllum residue, rice residues, and corn residues (2). Similarly, fermented materials of wheat remain, rice husks, corn residues, and sawdust was evaluated for their suitability as a bacteria growth medium. It was found that wheat and corn residues gave the best results. This was attributed to the medium content of nutritional requirements of bacteria such as carbon, nitrogen, phosphorous, potassium, and others (4).

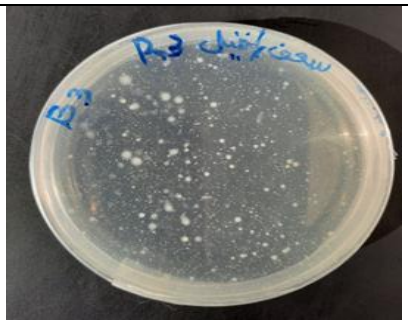




Bacillus + Wheat residue



Bacillus + Rice residue



Bacillus + palm fronds

Figure 1. Shows the effect of some plant residues on the growth of three types of *Bacillus* spp.

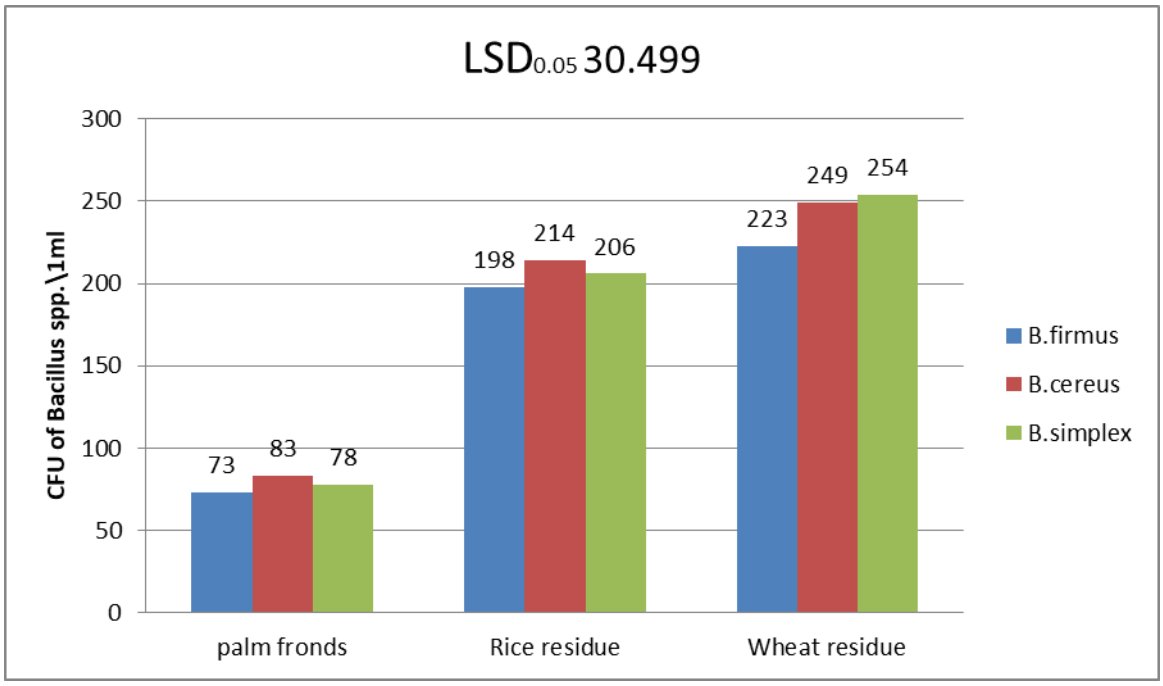


Figure 2. Effect of fermented plant residue of palm fronds, rice residues, and wheat residue on growth and development of *Bacillus firmus, B. cereus,* and *B. simplex*. CFU (10^8)/1 ml, LSD (P≤0.05)= 30.499.

Effect of interaction fermented plant residues media and carrier materials (sand or calcium sulfate) on the growth of the studied *Bacillus* spp.:

The results showed that palm fronds/calcium sulfate medium resulted in bacterial colonies of *B. sereus* and *B. simplex* of 60*10^8 CFU and 64*10^8 CFU which was higher than in the case of sand as a carrier (44.3 *10^8 CFU and 54.3*10^8 CFU). However, palm fronds/sand medium increased bacterial colonies of *B. firmus* up to 79.7*10^8 CFU than in the case of calcium sulfate and for the same medium (Table 1). On the other hand, in the case of rice residues, the number of colonies for all species was higher with the sand medium and ranged from 184.3*10^8 CFU to 196.3*10^8 CFU than in the case of calcium sulfate ranged from 203*10^8 CFU to

213*10^8 CFU. Regardless of the medium and the carrier, wheat residue resulted in the highest number of bacterial colonies among all the interactions. These results agreed with the findings of (Kumar. S., and A. Singh, 2015) who used calcium sulfate and talc as carriers for biocides production, and found that calcium sulfate was the most efficient carrier for bacterial inoculums compared to talc and kaolin. The reason for the increase in the number of reproductive units in treating sand as a carrier with wheat waste as a growth medium may be due to the carrier's suitability in improving the nutritional environment by increasing the available nutrients or changing the pH to suit the growth of bacteria or their ability to survive during storage.

Table 1. Effect of different media (palm fronds, rice residues, wheat residues) and carrier materials (sand and calcium sulfate) on the growth and development of Bacillus species under study

LSD 0.05	*B. simplex*	*B .cereus*	*B. firmus* *	carrier materials
LSD 0.05 = 30.499	78.0	83.0	73.0	palm fronds
	206.0	214.0	198.0	rice residues
	252.7	249.0	223.0	Wheat residues
LSD 0.05 = 92.508	54.3	44.3	79.7	palm fronds + sand
	189.0	196.3	184.3	Rice waste + sand
	243.0	173.0	200.3	Wheat waste + sand
LSD 0.05 = 63.679	64.0	60.0	68.7	Palm Leaves + Calcium Sulfate
	178.0	178.7	112.3	Rice waste + calcium sulfate
	219.0	213.0	203.7	Wheat waste + calcium sulfate
LSD (P≤0.05)= 64.17				

Each number is an average of three replicates. Bacteria numbers were measured by the number of CFU in 1ml*10^8.

Effect of different packaging materials and storage laboratory conditions on the efficiency of the manufactured biocide:

Regarding the effect of packing materials (plastic bags, paper bags, plastic containers) on the efficiency and vitality of bacteria in the biocidal content of the plant. It was noted (Table 2) that the highest bacterial colonies that ranged from 267*10^8 CFU to-253*10^8 CFU were higher in the case of the plastic container with a significant difference from the number of colonies in the case of paper bags (233-230) *10^8 CFU, which did not differ from their numbers (235-216*10^8 CFU) in the case of storage in plastic bags after 15 days of storage in laboratory conditions. It was also noted that the increase in the storage period, after 30, 60, and 90 days, the significant differences between the types of storage materials began to decrease regardless of bacteria species. In general, the highest numbers of bacterial colonies for each type of bacteria were recorded in the case of storage in plastic containers, followed by paper bags and then plastic bags, respectively.

Table 2. Number of reproductive units per ml 10^8 for three formulations of the manufactured pesticide that were intertwined with the packaging materials and stored at laboratory temperature

Period of stored	Stored Biocide formula	Plastic bag	Paper bag	Plastic container
15 Days	A **	216.0	233.3	267.0
	B	227.7	231.0	253.0
	C	235.7	230.0	254.7
30 Days	A	213.3	228.7	230.0
	B	214.0	213.7	231.3
	C	197.3	197.7	221.0
60 Days	A	192.7	202.3	217.3
	B	201.0	189.3	195.3
	C	197.0	186.0	197.3
90 Days	A	181.3	193.3	203.3
	B	192.0	190.7	205.0
	C	188.7	186.0	189.3
LSD 0.05= 28.75		LSD 0.05= 23.96	LSD 0.05=36.11	LSD 0.05=27.95

Each number is an average of three replicates. Bacteria were measured by the number of CFU in 1ml*10^8, ** (A) *Bacillus firmus*+wheat residue+ sand; (B) *Bacillus cereus*+wheat residue+sand; (C) *Bacillus simplex* +Wheat waste + sand.

Effect of different packaging materials and storage at 4°C on the efficiency of the manufactured biocide:

Regarding the effect of packing materials (plastic bags, paper bags, plastic containers) on the efficiency and vitality of bacteria in the biocidal content, it was noted that (Table 3) the highest bacterial colonies (265-250*10^8 CFU) were in the case of plastic packages with a significant difference from the number of colonies in the case of paper bags that ranged from 238 to-225*10^8 CFU, with no difference from their numbers (234-214*10^8 CFU) in the case of storage in plastic bags after 15 days of storage at 4°C conditions. It is also noted that with the increase in the storage period, i.e. after 30, 60, and 90 days, the significant differences between the types of storage materials began to decrease. Apparently, among the soring materials, these plastic containers were the best for storing the bacterial-manufactured biocide. It was also noted that storing the biocide in lab conditions did not differ much from storing it at 4°C conditions.

Table 3. Effect of different packaging materials and storage at 4°C on the efficiency of the manufactured biocide

Period of stored	Consist of biocide	*Plastic Bags	Paper bags	Plastic cans
15 Days	A**	214.0	238.7	265.3
	B	227.3	233.7	250.0
	C	234.3	225.0	253.3

	A	206.7	216.7	236.7
30	B	209.0	217.3	212.0
Days	C	190.3	202.0	213.0
	A	188.3	195.0	216.3
60	B	194.7	195.0	200.7
Days	C	195.0	188.0	210.0
	A	185.7	191.0	212.0
90	B	197.0	200.3	205.7
Days	C	186.3	181.3	198.0
LSD 0.05= 29.27		LSD 0.05 =24.75	LSD.0.05=37.26	LSD.0.05=27.47

Each number is an average of three replicates. Bacteria were measured by the number of CFU in 1ml*10^8, ** (A) *Bacillus firmus*+wheat residue+ sand; (B) *Bacillus cereus*+wheat residue+sand; (C) *Bacillus simplex* +Wheat waste + sand.

These results agreed with what was formerly found (M, S., F, E., and Leonetti, P. 2014) that storage conditions have a significant and effective effect on the shelf life of the biological control product and the need to improve storage conditions to support the survival of the bacterial cell for a longer term. they found that plastic containers with a high barrier were more appropriate. The long period of maintaining and viability of the bacterial cells in plastic containers and bags was assumed to be due to the ability of these packages to isolate oxygen and moisture regardless of temperature. (13). confirmed a long shelf life of 12 to 18 months a prerequisite for the successful commercial marketing of biological control compounds. On the other hand, the moisture content is a decisive factor affecting the success of the storage process and achieving the highest biological survival rates for the biological control factor at a moisture content of 38% of the total content of the biocidal composition. On the other hand, this study found there was no clear effect of storage conditions under laboratory conditions or at 4°C on the viability of the manufactured biocidal formulations. This was consistent with what was reached by (Vassilev N., and Mendes G. 2018) as it showed that storage at high temperatures compared to the average number of reproductive units for storage at low temperatures for any period. It may not give the desired results, especially if there are other influencing factors such as humidity, light, and exposure to oxygen.

Conclusion:

The results showed that three types of bacteria *Bacillus firmus*, *B. cerus*, and *B. simplex* did not differ among themselves in the growth of the same medium. While, the different media (palm fronds, rice residues, wheat residues) differed significantly among themselves via influencing the growth of bacterial colonies. The wheat residue was the best among the plant materials tested for supporting higher bacterial growth. It was also found that using sand as a carrier material is better than calcium sulfate and more suitable for biocide effectiveness. Long maintaining and viability of the bacterial cells in plastic containers was possible in both lab conditions and at 4°C.

Conflict of Interest

The authors have no conflict of interest.

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