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ANTIBACTERIAL ACTIVITY OF BACTERIOCIN STRAINS FROM *Bacillus thuringiensis (Bt)*, WITH DIFFERENT CONCENTRATIONS OF AMMONIUM SULFATE PRECIPITATE

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ABSTRACT

The antimicrobial activity of some bacteriocin strains produced by *Bacillus thuringiensis (Bt)* bacteria had been tested against *Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa* bacteria under different concentrations of ammonium sulfate. The *B*t strains were previously isolated from various habitats in Middle Tennessee (USA). Bacteriocin production and activity were defined by the agar well diffusing using the sensitivity of the standard reference bacteria as a measure of its activity. No inhibition was observed on *P. aeruginosa* upon incubation with many bacteriocins in 20% ammonium sulfate precipitate, while a medium inhibition zone was observed for other bacteriocins under these same conditions. A strong inhibition zone was shown for some strains against *S. aureus* with 20%, 30% and 40% of ammonium sulfate, while no inhibition zone observed for bacteriocin produced by other strains with 20% ammonium sulfate precipitate against *B. cereus*. Increasing concentrations of ammonium sulfate adequately increase the antimicrobial activity of bacteriocins.

Keywords: *Bacillus thuringiensis (Bt)*; Bacteriocins, Antibacterial, Ammonium sulfate

1. INTRODUCTION

Many bacteria such as *Bacillus thuringiensis (Bt)*, lactic acid bacteria, and some others produce certain antimicrobial peptides (AMPs) or small proteins (3-10 kDa) that are classified as bacteriocins **[1, 2]**. Bacteriocins have several

characteristics, including their large diversity and high abundance **[3]**. These bacteriocins had been used in industry and medical fields. Some studies reported there are four main classes of bacteriocins, class I, class II, class III, and class IV **[4, 5]**. These four classes are distinguished by the existence of disulfide and sulfide (lanthionine) bonds and other factors, including sensitivity of enzymes, thermo-stability, mode of action, as well as molecular mass **[6]**. However, there are no appropriate classification criteria for them **[7]**.

Bacteriocins sensitivity to enzymes, temperature, pH, and chemicals was described. Amino acid sequence of peptide formation determines the susceptibility of the bacteriocins to certain enzymes such as proteinase K **[8]**.Their degree of degradation towards different proteolytic enzymes also differs according to their amino acid sequences **[9]**. However, many environmental factors may affect or change bacteriocin titers **[10]**.

Bacteriocins are harmless to the environment, and the human body, so are used in preserving foods due to their inherent ability to inhibit microorganism contamination on the onset of the production process **[11]**.

Currently, there are about eighteen bacteriocins that are produced by various strains of *Bacillus thuringiensis* (*Bt*) **[12]**, they produce and synthesize insecticidal proteins namely Cyt and Cry toxins **[13]**. Due to the above reasons, *Bt* can be used in controlling insect pests and disease vectors in agriculture and public health, respectively **[14]**. The utilization of microorganisms in controlling pests is a concept that has been in existence for decades. *Bt*-based insecticides have been in use since 1938 **[3]**.

The aim of this study was to highlight the antimicrobial activity of bacteriocn strains produced by *Bacillus thuringiensis*is (Bt) against some other bacteria types, under different concentrations of ammonium sulfate onto the culture media.

2. METHODS

2.1 Preparation of Culture Broth

The isolates of *Bacillus thuringiensis* stock culture were previously isolated from various habitats in Middle Tennessee, USA, classified by **Ejiofor & Johnson (2002) [15]**. The isolate had been maintained and frequently sub-cultured on Luria-Bertani (LB) agar slants at 4^{o}C. Then they were cultured for 24 to 48 hours at 30^{o}C. Pure cultures were then cultivated in 250-mL baffled Erlenmeyer flasks containing 50 mL of LB broth.

2.2 Agar Well Diffusing Assay

The production of bacteriocins by the *Bt* strains was define by the agar well

diffusing using the sensitivity of standard reference bacteria as a measure of its activity. The reference strains were *Staphylococcus aureus* (CB155554A), *Pseudomonas aeruginosa* (CB155250A), and Bacillus cereus (CB154870A). Agar plates were prepared for each indicator organisms, and the wells were cut by using a sterile cork borer. To allow diffusion, the plates were left at room temperature for 2 hours then the plates incubated face down overnight at 30°C **[16]**.

2.3 Precipitation and Concentration of Bacteriocin from Broth Culture

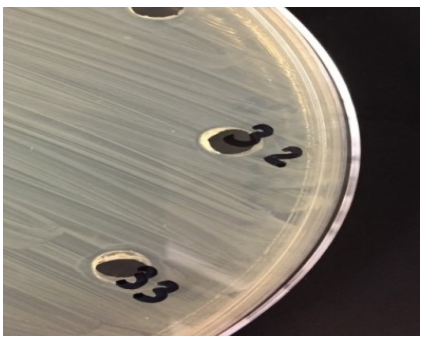
To ensure that any produced bacteriocin was detected, a modification of the precipitation method **[17]** was used. 20g of ammonium sulfate were added to each solution and the solutions were then incubated at 4°C for 1 hour with moderate shaking. The bacteriocin concentrates were stored at -20°C until used for bacteriocin-sensitivity tests against the reference strains. We measure the antimicrobial activity zone in millimeters. A measure of the inhibition zone in bacteriocin strains was as follows: weak inhibition zone (7-9mm), medium inhibition zone (10-12mm), strong inhibition zone (13-21mm), and no inhibition zone (0 mm).

3. RESULS & DISCUSSION

3.1 Activity of *Bt* bacteriocin before treatment with ammonium sulfate:

Bacillus thuringiensis strains *Bt* 71, 70, 33 and *Bt k*, without ammonium sulfate, have antimicrobial activity after 24 hours of incubation against different indicators. The inhibition zone of *P. aeruginosa*, *B. cereus* and *S. aureus*, before treatment with ammonium sulfate with these bacteriocins were shown in **Figures (1, 2 and 3)**.

a



b

Figure 1: Showing zone of inhibition on a lawn of *Staphylococcus* by Bt71and Bt 33 after 24 hour of incubation without ammonium sulfate. (a) The inhibition zone was (20mm) by Bt 71. There was no bacteriocin activity detected on the other Bt strains

Figure 2: Showing zone of inhibition on a lawn of *P. aeruginosa* by *Bt k and Bt* 71after 24 hour of incubation without ammonium sulfate. (a)The inhibition zone was (12mm) by *Bt k* and there was antimicrobial activity around *Bt* 71 and the size was around (12 mm). There was no bacteriocin activity detected on the other *Bt* strains

Figure 3: Showing zone of inhibition on a lawn of *Bacillus cereus* by *Bt* 70 *and Bt* 71 after 24 hour of incubation without ammonium sulfate. (a)The inhibition zone was (12mm) in both *Bt* strains

3.2 Activity of *Bt* bacteriocin with ammonium sulfate:

3.2.1 Antibacterial activity against *Pseudomonas aeruginosa*

The inhibition of bacterial activity by bacteriocins against *Pseudomonas aeruginosa* after treatment with 20%, 30% and 40% ammonium sulfate precipitate showed in **(Table 1)**.

No inhibition was observed on *Pseudomonas aeruginosa* upon incubation with Bacteriocin 1-9, 11-17, 19-20, 23-25, 27-28, 30-31, 33-43, 45-56, 58-60, 67-71 and *Bt* I in 20% ammonium sulfate precipitate. A medium inhibition zone was observed for bacteriocin*Bt*10, 21, 22, 57 and 72 under these same conditions. A weak inhibition zone was mostly observed for strains 18, 26, 29, 32, 44, 62, 63, 65, and 32. Incubation with 30% ammonium sulfate showed the following results: No inhibition zone was observed for strains2 through 9, 11-17, 19-20, 23-25, 28, 31, 33-43, 45-56, 58, 60, 67-70 and *Bt* I, a medium inhibition zone was observed for strains 1, 10, 21-22, 27, 29, 57, 65and 72 while a weak inhibition zone was shown for strains 18, 26, 30,32, 44, 62 and 63. Under 40% ammonium sulfate precipitate conditions, the following was observed: no inhibition zone for strains 2, 6-9, 11, 14, 17, 19, 23, 24, 28, 34, 39-43, 54-56, 58, 60, 67-70 and *Bt* I, a medium inhibition zone for strains 1, 3-5, 10, 21-22, 27, 29-30, 38, 57, 62 and

strain 65. A weak inhibition zone on *Pseudomonas aeruginosa* was observed for strains 12-13, 15 to 16, 18, 20, 25, 26, 32 to 33, 44 and strain 63.

3.2.2 Antibacterial activity against *Staphylococcus aureus*

For *Staphylococcus aureus*, the antibacterial activity of the different bacteriocins strains on the inhibition of the growth of this bacterium is shown in **(Table 2).** The results were as follows: with 20%, 30% and 40% of ammonium sulfate there was no inhibition zone shown for bacteriocin produced by strains 2-11, 14, 15, 17-19 23, 24, 27, 28, 32-41, 43, 45-48, 50-51, 56, 58-60, 67-70 and *Bt I.* A medium inhibition zone on the growth of this bacteria was observed to be present after treatment with the following bacteriocin strains: strain 1, 25, 26, 49, 52, 57 and 72. A weak inhibition zone was predominantly observed for strains 13, 16, 20, 21, 29, 42, 44, 53, 62, 63 and 65. A strong inhibition zone was shown for strains 71, 72 and *Bt k.*

3.2.3 Antibacterial activity against *Bacillus cereus*

For *Bacillus cereus,* the antibacterial activity of the different bacteriocins strains on the inhibition of the growth of this bacterium is shown in **(Table 3)**. The results were as follows: no inhibition zone showed for bacteriocin produced by strains 1-9, 11-17, 19-20, 23-25, 27-28, 30-32, 34-43, 45-56, 58-60, 67-71 and *Bt*I after incubation with 20% ammonium sulfate precipitate. A medium inhibition zone on the growth of this bacteria was observed to be present after treatment with the following bacteriocin strains: strain 10, 21, 57 and 72. A weak inhibition zone was predominantly observed for strains 18, 26, 29, 33, 44 and 62-65. A measurement of bacteriocin activity after incubation with 30% ammonium sulfate on the inhibition of growth was followed: no inhibition zone was observed for strains 2 to 9, 11 to 17, 19, 20, 23 to 25, 28, 32, 34-43, 45-56, 58-60, 67-70, and *Bt I.* A medium inhibition zone with 30% and 40% ammonium sulfate was shown for strains 1, 10, 21, 57 and 65. A weak inhibition zone was demonstrated for bacteriocin strains 18, 26, 30, 33 and 44. A strong inhibition zone after adding 30% and 40% ammonium sulfate was with 71, 72 and *Bt k.*

3.2.4 Effect of ammonium sulfate

Ammonium sulfate precipitation has been used as a method of protein purification of bacteriocins isolated from bacterial strains. Bacteriocins in the supernatant can be concentrated by employing a method of salting out using ammonium sulfate **[18]**. Bacteriocin concentration with 80% ammonium sulfate was shown to produce strong zones of inhibition in *A. hydrophila*

and *P. stutzeri* **[2]**. 20% ammonium sulfate precipitation was observed in *Pseudomonas aeruginosa* to cause a low frequency of zone inhibition with medium zone inhibition only being recorded for five out of 66 samples. This frequency increased to nine out of 66 samples for 30% ammonium sulfate precipitate and 14 out of 66 samples in 40% ammonium sulfate precipitation of the bacteriocins **[19]**. The same trend was also observed in *Staphylococcus aureus* and *B. cereus.* Increasing the concentration of ammonium sulfate to about 80% might lead to a stronger inhibition zone formation as it has been previously described for other bacteriocins produced by other bacterial strains.

Table 1: Antibacterial activity of bacteriocin with well diffusion against *P. aeruginosa* (20%, 30% and 40% ammonium sulfate precipitate)

Bt	20%	30%	40%	*Bt*	20%	30%	40%
Bt 1	N	M	M	*Bt 34*	N	N	N
Bt 2	N	N	N	*Bt 38*	N	N	M
Bt 3	N	N	M	*Bt 39*	N	N	N
Bt 4	N	N	M	*Bt 40*	N	N	N
Bt 5	N	N	M	*Bt 41*	N	N	N
Bt 6	N	N	N	*Bt 42*	N	N	N
Bt 7	N	N	N	*Bt 43*	N	N	N
Bt 8	N	N	N	*Bt 44*	W	W	W
Bt 9	N	N	N	*Bt 45*	N	N	N
Bt 10	M	M	M	*Bt 46*	N	N	N
Bt 11	N	N	N	*Bt 47*	N	N	N
Bt 12	N	N	W	*Bt 48*	N	N	N
Bt 13	N	N	W	*Bt 49*	N	N	N
Bt 14	N	N	N	*Bt 50*	N	N	N
Bt 15	N	N	W	*Bt 51*	N	N	N
Bt 16	N	N	W	*Bt 52*	N	N	N
Bt 17	N	N	N	*Bt 53*	N	N	N
Bt 18	W	W	W	*Bt 54*	N	N	N
Bt 19	N	N	N	*Bt 56*	N	N	N
Bt 20	N	N	W	*Bt 57*	M	M	M
Bt 21	M	M	M	*Bt 58*	N	N	N
Bt 22	M	M	M	*Bt 60*	N	N	N
Bt 23	N	N	N	*Bt 62*	W	W	M
Bt 24	N	N	N	*Bt 63*	W	W	W
Bt 25	N	N	W	*Bt 65*	W	M	M
Bt 26	W	W	W	*Bt 67*	N	N	N
Bt 27	N	M	M	*Bt 69*	N	N	N
Bt 28	N	N	N	*Bt 70*	N	N	N
Bt 29	W	M	M	*Bt 71*	N	S	S
Bt 30	N	W	M	*Bt 72*	M	M	S
Bt 32	W	W	W	*Bt K*	S	S	S
Bt 33	N	N	W	*Bt I*	N	N	N

Degree of inhibition zone: Week (7-9), Medium (10-12mm), Strong (13-21mm) and No inhibition (0).

Table 2: Antibacterial activity of bacteriocin with well diffusion against *S.aureus* (20%, 30% and 40% ammonium sulfate precipitate)

Bt	20%	30%	40%	*Bt*	20%	30%	40%
Bt 1	N	M	M	*Bt 34*	N	N	N
Bt 2	N	N	N	*Bt 38*	N	N	N
Bt 3	N	N	N	*Bt 39*	N	N	N
Bt 4	N	N	N	*Bt 40*	N	N	N
Bt 5	N	N	N	*Bt 41*	N	N	N
Bt 6	N	N	N	*Bt 42*	W	W	N

Bt 7	N	N	N	*Bt 43*	N	N	N
Bt 8	N	N	N	*Bt 44*	W	W	W
Bt 9	N	N	N	*Bt 45*	N	N	N
Bt 10	N	N	N	*Bt 46*	N	N	N
Bt 11	N	N	N	*Bt 47*	N	N	N
Bt 12	N	N	N	*Bt 48*	N	N	N
Bt 13	W	W	W	*Bt 49*	M	M	M
Bt 14	N	N	N	*Bt 50*	N	N	N
Bt 15	N	N	N	*Bt 51*	N	N	N
Bt 16	W	W	W	*Bt 52*	M	M	M
Bt 17	N	N	N	*Bt 53*	W	W	W
Bt 18	N	N	N	*Bt 54*	N	N	M
Bt 19	N	N	N	*Bt 56*	N	N	N
Bt 20	W	W	W	*Bt 57*	M	M	M
Bt 21	N	W	W	*Bt 58*	N	N	N
Bt 22	N	N	W	*Bt 60*	N	N	N
Bt 23	N	N	N	*Bt 62*	W	W	W
Bt 24	N	N	N	*Bt 63*	N	N	W
Bt 25	W	M	M	*Bt 65*	N	N	W
Bt 26	M	M	M	*Bt 67*	N	N	N
Bt 27	N	N	N	*Bt 69*	N	N	N
Bt 28	N	N	N	*Bt 70*	N	N	N
Bt 29	W	W	W	*Bt 71*	S	S	S
Bt 30	N	N	W	*Bt 72*	N	M	S
Bt 32	N	N	N	*Bt K*	S	S	S
Bt 33	N	N	N	*Bt I*	N	N	N

Degree of inhibition zone: Week (7-9), Medium (10-12mm), Strong (13-21mm) and No inhibition (0).

Table 3: Antimicrobial activity of bacteriocin with well diffusion against *B. cereus* (20%, 30% and 40% ammonium sulfate precipitate)

Bt	20%	30%	40%	*Bt*	20%	30%	40%
Bt 1	N	M	M	*Bt 34*	N	N	N
Bt 2	N	N	N	*Bt 38*	N	N	M
Bt 3	N	N	M	*Bt 39*	N	N	N
Bt 4	N	N	M	*Bt 40*	N	N	N
Bt 5	N	N	M	*Bt 41*	N	N	N
Bt 6	N	N	N	*Bt 42*	N	N	N
Bt 7	N	N	N	*Bt 43*	N	N	N
Bt 8	N	N	N	*Bt 44*	W	W	W
Bt 9	N	N	N	*Bt 45*	N	N	N
Bt 10	M	M	M	*Bt 46*	N	N	N
Bt 11	N	N	N	*Bt 47*	N	N	N
Bt 12	N	N	W	*Bt 48*	N	N	N
Bt 13	N	N	W	*Bt 49*	N	N	N
Bt 14	N	N	N	*Bt 50*	N	N	N
Bt 15	N	N	N	*Bt 51*	N	N	N
Bt 16	N	N	N	*Bt 52*	N	N	N
Bt 17	N	N	N	*Bt 53*	N	N	N
Bt 18	W	W	W	*Bt 54*	N	N	N
Bt 19	N	N	N	*Bt 56*	N	N	N
Bt 20	N	N	W	*Bt 57*	M	M	M
Bt 21	M	M	M	*Bt 58*	N	N	N
Bt 22	M	M	M	*Bt 60*	N	N	N
Bt 23	N	N	N	*Bt 62*	W	W	W
Bt 24	N	N	N	*Bt 63*	W	W	W
Bt 25	N	N	W	*Bt 65*	W	M	M
Bt 26	W	W	W	*Bt 67*	N	N	N
Bt 27	N	M	M	*Bt 69*	N	N	N
Bt 28	N	N	N	*Bt 70*	N	N	N
Bt 29	W	M	M	*Bt 71*	N	S	S
Bt 30	N	W	W	*Bt 72*	M	S	S
Bt 32	N	N	N	*Bt K*	S	S	S
Bt 33	W	W	W	*Bt I*	N	N	N

Degree of inhibition zone: Week (7-9), Medium (10-12mm), Strong (13-21mm) and No inhibition (0).

4. CONCLUSION

The effect of varying concentrations of ammonium sulfate precipitate on zones of antimicrobial activity of bacteriocin strains from *Bacillus thuringiensis* (Bt) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and *Bacillus cereus*was studied.Our results demonstrated that increasing concentrations of ammonium sulfate adequately concentrates the bacteriocins produced by most of *Bacillus thuringiensis* strains, and consequently increase their antibacterial activity.

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