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ISOLATION AND CHARACTERIZATION OF PARTIALLY FURIFIED BACTERIOCIN OF *Bacillus cereus* (CF1) SOIL ISOLATE

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ABSTRACT
Bacteriocins are proteinaceous antimicrobial substances produce by bacteria against closely related bacterial species. However their broad spectrum inhibitory activity has been observed against other microorganisms such as fungi. The aim of this study was to isolate and determine physical, chemical and biological characteristics of bacteriocin from B. cereus of soil origin. A Gram-positive spore forming bacilli coded as CF1 was isolated by pour plating technique from the soil sample of the cereal farm land of Kura local government area, Kano State Nigeria and identified as Bacillus cereus based on cultural, microscopic and biochemical characteristics. Bacteriocin was isolated from this B. cereus and partially purified by solvent method (cold acetone) and had broad spectrum inhibitory activity with MIC of 64 AU/ ml. This bacteriocin was thermo stable up to 121°C for 15 minutes and its activity was maintained at wider pH value of 2-12. Although, the bacteriocin isolated by this B. cereus was not affected by some chemical such as Urea, EDTA and Tween 80, other chemical such as SDS and trypsin enzyme deactivated its activity. It is therefore recommended that further studies be carryout on this bacteriocin to purify the bacteriocin using Poly Acryl Amide Gel Electrophoresis PAGE and to elucidate its sequence, to determine its molecular conformation and antibacterial activity on multi drug resistant pathogens.
Key words: Bacteriocin, Bacillus cereus, Broad spectrum, Tween 80 and Sodium Dodecyl Sulphate.

INTRODUCTION

There are five members of *Bacillus cereus* group including, *B. cereus* sensu stricto, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, and *B. weihenstephanensis* (Lechner *et al.,* 1998, Schoeni and Wong, 2005, Tourasse *et al.,* 2006). And the distinguishing factor for these members is attributed to mobile plasmids (Rasko *et al.,* 2005, Thomas *et al.,* 2000, Tourasse *et al.* 2006, Vander Auwera *et al*., 2007). The plasmid-borne emetic toxin is found in *B. cereus* (Ehling—Schlz *et al.,* 2004, Horwood *et al.,* 2004, Hoton, *et al.,* 2005), while *B. thuringiensis* carries insecticidal crystal protein (ICP) (*cry*) genes on one or more plasmids (Carlson, *et al.* 1994, Ben-Dov *et al.,* 1997 and Chattopadhyay *et al.,* 2004). Therefore, differentiation of *B. cereus* group members using molecular techniques is not routine in food-poisoning diagnostic methods and may cause underreporting of species such as *B. thuringiensis* (Arnesen *et al*., 2008, EFSA, 2005). *Bacillus* species are Gram-positive, endospore-forming, chemo heterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative anaerobic and catalase positive (Arnesen *et al*., 2008). Members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms (Bavykin *et al.,* 2004).

Many *Bacillus* species are of remarkable importance because they construct antibiotics (Arnesen *et al*., 2008). The potential of *Bacillus* species to synthesize a wide variety of metabolites with antimicrobial activity has been widely used in medicine and pharmaceutical industry; one of its abilities is to control various diseases in animals, humans and plants when applied as a biological control agent (Carlson, *et al.* 1994, Ben-Dov *et al.,* 1997 and Brenstein *et al.,* 1999). Owing to the fact that *Bacillus* species have constructed antibiotics in the soluble protein structure and that these antibiotics have been inexpensive and more effective in studies accomplished to date, these microorganisms are desirable for commercial production (Chattopadhyay *et al.,* 2004, Ehlinn-Schulz *et al.,* 2004).

In recent years, many investigations have utilized the antimicrobial properties of *Bacillus* strains (Health Products and Food Branch, Government of Canada (HPFBGC), 2003, Health Canada (HC), 2005; Hansen and Hendricsen, 2001; European Parliament (EP), 1998; European Food Safety Authority (EFSA), 2005). In a study, Al-Ajlani determined that 54 of the 118 *Bacillus* strains isolated from soil samples demonstrated antagonistic activities against at least two or more strain from a panel of pathogenic and nonpathogenic microorganisms (Horwood *et* al., 2004). Therefore, the present investigation is aimed at isolation and characterization of bacteriocin from *Bacillus cereus* of soil origin, so as to determine the effect of physical, chemical and biological parameters on the bacteriocin isolated.

MATERIAL AND METHODS

Collection and Preparation of Soil Samples

Soil samples were collected as described by Abdulkadir and Waliyu, (2012) in which 5g of soil sample was collected in sterile Petri dishes from four different locations of the cereal farm land (CF) kura local Government Area. Samples were collected from the upper layer where most bacterial population is concentrated. Four samples were randomly collected mixed together to make a composite sample from which 1g of the composite soil sample was used to prepare soil suspension in normal saline.

Isolation and identification of the *Bacillus cereus* from the Soil Sample

The soil suspensions prepared above was serially diluted with normal saline up to 10^{-5} to reduce the bacterial load and used for the isolation of the bacterial specie according to the method of Baserisalehi and Bahador (2013) and Naz and Rasool (2013). One milliliter of 10^{-5} of the serially diluted soil suspension was poured aseptically into a sterile petridish and 15 ml of a molten nutrient agar medium cooled to 45 ^{o}C was added, mixed gently and allowed to gel. The plates were then incubated for 24 hrs at 35^{o}C. All discrete colonies were selected based on their cultural characteristics (Ogundana, 1996), coded and subjected to Gram's staining (Kumari and Ichhpujani, 2000; Cheesbrough, 2004). Gram positive spore forming bacilli with isolation code CF1 was selected and kept on a slant at 4^{o}C for further investigation. The isolate was also subjected to biochemical identification as described by Bergey's Manual of Determinative Bacteriology (George *et al.;* 2001).

Indicator Organisms

The indicator organisms used for the bacteriocins detection test in this work were *Staphylococcus aureus* MCB/BCC/0018/BUK (a Gram-positive bacterium) and *Eschericia coli* MBC/BCC/0017/BUK (Gram-negative bacteria) available at the Laboratory of the Department of Microbiology, Bayero University Kano.

Production and Purification of Crude Bacteriocin

The isolate identified as *Bacillus cereus* (CF1) was inoculated into 250ml of De-Man Ragosa Sharpe (MRS) broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.0) at 5% inoculum of standardize overnight culture and incubated at 35 ºC for 48 hrs. After incubation, cells were removed from the growth medium by centrifugation (10,000 rpm for 15 min, 4ºC). Cell-free supernatants was adjusted to pH 7.0 using 1N NaOH so as to remove the effects of other inhibitory substances such as organic acid and then filter sterilized using 0.25µm syringe filter; Ogunbanwo *et al.,* 2003). The crude bacteriocin was partially purified by precipitation from the cell-free supernatant using cooled acetone in 1:3 (broth: acetone) and the resulting precipitate was collected by centrifugation at 10,000 rpm for 15 min and dissolved in 20 ml of 50 mM sodium phosphate buffer (pH 5.6) and kept in refrigerator at 4ºC for further analysis.

Bacteriocin Bioassay

The inhibitory effect of the bacteriocin extracted from *Bacillus cereus* (CF1) isolated from the soil sample was tested using agar well diffusion assay as described by (Toba *et al.,* 1996; Ivanova *et al*., 2000; Mohankumar and Murugalatha; 2011). One hundred (100) micro-liter (0.1ml) of the partially purified bacteriocin was introduced into the well (Θ=5mm) bored in the nutrient agar previously inoculated with indicator organisms and incubated at 37ºC for 24hrs. After incubation period the inhibition zone produced around the well was recorded in mm using standard ruler.

Determination of Bacteriocin Titer (Minimum Inhibitory Concentration)

To determine the bacteriocin titer, the method of Graciela *et al.,* (1995) was used as adopted by Ogunbanwo *et* al., (2003). Two fold serial dilution of each of the bacteriocin was prepared with normal saline. Briefly, ten sterile test-tubes containing 1ml each of normal saline, were arranged serially and labeled 1/2, 1/4 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/562 and1/1124. One milliliter each of the purified bacteriocins, respectively, was transferred into the first tube and mixed well. From the first tube, 1ml was transferred to the second tube and mixed well and from the second tube 1ml was transferred to the third tube, and the process continued to the last test-tube.

Then 100µl of each dilution was tested with indicator strain incubated for 18-24 hrs at 37ºC and examined for the presence of 2 mm or larger zone of inhibition around the wells. The antibacterial activity of the bacteriocin expressed in activity units per milliliter ($AU^{-1}ml$) was defined as the reciprocal of the highest dilution showing the inhibition zone (Graciela *et al.,* 1995).

Bacteriocin Characterization

Physical Properties of the Bacteriocin Isolated

To determine the physical properties of the bacteriocin isolated, effect of pH and temperature against the bacteriocin were tested according to the method of Ivanova *et al*., 2000; and Baserisalehi and Bahador, 2013. Briefly, for pH stability, 1ml of the crude bacteriocin preparation was treated at different pH of (2, 4, 6, 8, 10, and 12) values at 30°C for 3 h. Each of the pH was then adjusted to7.0 and the solutions were assayed for residual activity. And to examine heat stability, 1ml of the crude bacteriocin preparation was heated at temperature of 40°C, 60°C, 80°C, 100°C, and 121°C for 15 min followed by assaying bacteriocin solutions for residual activity (Ivanova *et al*., 2000; Maja *et al.,* 2010; Baserisalehi and Bahador, 2013).

Chemical Properties of the Bacteriocin Isolated

The chemical tested include: surfactants such as EDTA, Tween 80 and urea; whereas Sodium Dodecyl Sulphate (SDS) was used as a typical detergent at final concentration 0.1, 1, and 5%. Sample and control (active supernatant) were incubated at 30ºC for 5 h and tested for residual activity as previously described (Ivanova *et al*., 2000; Rajaram *et al.,* 2010).

Biochemical Properties of the Bacteriocin Isolated

The sensitivity of the bacteriocin produced to proteolytic enzyme trypsin was tested on cell-free supernatant (pH 7.0) at 30ºC for 2 h with 0.1 mg/ ml and 1.0 mg/ ml final concentration of the enzyme. After treatment residual activity was tested (Ivanova *et al*., 2000; Rajaram *et al.,* 2010).

RESULTS

Result of Isolation and Identification of *Bacillus cereus* from Soil

The results of this study showed that, culturally, the isolate coded CF1 was creamy in colour; none mucoid and none pigmented colonies. The elevation of the colonies was flat with irregular shape of the margin. Microscopically, the isolate was Gram-positive spore forming bacilli whose cells were arranged in chain. Biochemical characteristics of the isolate showed that the isolate was positive for starch hydrolysis, citrate utilization, and acid production from glucose and vouges proskeur test was also positive. The isolate was also found to be motile.

Bacteriocin Production and Potency Determination

The result of the study showed that thirty five (15) ml of bacteriocin (deposit) was recovered from 250ml of liquid medium which gave 14% as the percentage yield of the bacteriocin. And the bacteriocin obtained inhibited the growth of both *Staphylococcus aureus* and *E. coli* with inhibition zone of 17.00±00 mm each, which indicated that the bacteriocin obtained in this study had wide or broad inhibitory activity. The result also showed that the inhibitory activity of the bacteriocin obtained on the two organisms was similar (17.00±00 mm) despite the variation in cell wall property of the organisms tested. Table 1: give the inhibition zones of the *Bacillus cereus* CF1soil isolate bacteriocin on *Staphylococcus aureus* and *E. coli* and the percentage recovery of the bacteriocin isolated.

Table 1: Inhibitory Activity and Percentage Recovery of Bacteriocin Isolated from *B. cereus* CF1 Soil Isolate.

Zones of Inhibition (mm)		Percentage Recovery of Bacteriocin Isolated		
S. aureus	E. coli	V1	V2	%R
17.00±00	17.00±00	250 ml	15 ml	14

Key: V1= Volume of Medium used, V2= Volume of Bacteriocin Deposit Obtained and %R= Percentage Recovery of the Bacteriocin Obtained.

Determination of Bacteriocin Titer (Minimum Inhibitory Concentration)

The result of Minimum Inhibitory Concentration (MIC) of bacteriocin obtained in this study is given in Table 2. From the Table, it can be seen that the bacteriocin extracted from the *B. cereus* CF1 soil isolate had minimum inhibitory concentration of 64 AU^{-1}ml which was found to be the same on both *S. aureus* and *E. coli*.

Table 2: Minimum Inhibitory Concentration of Bacteriocin Isolated from *B. cereus* CF1 (AU^{-1}ml).

Indicator organism	Bacteriocin Titer (MIC) AU^{-1}ml)
E. coli	64.00±0.00
S. aureus	64.00±0.00

Physical, Chemical and Biochemical Characteristics of Bacteriocin Produced by *B. cereus* CF1 Soil Isolate

The results of this study showed that the bacteriocin produced by the *B. cereus* CF1 soil isolate was heat stable at temperature of 40, 60, 80, 100 and 121 °C when treated for a period of 15 minutes as shown in Table 3. Also Table 3 shows the result of pH stability of the bacteriocin isolated in this study and the result indicated that the bacteriocin isolated had activity at wide pH range of 2-12 when treated for a period of 3hr. Table 4 showed the results of effect of urea treatment on the bacteriocin produced in this study and the result indicated that urea had no effect on the bacteriocin extracted from *B. cereus* CF1 soil isolate at the test concentration of 0.1, 1.0 and 5 % when treated for a period of 5hr. However, Sodium Dodecyl Sulphate (SDS), at concentrations of 0.1, 1 and 5% deactivated the bacteriocin produced by *B. cereus* CF1 soil isolate after treatment for a period of 3hr hence no residual activity was observed against the indicator bacteria as shown in Table 8. The result also showed that the activity of bacteriocin of *B. cereus* CFI isolate was not affected by EDTA and Tween 80 at concentration of 0.1, 1.0 and 5% respectively as shown in table 4. However, Table 5 presented the effect of the enzyme trypsin on the bacteriocin obtained in this study and the findings showed that, the enzyme trypsin deactivated the bacteriocins produced by the *B. cereus* CF1 soil isolate at all concentrations used when treated for a period of 2hr.

Table 3: Effect of Physical parameter on Activity of *B. cereus* CF1 bacteriocin

Residual Activity (mm)											
Temperature °C					Control	pH					
40	60	80	100	121		2	4	6	8	10	12
17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0	17± 0.0

Note: (1) *Staphylococcus aureus* was used as indicator organism to determine the above data.
(2) The data above are mean of three consecutive readings.

Table 4: Effect of Chemical parameter on Activity of *B. cereus* CF1 bacteriocin

	Residual Activity (mm±SD)			
Concentration (%)	Urea	SDS	EDTA	Tween 80
0.1	17.00±0.00a	0.00±0.00b	17.00±0.00a	17.00±0.00a
1.0	17.00±0.00a	0.00±0.00b	17.00±0.00a	17.00±0.00a
5.0	17.00±0.00a	0.00±0.00b	17.00±0.00a	17.00±0.00a
Control	17.00±0.00a	17.00±0.00a	17.00±0.00a	17.00±0.00a

Note: (1) *Staphylococcus aureus* was used as indicator organism to determine the above data.
(2) The data above are mean of three consecutive readings.

Table 5: Effect of Proteolytic Enzyme on Activity of Bacteriocin Isolated from *B. cereus* CF1 Soil Isolate.

Isolation Code	Enzyme Concentration (mg/ml)/ Inhibition zone (mm)			
	0.1	0.5	1	Control
S. aureus	0.00±0.00	0.00±0.00	0.00±0.00	17.00±0.00

Note: (1) *Staphylococcus aureus* was used as indicator organism to determine the above data.
(2) The data above are mean of three consecutive readings.

DISCUSSION

The findings from this study showed that, the bacterial isolate obtained from the soil coded as CF1 was identified as *Bacillus cereus.* This finding is in agreement with the work of other researchers that, *Bacillus cereus* is found frequently as a saprophyte in soil (Becker *et al.,* 1994; Notermans *et al.,* 1997).

Bacillus cereus CF1 isolated in this study was capable of producing bacteriocin a proteinaceous antibacterial compound. This is similar with the findings of Bizani and Brandelli, (2002), where they isolated bacteriocin producing *Bacillus cereus* 8 A from native soil of south Brazil. It was also similar to the work of Kong *et al.* (2016), however their isolate was *Bacillus amyloliquefacience* RX7 from soil. Another bacteriocin producing *Bacillus* strain (*B. subtilis* KKU213) was also isolated from local soil of Thailand (Nalisa *et al.,* 2015). Similarly, Karmen and Bojana; 2003, isolated bacteriocin producing *B. cereus* from food such as milk and milk products. Moreover, bacteriocin producing *B. cereus* was also isolated from buffalo milk (Senbagam *et al.,* 2013).

The result of the antibacterial activity of the bacteriocin obtained in this study on the indicator organisms showed that the bacteriocin obtained is broad spectrum in nature due to its inhibitory activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Eschericia coli*) bacteria. This is in agreement with the findings of Nalisa *et al.,* 2015 and Kong *et al.,* 2016 respectively, where they isolated bacteriocins from *Bacillus* strains of soil origin which inhibit the growth of both Gram-positive and Gram-negative bacteria. Similarly, this finding agrees with the report of Karmen and Bojana, (2003), Senbagam *et al.* (2013), where they isolated bacteriocins active against both Gram-positive and Gram-negative bacterial isolates. On the other hand, the findings from this study is contrary to the report of other researchers where they isolated bacteriocins with narrow spectrum inhibitory activities i.e inhibiting only gram-positive or gram-negative group of bacteria (Stevens *et al.,* 1991; Rodriguez, 1996; Messi *et al.,* 2001; Caridi, 2002; Lade *et al*., 2006).

The Minimum Inhibitory Concentration (MIC) of the bacteriocin obtained in this study was low (64 AU/ml) compared to the MIC (51200 AU/ml) of bacteriocin isolated from *Bacillus cereus* isolated from buffalo milk.

Findings from the physical properties of the bacteriocin obtained in this study indicated that this bacteriocin is relatively thermo- stable up to temperature of 100°C and 121°C. This is in agreement with the report of (Adedire and Odeniyi, 2017, Senbagam *et al.,* 2013, Nalisa *et al.,* 2015) where they isolated thermo stable bacteriocins from *B. cereus*. Similarly the result agrees with the report of Vinod *et al.* (2006) where the bacteriocins they isolated withstand temperature of 121°C. The bacteriocin obtained in this study retained its activity at wider pH range of 2-12. This finding is similar with the report of (Cherif *et al.,* 2001; Torkar and Matijastic, 2003; Sehar and Sheikh, 2013) where they isolated bacteriocin active at wide pH range of 2-12. However, the results obtained in this study was slightly contrary to the work of other authors; Adedire and Odeniyi, (2017) who isolated bacteriocins with activity at pH range of 4-8, and Nalisa *et al,* (2015) isolated bacteriocins with activity at pH range of 4-10, similarly, Senbagam *et al,* (2013) isolated bacteriocins with activity at wide pH range of 3-10.

Bacteriocin isolated in study maintained its inhibitory activities when treated with Urea, Tween 80 and EDTA at concentrations of 0.1, 1.0 and 5%. This show that Urea, Tween 80 and EDTA have no detrimental effect on the bacteriocins isolated which agrees with the finding of other authors (Adedire and Odeniyi, 2017), although in their finding the activity of the bacteriocin-like extracellular metabolite increases by the addition of EDTA at 30%. However, the results of effect of proteolytic enzyme trypsin, and SDS on the bacteriocin obtained in this study showed that proteolytic enzyme trysin and SDS deactivated this bacteriocin. This finding agrees with the work of Kong *et al.,* (2016) who isolated bacteriocin from *B. amyloleqefaciens* RX7 which was inactivated by proteolytic enzyme, however the result is contrary to the findings of other author who isolated bacteriocin from *Bacillus cereus* that has retained its activity after treatment with proteolytic enzyme trypsin and pepsin (Nalisa *et al.,* 2015), similarly bacteriocin produced by *B. cereus* 8A was resistant to proteolytic enzyme trypsin (Bizani and Brandelli, 2002).

Results of the effect of the chemical parameters on the activity of bacteriocin obtained in this study was analysed by Two-way ANOVA and statistically, there is no significance difference for the effect of Urea, EDTA and Tween 80 at concentration of 0.1, 1.0 and 5.0 when compared to the control treatment. However, the result of the effect of SDS on the activity of the bacteriocin obtained in this at concentration of 0.1, 1.0 and 5.0 showed a significance difference when compared with control treatment.

CONCLUSION

A broad spectrum bacteriocin was isolated from *Bacillus cereus* CF1 soil isolate. The bacteriocin obtained was thermo stable and remained active at wide pH range of 2-12. Treatment of this bacteriocin with EDTA and urea did not affect the bacteriocin activity, although trypsin and SDS treatment deactivated its activity. The MIC of the bacteriocin obtained was found to be 64AU/ml against the indicator organisms used. It is therefore recommended that further research be conducted to determine the molecular weight of the bacteriocin extracted and to purify the bacteriocin obtained by polyacrylamide gel electrophoresis (PAGE); and their structures elucidated by Liquid Chromatography Mass Spectrophotometry (LCMS). Finally, the inhibitory effect of this bacteriocin should be check against multidrug resistant clinical bacterial isolates.

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