International Journal of Current Engineering and Technology
E-ISSN 2277 – 4106, P-ISSN 2347 - 5161

Available at http://inpressco.com/category/ijcet

Research Article

Effects of Crude and Purified Bacteriocin of *Pediococcus pentosaceus* on the Growth and Zearalenone Production by *Fusarium graminearum*

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Accepted 25 November 2013, Available online 01February 2014, **Vol.4, No.1 (February 2014)**

Abstract

Bacteriocins were metabolized products of food – grad , microorganisms and some of which were used as food preservative to improve the food safety. A bacteriocin producing strain Pediococcus pentosaceus was isolated from vegetable food and human stool in the previous study .The purification technologies and the activity determination and the characteristics of the bacteriocin was also explained .The results suggested that the antifungal substance was not acid metabolization and it was bacteriocin . The activity was determined by the method of micro titer plate well . The yield of bacteriocin was (9.08%) and the specific activity was (27540) Au/mg . The specific activity was increased by (14123) fold , The protein band was observed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a single band with a molecular mass 38 KDa. The research presented here mainly concerns the inhibition of fungal growth Fusarium graminearum and Zearalenone (ZEN) production in liquid medium by crude and purified bacteriocin of Pediococcuspentosaceus. The dry weight of Fusarium graminearum was evaluated (gm) and the results are presented in inhibition of fungal growth and reduced the level of Zearalenone production in liquid medium specially at higher concentrations of bacteriocin .

Keywords:*Bacteriocin, Metabolization, Pediococcuspentosaceus etc.*

Introduction

[1] *Pediococcus pentosaceus* is currently one of many bacteria being studied as preservative and this species is considered by EFSA suitable for the qualified presumption of safety (QPS) approach to safety assessment. Among antimicrobialsubstance produced by *Pediococcus pentosaceus* bateriocins have gained increasing interest as possible natural food preservatives (Magnuson &Schnurer,2001) . They have been used as biopreservatives in model food systems and shown to be effective in inhibiting the pathogenic and food spoilage microorganisms (Schnurer, &Magnusson , 2005) . Food preservation is achieved by using either a bacteriocin producing starter culture or by applying the bacteriocin itself as food additive in its relatively pure form. Molds and yeast are important spoilage organisms in different food and feed system.

Zearalenone (ZEN) is a secondary fungal metabolite produced by different species of Fusarium such as *Fusarium culmorum* ,*Fusarium graminearum* , which are regular contaminants of cereal crops worldwide (Bennette&Klich ,2003) . Zearalenone is a resorcyctic acid lactone , chemically described as 6- (10- hydroxy-6-oxo-trans-1-undecenyl) – B- resorcyclic acid lactone , ZEN is a strong estrogenic compound which causes reproductive problems in animals , ZEN may affect the uterus by decreasing progesterone secretion and altering the morphology of uterine tissues (Utermark and karlovisky,2007.) .ZEN has reported to be genotoxic and to induce DNA adduct formation (Ouanes*et al*., 2005). In addition, ZEN was able shown to be hepatotoxic (Shekhany , 2008) .

Physical and chemical methods have been developed to control the occurrence of fungi and their toxins , but no efficient strategy has yet been proposed to reduce the presence of mycotoxins . Among natural biological antigonists , Lactic Acid Bacteria group (LAB) which are mainly divided into four genera *: Lactococ*cus, *Lactobacillus* , *Leuconostoc* and *Pediococcus*, they have several potenial applications and are widely used for the production of fermented foods and are also part of intestinal micro flora (Rattanachaikunsopon&Phumkhachorn ,2010) . These bacteria have a long history of use in foods, they produce some antagonistic compounds able to control pathogenic bacteria and undesirable spoilage micro flora , in particular using LAB to control mold growth could be an interesting alternative to physical and chemical methods because these bacteria have been reported to have strong antimicrobial properties. However the antifungal activity

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of lactic strains remains to be elucidated (Al-Nezami , 2004 ; Zinedine*et al*., 2005 ; Mandal&Mandal , 2007) . Some reports indicate that different bacteria such as some probiotic species of Lactobacillus or yeast may cause degradation or removal of mycotoxins from the growth medium (Niderkorn*etal* ., 2009) and some authors have reported the possibility of degradation or biotransformation of mycotoxin by microorganisms (Hassan &Bullerman , 2008). Rouse and others, 2008 investigate the antifungal activity produced by lactic acid bacteria (LAB) isolated from malted cereals and determined the ability of LAB to prevent fungal growth in a particular food model system. Numerous studies have reported that these molecules are inactive against Gram-negative bacteria and eucaryotic microorganisms such as yeasts or moulds (Batish& Grover, 1997). Moreover, the action of the antifungal properties of LAB on some mycotoxinogenic moulds have also been reported by a few authors. As of this time, the main LAB recognized for their ability to prevent or limit mycotoxinogenicmould growth belong to the genera *Lactococcus* and *Lactobacillus* and, to a lesser extent, to *Pediococcus* and *Leuconostoc*(Dalie*et al.,* 2010).

The aim of the this research was to desecribe the antifungal spectrum , the basal biochemical characteristics and the production conditions for the fungal inhibitors compound (Bacteriocin) from *Pediococcus pentosaceous* and tested their ability to inhibit the growth and zearalenone production of *Fusarium graminearum* .

Material and Methods

Microorganism and medium

Accessions of fungi: *fusarium graminearum* was isolated from poultry feed and produced of mycotoxinZearalenone **.** Potato Dextrose Agar (PDA) was used for fungal culture and *Pediococcus pentosaceus* isolates were previously isolated from human and food source in Baghdad's university laboratories , and the isolates were determined by using the Ap1 50 CHL micro-identification system and subjected to microbiological and biochemical assays and characterized according to the scheme of (Holt *et al*., 1994).

Bacteriocin production

Ten isolate of *Pediococcuspentosaceus* was grown in MRS at 30 ºC for 24 h. The culture supernatant was then collected adjusted to pH 6.5 , Filter through 0.22 µm pore size filters , concentrated to 0.1 volume by polyethylene glycol dialysis (Sigma PEG 20000) and again filter – sterilized . This material was designated crude bacteriocin and was frozen at -20 ºC when not used immediately (Piva&Headon , 1994).

Purification of bacteriocin

The crude bacteriocin was pruified according to (Piva&Headon , 1994) , Recovers of the purified protein from the butanol extract was achieved by Electroendosomatic preparative electrophoresis (EPE) according to (Curioni*et al.,* 1988) and the resulting sample was freeze – dried .

Protein concentration

Protein concentration was determined by using the dye – binding method (Bradford , 1976).

Sodium Dodecyl sulphate – polyacrslamide Gel Electrophoresis (SDS-PAGE)

The purity and molecular mass of purified bacteriocin were determined using 15% polyacrylamid SDS-PAGE according to the method of (Laemmli , 1970) . low molecular weight protein kits (Pharmacia chemical Co.) were used as markers .

Effect of heat and pH on enzymes

The sensitivity of bacteriocin to enzyme was tested using the purified bacteriocin which was treated with the following enzymes papien , trypsin , proteinuse k, pronase E , α- amylase , β-glucuronidase , β-galactosidase and β-N-acetylglucosaminidase . Samples were then boild for 2 min. to inactivate the enzyme . To analyse thermal stability of bacteriocin were exposed to temperatures ranging from 25-90 ºC for 30 min. , 100 ºC for 1-5 min. , 121 ºC (103.5 Kpa) for 15 min. and frozen for up to 30 days . The activity of bacteriocin at different pH values was estimated by adjusting the pH of supernatant samples to pH 3-11. After the treatments the samples were tested for antifungal activity against *Fusarium graminearum* .

Antifungal activity assay

The micro titer plate well assay were used to detect antifungal activity according to(Magnusson and Schnurer, 2001).

Effect of crude and purified of Pediococcuspentosaceusbacteriocin on dry mycelial weight of Fusarium graminearum in liquid media

The crude and purified bacteriocin extracts of *Pediococcuspentosaceus* were screened for antifungal activity against *Fusarium graminearum* . 50 ml of YES medium treated with different concentration (0.0, 125 , 250 , 500, 1000) mg/ml from the crude and purified of *Pediococcus pentosaceus* bacteriocin . which was taken in a 100 ml Erlenmeyer conical flask and sterilized at 121°C, 15 lb/inch2 pressure for 15 minutes and allowed to cool. The flasks were inoculated with 7 mm diameter mycelia disc of *Fusarium graminearum* taken from 7 days old culture , After incubation at 25 ±2 ºC for 14 days , the content of the each flask were poured into a pre weighed Whatmman No. 1 filter paper. The filter paper with the mycelial mat was dried in an oven at 60 ºC until a constant weight was reached. The dry weight of the mycelia was determined by subtracting the weight of the filter paper from the total weight of the filter paper with mycelia. Three replicates were maintained for each treatment

(Kumar &Prasad , 1992) . calculated using the formula:- inhibition ratio = C – T / C X 100 where C = Mycelial weight in control and T = Mycelial weight in treatment.

Testing for ZEN detoxification activity

This study was conducted the ability of different concentration (0.0, 125 , 250 , 500, 1000) mg/ml from the crude and purified of *Pediococcus pentosaceus* bacteriocin in removing or destruction ZEN by using produced isolate *Fusarium graminearum* after growing on a specific medium for ZEN production YES according to method (Stancic*et al*., 2009) . The samples were extracted on the 14th day of culture according to the method described earlier **with some** modifications (Liao *et al.*, 2009) by using 50 ml of extraction solution (acetonitile / water = 90 / 10 , v/v) then added 1.5 ml from fungal filteration with 7.5 ml extraction solution , the mixture was centrifuged for 30 min. at 3000 rpm . The extract was filter through milipore filter (0.22 µm) in diameter of pore.

High performance liquid Chromatography (HPLC) / Analysis

The HPLC system consisted of a Shimadzu HPLC system model LC-2010 AHT , the colume Hyper Clone 5µCN 120A (250 ×4.6mm) Phenomenex . Injection volume was 0.2 µl . The detector wavelength was UV . 218 nm . The mobile phase, acetonitrile / water (50/50) v/v , was pumped at a flow rate of 0.5 ml/min., according to ECC , 1992 calculated the concentration of ZEN by comparing curve area for samples with standard solution of ZEN (ECC). Tested percent of ZEN , then The Percent Reduction of Zearalenone was calculated using the formula:-
Ratio ofReduction = concentration of control – concentration of sample / concentration of control X 100 .

Results and Discussion

Isolation of Bacteriocin Producing Strains

A total of 11 *Pediococcuspentosaceus* strains were isolated from 11 samples of human and food source , after preliminary identification (Reuter and Goldberg ,2002 ; Silvester and Dicks , 2003) of these strains were found to produce antimicrobial substances and the isolate *Pediococcus pentosaceus* p.10 showed the highest antimicrobial activity .Bacteriocin activity was purified up to 14123 folds , the overall yield and activity are summarized in (Table-1).

Table 1 Purification of bacteriocin produced by *Pediococcuspentosaceus*

Purification stage	Culture stage	Dialysis against PEG	Butanol extraction	EPE
Vol(ml)	**1000**	**100**	**18**	**0.6**
Total activity (AU)	**37000**	**35000**	**7120**	**3360**
Bacteriocin activity(AU/ml)	**37**	**350**	**395.55**	**5600**
Total protein (mg)	**18900**	**147.50**	**0.83**	**0.2**
Protein conc. (mg/ml)	**18.9**	**147.5**	**0.83**	**0.2**
Specific activity (AU/mg)	**0.195**	**2.372**	**474.66**	**275.40**
Activity recovered %	**100**	**94.59**	**19.24**	**9.08**
Purification *(fold)*	**1**	**12.59**	**2434.1**	**14123**

Effect of Enzymes, heat and pH on antifungal activity

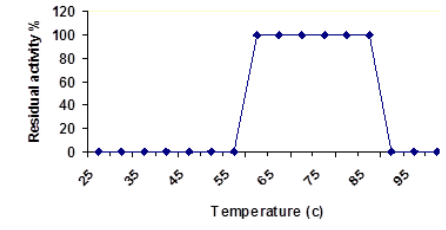
In order to test the effect of proteolyticenzymes , 5600 Au/ml of bacteriocin were tested for sensibility to papien , Trypsin , proteniase K. and pronase E. The bacteriocin was sensetive to proteinase K and pronase E. (Table-2) and was not sensible to other enzymes α – amylase , β-glucuronidase, β-galactosidase and β- N-acetylglucosaminidase.

Table 2 Effect of enzymes on bacteriocin activity

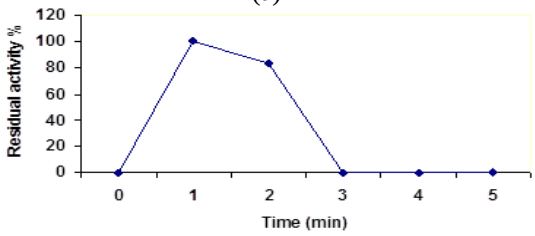
Treatment	Residual activity %
Untreated bacteriocin	100
α amylase	100
β-glucuronidase	100
β-galactosidase	100
β-N-acetylglucosaminidase	100
Proteinase k	0.0
Pronase E	0.0

Note: Control with each enzymes were performed

The bacteriocin was incubated for 30 min at different temperatures and residual activity was measured, it was stable at 80 ºC but the activity gradually decreased with the increase in temperature. The residual activity was 90% after incubation at 82 ◦C for 30 min. and total loss of activity was observed after incubation at 85 ºC (Figure-1a) and (Table-3). When the bacteriocin was incubated at 100 ºC , the residual activity was 80% after 2-3 min. but it was completely absent after 3 min.incubation (Figure-1b) . Bacteriocin activity was not lost by cooling and freezing storage.



(a)



(b)

Figure- 1 Thermal stability of bacteriocin 5600 Au/ ml was incubated at various temperature for 30 min. (a) or at 100 ºC for different times (b) and residual activity was measured

Activity is expressed as the percentage of residual activity determined against *Fusarium graminearum*.

Table 3 Thermal stability of bacteriocin on different conditions

Treatment	Residual activity
100 ºC / 15 min.	0.0
121 ºC / 103.5 KPa / 15 min	0.0
4 ºC / 30 d.	100
-20 ºC / 30d	100
Freeze-dried	100

The bacteriocin was active over a pH range of (4.0-7.5) but it was inactivated when incubated outside of these limits (Figure-2).

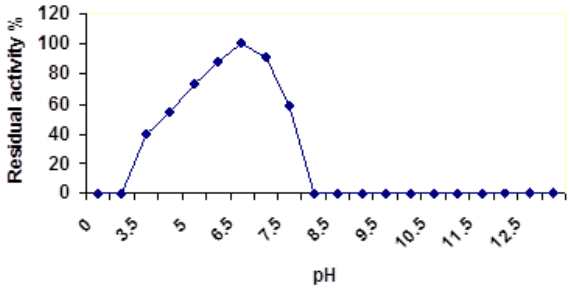


Figure 2 Effect of pH on the bacteriocin activity

The bacteriocin preparation from the isolate showed distinctive characteristics with respect temp., it completely lost it's activity at pH 2 and above 8 and the residual activity was 90% after incubation at 82◦C , and total loss of activity was observed after incubation at 85 ◦C and 30 min. and it completely lost its activity at 100 ◦C after 3 min. The bacteriocin from *Pediococcus pentosaceus* CFR S111 was found to be heat stable as it showed resistance after the treatment at 121 c for 3 min. Similarly more than 80% activity of the bacteriocin of *Pediococcus pentosaceus* ACCEL was left after 15 min. of heating at 121 ◦C (Wu et al., 2004) , and bacteriocin produced by *Pediococcuspentosaceus* CFRB19 showed resistance when subjected to similar treatment (Venkateshwari*et al*., 2010) .SDS-PAGE analysis revealed band corresponding to an apparent M.wt. 38KDa. Similarly , production of bacteriocin with a MW. Of 4.8 KDa by *Pediococcuspentosaceus* CFRB19 (Venkateshwari*et al*., 2010) , However production of bacteriocin having MW. Of 17.5 KDa by *Pediococcus pentosaceus* ACCEL was also reported (Wu *et al.,* 2004). Have characterized another bacteriocin from *Pediococcus pentosaceus* with MW.80KDa. (Wu *et al.,*2004) . A review of the literature indicates that the bacteriocin in the present work is a new proteinaceous Compound from *Pediococcus pentosaceus* active against *Fusarium graminearum* that has a molecular mass of 38 KDa and has not been studied earlier .

Effect ofPediococcus pentosaceus bateriocin on dry mycelial weight of Fusarium graminearum

The results showed the ability of the crude bacteriocin*Pediococcus pentosaceus* bateriocin of antifungal agent . 4th concentration (125 , 250 , 500 , 1000) mg/ml of both crude and purified *Pediococcus pentosaceus*bateriocin (Table-4) generally have a weak fungistatic effect on the dry weigh of *Fusarium graminearum* . In fact that growth was slower in the presence of purified bacteriocin in 4th concentration than in crude bacteriocin ,espicially at higher concentration (250, 500 , 1000) mg/ml the inhibition ratio were 0.0% equal to control treatment .

Table 4 Effect of crude and purified *Pediococcus pentosaceus* bateriocin on dry weight of *Fusarium graminearum*

Crude bacteriocin		
Concentrations (mg/ml)	Dry weight (gm)	Inhibition ratio
0.0	0.5	0.0
125	0.7	40
250	0.6	20
500	0.7	40
1000	0.4	20
Purified bacteriocin		
0.0	0.5	0.0
125	0.6	20
250	0.5	0.0
500	0.5	0.0
1000	0.5	0.0

These findings are similar to those of (Hassan and Bullerman ,2008) who showed that *Lactobacillus paracasasei* isolated from a sourdough bread culture showed a promising ability of inhibiting wide spectrum of *Fusarium* species because these have been reported to have strong antimicrobial properties . However the antifungal activity of lactic strains remains to be elucidated (Gwiazdowska*et al.*, 2008).

Testing for mycotoxin detoxification activity , the result showed in (Table-5) the ability of crude and purified *Pediococcus pentosaceus* bacteriocin to reduce ZEN concentration in liquid media (YES) but this reduction was increasing at low concentrations specially with crude bacteriocin, while all concentration of purified bacteriocinwas may cause degradation or removal of ZEN from the liquid media .

Table5 Effect of crude and purified bacteriocins *Pediococcus pentosaceus* bacteriocin on Zearalenone production

Crude bacteriocin		
Concentrations (mg/ml)	Zearalenoneconcentation(ng/ml)	Reduction ratio %
0.0	2.57	0.0
125	0.075	97.08
250	0.109	95.72
500	0.201	92.16
1000	0.339	96.80
Purified bacteriocin		
0.0	2.57	0.0
125	0.066	97.43
250	0.078	96.96
500	0.072	97.19
1000	0.068	97.35

The results revealed that extracellular metabolites of *Pediococcus* can effectively inhibit fungal growth and ZEN production in liquid medium. Numerous investigations have reported the antimycotic and antimycotoxigenic activity of lactic acid bacteria (Gourman&Bullerman , 1995 ; El-Nezami *et al*., 2002 described significant reduction in the concentration of ZEN in Liquid media.

Morever , the action of the antifungal properties of lactic acid bacteria (LAB) on some mycotoxigenic molds have been reported by a few authors . As of this time , the main LAB recognized for their ability to prevent or limit mycotoxigenic mold growth belong to the genera *Lactobacillus* and *Lactococcu*s and to a lesser extent , to *Pediococcus* and *Leuconostoc.*

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