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Short paper

Effects of partially purified enterocins from *Enterococcus faecalis* strains on the growth of some phytopathogenic fungi

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Abstract. Plant protection is an important area which needs attention since most of the hazardous inputs added into agricultural systems are in form of synthetic chemicals. The inhibitory activity of partially purified enterocins (PPEs) produced by *Enterococcus faecalis* strains on plant pathogenic fungi was investigated in this study. The PPEs were preliminarily screened against bacteria using agar-well diffusion method. The active extracts were partially purified using ion exchange chromatography. The *in vitro* anti-fungal properties of the PPEs were determined using agar dilution and broth dilution techniques. The PPEs tested in this study inhibited the growth of *Botryodiplodia theobromae*, *Aspergillus niger*, *Pythium ultimum*, *Penicillium expansum* and *Fusarium oxysporum*. At different concentrations PPEs had varying inhibitory effects on the dry mycelial weight of *Pythium ultimum* and *F. oxysporum*. At the 96th hour of the experiment, enterocin UNAD 012 had higher percentage inhibition ranging between 37.63 and 84.11% than enterocin UNAD 046 with percentage inhibition ranging between 28.77% and 67.27% on the test fungi. This inhibitory activity of enterocins produced by *E. faecalis* on fungi makes them as potential biocontrol agents due to their ability in suppressing their growth.

Keywords. Bacteriocin, enterocins, *Enterococcus faecalis*, fungi, phytopathogens.

1 Introduction

Phytopathogenic fungi are capable of causing infectious diseases in plants. They damage plants and plant product on which human beings depend for

food, clothing, shelter, furniture and the environment. Most of them belong to the family Ascomycetes and Basidiomycetes. Common species include *Pythium ultimum, Penicillium expansum, Fusarium oxysporum. Aspergillus fumigatus, Botryodiplodia theobromae* and *Phytophthora* spp. (Aderiye *et al.* 1996, Fagbohun *et al*. 2008).

Enterococcus is a lactic acid bacteria (LAB) found in gastrointestinal flora, oral cavity and human vagina. They are widespread in nature and have been detected in the fecal samples from humans, lower vertebrates and insects (David *et al*. 2012). Enterococci has been reported to produce bacterocins; an extracellular macro-molecular protein/peptides which exert a lethal effect on bacteria or the related groups (Papagiani *et al*. 2004). Bacteriocins as antimicrobial peptides could be a better replacement to chemical fungicides. All species of enterococci are capable of producing bioactive bacteriocins named as enterocin (Gilmore *et al.*, 2002).

Bacteriocins have been reported to act against both related species and distantly related genera (Vidaver *et al.* 1972, Okkers *et al.* 1999). They act on food-borne pathogenic and spoilage micro-organisms and in the recent time, their activity against plant pathogens was reported (Schillinger *et al.* 1996). The potential of bacteriocin from *B. subtilis* has antagonistic and bactericidal effects on *Agrobacterium* spp., the causative agent of crown gall. The proprieties of bacteriocins indicated that they have a strong potential to be used in biological control of crown gall disease (Hammami *et al.* 2009).

Fungi have been reported to cause numerous diseases in plants. Some of the chemicals used to control these diseases bio-accumulate in the plants and eventually enter food chains. Current campaign for the fungicide-free fruits and vegetables products, and rise in fungal resistance to common chemo-control agents necessitate the search for alternative control methods for myco-phyto-pathogens. In this study we evaluated the anti-fungal ability of entrocins produced by two strains of *Enterococcus faecalis*. The antimicrobial spectrum and some properties of the bacteriocins are described and their anti-fungal properties against some phytopathogenic fungi were also studied.

2 Materials and Methods

2.1 Preparation of Cell Free Supernatant (CFS)

Two *Enterococcus faecalis* strains were collected from the stock cultures maintained in the Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria. The organisms were separately revived in de Man Rogosa and Sharpe (MRS) broth. The broth was incubated at 37ºC for 24 h after which it was centrifuged for 10 min at 10,000 *g* at 4ºC. The supernatant was decanted gently and later filtered through a membrane filter with a pore size of 0.22

μm. The interfering effects of peroxides and organic acids in the CFS were eliminated by addition of 1 N NaOH and 130 U/mL of catalase (Sigma Chemical Co., St. Louis, MO, USA) respectively.

2.2 Determination of antibacterial activity of CFS

The bacteria used in the primary screening of the enterocin include *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. They were collected from the Department of Microbiology, Ekiti State University, Ado-Ekiti. The organisms were grown for 18 h at 37ºC and the turbidity adjusted to 0.5 McFarland Standard. Antibacterial potential of the CFS was determined using agar-well diffusion assay. The reciprocal of least serial dilution of CFS with antibacterial activity was taken to be activity unit (AU) as described by David *et al.* (2017).

2.3 Partial Purification of enterocin by Ammonium sulphate precipitation

To saturation level, ammonium suphate (ranging between 60 and 90 %) was added to 50 ml of CSF with constant stirring and kept overnight at 4ºC. The solution was centrifuged at 10,000 *g* for 20 min at 4ºC and later dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 5.0). The supernatant was stored at 4ºC until used.

2.4 Determination of protein content of the enterocin produced

The protein content of the CFS was determined according to Bradford (1976). The optical density of each of the samples was calculated from the bestfit equation line obtained from the graph of the Bovine Serum Albumin (BSA) standard curve.

2.5 Source of phytopathogenic fungi

Fungi isolates primarily isolated from infected plants were collected from the stock cultures maintained at the Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria. The fungi include *Aspergillus niger, Botrydiplodia theobromae, Fusarium oxysporum, Penicillium expansum* and *Pythium ultimum*. The test fungi were maintained on slants of Potato Dextrose Agar at 4ºC until use.

2.6 Determination of antifungal property of enterocin

The partially purified enterocin was sterilized by filtering it through filters with 0.22 μm pore size and the 2 ml of the filtrate was added into 10 ml of sterile potato dextrose broth. The broth was incubated at 37°C for 24 h and the sterile enterocin did not produce any turbidity. The anti-fungal activity of the different extracts of the partially purified enterocins was determined according to poisoned food assay method described by Nene and Thapilyal (2002). At the right concentrations, the sterile extract was mixed with sterilized Potato Dextrose Agar (PDA) just before the setting of the agar. Agar plug (10 mm) from the advancing edge of five-day culture of each of the test fungi was inverted on the center of each plate and incubated at 25°C for 96 h. The PDA plate without enterocin was also maintained at the same condition to serve as the control and the experiment was performed in triplicate. The diameter of fungal colony was measured to the nearest centimeter.

2.7 Analysis of data

Results of this study were presented as the mean values of the replicates. One-way analysis of variance (ANOVA) was carried out using SPSS 16.0. Significance was accepted at $P \leq 0.05$.

3 Results and Discussion

Out of four isolates screened for bacteriocinogenic potential, only two of the bacteriocin-producing strains (UNAD 012 and UNAD 046) showed a prominent activity against the test organisms (Table 1).

Table 1. Antibacterial activity (inhibition zones in mm) of crude enterocin produced by strains of *Enterococcus faecalis*.

Test organisms		**Enterocins from *E. faecalis* strains**			
		UNAD 012	UNAD 046	UNAD 019	UNAD 033
Gram positive	*B. subtilis*	10	36	10	-
	S. aureus	10	15	10	10
Gram negative	*E. coli*	15	16	10	-
	K. pneumoniae	19	10	10	-

Table 2. Activity, protein concentration, yield and fold of selected enterocin.

Parameter	Purification steps					
	Crude		$(NH_4)_2SO_4$		Ion Exchange	
	UNAD 012	UNAD 046	UNAD 012	UNAD 046	UNAD 012	UNAD 046
Volume (ml)	10	10	5	6	5	2
Activity (AU/ml)	122	158	145	207	95	125
Protein conc. (mg/ml)	15.8	17.2	6.2	6.0	1.4	1.9
Total Activity (AU)	1220	1570	725	1102	285	263
Total Protein (mg)	158	148	31	48	4.2	5.2
Specific activity (AU/mg)	7.7	9.9	23.3	20.3	67.8	40.4
Yield %	100	100	25.4	59.0	3.4	18.4
Purification fold	1	1	3	2.04	8.7	2.9

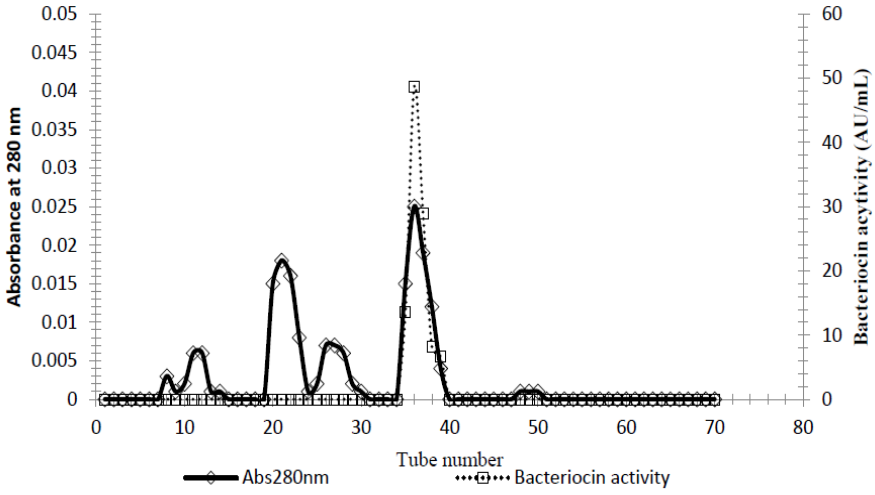

Fig. 1. **Elution profile of bacteriocin UNAD 012 deduced from the determination of bacteriocin activity**.

The zone of inhibition ranged between 19 and 36 mm against the test organisms. Enterocins UNAD 012 and UNAD 046 have better activity against Gram negative and Gram positive bacteria, respectively. Enterocin produced by strain UNAD 033 had the least effect on the isolates. Enterocins have been reported to inhibit bacteria (Laukova *et al*. 1993, Casula and Cutting 2002, Foulquie *et al.* 2003). In this study, four test bacteria were used at the primary screening stage of enterocin production. The bacteria were used to determine

the potency of the bacteriocins produced by the strains of *E. faecalis.* Table 2 shows the effects of purification on specific activity of two promising enterocinogenic producing *E. faecalis.* The specific activity increased with purification processes while a decrease was noticed in the yield, protein concentration and total protein of the enterocins. This observation was comparable to the findings of Whitford *et al.* (2001). The elution profile of bacteriocins deduced from the determination of bacteriocin activity was represented in Figures 1 and 2.

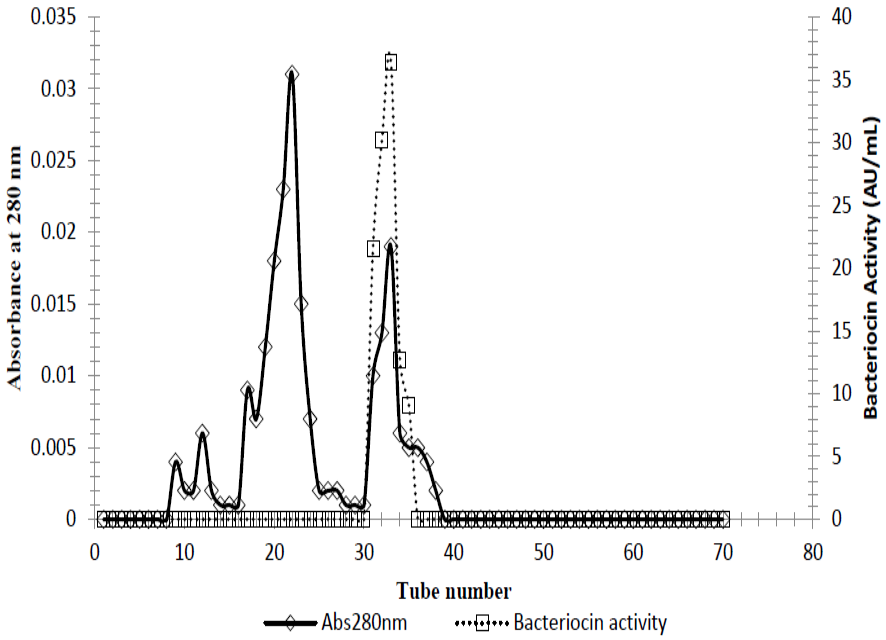

Fig. 2. Elution profile of bacteriocin UNAD 046 deduced from the determination of bacteriocin activity.

Compared with the control, enterocin UNAD 012 had a significant effect on *P. ultimum* at $P<0.05$. At $P<0.05$ significant level, the growth of the fungi at 24h differs significantly from those of 72h and at 96h. At the same significant level, the difference of the growth of the fungi at 48 h differs from the growth at 96h. There was a significant difference ($P<0.05$) on the percentage inhibition of the effects on the enterocin on the test fungi except UNAD 012 on *P. ultimum* (Table 3). As shown in Table 4, the percentage inhibition of the fungi increased with time of exposure to the enterocins.

Table 3. Antifungal activities of enterocins, at their arbitrary units (AU), on selected fungi isolates (radial mycelial in cm).

Test organisms	Enterocins	Time (h)			
		24	48	72	96
Control		3.30±1.79	5.85±1.78	9.80±3.78	12.65±3.31
P. expansum	UNAD 012	2.85±1.02	3.25±1.03	7.80±2.56	7.89±3.97
	UNAD 046	1.85±0.45	4.25±1.99	8.80±2.78	9.01±3.78
B. theobromae	UNAD 012	1.59±0.89	2.07±1.02	2.90±1.45	3.02±1.34
	UNAD 046	2.73±1.06	3.00±1.74	8.56±3.97	9.00±3.45
A. niger	UNAD 012	1.10 ±0.98	2.50±1.52	3.50±0.46	3.52±1.49
	UNAD 046	2.10±1.16	3.75±1.48	4.65±1.66	5.97±3.34
F. oxysporum	UNAD 012	1.50±0.48.	2.20±1.93	4.50±2.09	5.81±3.39
	UNAD 046	1.10 ±0.41	2.05±1.68	2.65±1.88	4.14±1.59
Py. ultimum	UNAD 012	1.50±0.56	1.50±1.46	1.52±1.34	2.01±1.78
	UNAD 046	1.50±0.91	4.00±1.33	4.80±2.94	5.17±3.97

Table 4. Percentage inhibition of the enterocins on test fungi.

Test organisms	Enterocins	Time (h)			
		24	**48**	**72**	**96**
Control		0	0	0	0
P. expansum	UNAD 012	13.64	44.44	20.41	37.63
	UNAD 046	43.94	27.35	10.20	28.77
B. theobromae	UNAD 012	51.82	64.62	70.41	76.13
	UNAD 046	17.27	48.72	12.65	28.85
A. niger	UNAD 012	66.67	57.26	64.29	72.17
	UNAD 046	36.36	35.90	52.55	52.81
F. oxysporum	UNAD 012	54.55	62.39	54.08	54.07
	UNAD 046	66.67	64.96	72.96	67.27
P. ultimum	UNAD 012	54.55	74.36	84.49	84.11
	UNAD 046	54.55	31.62	51.02	59.13

Compared with the control, UNAD 046 had a better inhibitory effect on *B. theobromae, A. niger, Penicillium expansum* and *P. ultimum* than UNAD 042. On the other hand, *F. oxysporum* was more susceptible to UNAD 046 than UNAD 012. These results were similar to those of Aruna and Madhuri (2016), Schillinger *et al.* (1996) reporting the susceptibility of different fungi (spoilage and pathogenic) to enterocins. Smaoui *et al.* (2010) reported that *Lactobacillus* spp. produce bacteriocins that are active against Gram-negative bacteria and also particularly inhibit fungi. Bacteriocin has been proposed to be a promising treatment of plant infections, and its application has been reported to be safe to animals and humans (Cleveland *et al*. 2001, Ogunbanwo

et al. 2004, Cole *et al*. 2006). Very few bacteriocins with antifungal properties have been reported and most enterocins studied have bacteriostatic and bacteriocidal activities on food-borne bacteria pathogens and not mould (Suzuki *et al.* 1991).

4 Conclusion

From this study, we observed that partially purified enterocins produced by *E. faecalis* had inhibitory spectrum on selected phytopathogenic fungi. Enterocin from *Enterococcus faecalis* could be a good candidate for biocontrol of phytopathogenic fungi. Nevertheless, more studies need to be done to further validate the results of this report.

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