*British Biotechnology Journal*
*3(3): 359-366, 2013*

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# Growth Inhibition of Some Phytopathogenic Bacteria by Cell-Free Extracts from *Enterococcus* sp

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***Authors' contributions***

*This work was carried out in a collaborative framework between Universidad Autónoma de Coahuila, Universidad Autónoma Agraria Antonio Narro and Grupo Bioindustrial del Norte. Authors CNA, DHC, RRH and CC designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author LCM developed this study as part of his research thesis. All authors read and approved the final manuscript.*

***Short Communication***

***Received 31st December 2012***
***Accepted 26th April 2013***
***Published 15th May 2013***

## ABSTRACT

**Aims:** To inhibit of bacterial growth of three important phyto-pathogenic bacteria: *Erwinia carotovora, Clavibacter michiganensis sp. michiganensis* and *Xanthomonas axonopodis* by cell-free extracts from submerged cultures of two strains of *Enterococcus* sp. was tested.
**Study Design:** A complete randomized experimental design with factorial fix was used to evaluate the efficiency of growth inhibition against the phytopathogenic bacteria.
**Place and Duration of Study:** Laboratory of Bioprocesses, Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Mexico, between December 2011 and July 2012.
**Methodology:** Enterococci strains were isolated from goat milk, buttermilk and whey by typical microbiological procedures and primarily identified based on biochemical tests.

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Strains were subsequently activated in MRS broth and cells were separated by centrifugation and filtration. Cell-free extracts were tested against plant pathogenic bacteria to determine their growth inhibition potential.
**Results:** Strains of *Enterococcus* MII-1 and MIV-2 were able to inhibit the growth of three pathogenic bacteria, demonstrating to be an attractive alternative for biological control assays.
**Conclusion:** The cell-free extracts of *Enterococcus spp.* show inhibition potential to inhibit phytopathogenic bacteria that cause diseases in horticultural crops. Further studies are needed to completely evidence the high potential of use of cell-free extracts from *Enterococcus* MII-1 and MIV-2.

*Keywords: Growth inhibition; Erwinia carotovora; Clavibacter michiganensis sp. Michiganensis; Xanthomonas axonopodis.*

## 1. INTRODUCTION

Currently, there are important economic losses in the Mexican agricultural sector and the rest of Latin-American Countries. Particularly, the losses are caused by diseases provoked by plant pathogenic bacteria in wheat, one of the four most important crops around the world [1-4]. Dry matter production and crop yield can significantly decrease, from the seedling stage to harvest, by presence of foliar diseases caused by fungi, viruses and/or bacteria, which can also affect seed viability and quality [5-7]. Worldwide, these losses range from 5 to 50% [5,8-10], and between 5 and 20% in Mexico (9). Phytopathogenic bacteria produce spots and leaf blights, soft rots of fruits, roots of storage organs, wilting, overgrowth, scabies, tumors and other symptoms [11]. Trying to reduce and control crop diseases (fungi, viruses and bacteria), different methods and combinations of them have been used to control of phytophatogens, including use of synthetic chemicals, however this methods is controversial, especially in orchards, where some synthetic chemicals may be harmful to health, so use of these chemical should be diminish to a minimum and if necessary, use of the least toxic synthetic chemical [12].

Antagonistic microorganisms (bacteria, yeasts and fungi) are able to exert an effect on biological control of different pathogens of agronomical interest and have been used for controlling various plant diseases of fruit []13,14]. The mechanisms by which antagonistic microorganisms affect pathogen populations are not always clear, but usually are attributed to the following effects: direct parasitism and death of pathogen; competition with pathogens for space or nutrients; direct toxic effects on the pathogenic agent for antibiotic substances released by the antagonist and toxic effects on pathogens by volatile compound such as ethylene released as result of metabolic activities of the antagonist [11]. This paper describes the isolation of *Enterococcus* strains, and the evaluation of the growth inhibition potential of their cell-free extracts on three important phytopathogenic bacteria (*Clavibacter michiganensis, Erwinia carotovora* and *Xanthomona axonopodis)*.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Enterococcical strains

Samples of goat milk, whey and buttermilk were used as sources for isolation of *Enterococcus* strains, codes in Table 1. The samples were provided by the Laboratory of

Food Technology at the Universidad Autónoma Agraria Antonio Narro (UAAAN). Samples were inoculated onto Man, Rogosa & Sharpe (MRS) agar plates for isolation *Enterococcus* sp strains. In this case, plates were incubated at 37ºC for 24h. For morphological analysis a US Micro-optical solution microscope Model 0827769 was used. All Samples were analyzed at 100x with immersion oil. Enterococci are Gram-positive cocci that often occur as diplococci or short chains. After biochemical characterization of microorganisms, subsequently 4 bacterial strains were selected from each sample according to cell morphology belonging to the *Enterococcus* genus, and then strains were inoculated on MRS agar plates.

**Table 1. Codes of the isolated strains from the samples on MRS agar plates**

| Sample | Strains (codes) |
|---|---|
| Whey (MI) | MI-1 |
| | MI-2 |
| | MI-3 |
| Buttermilk (MII) | MII-1 |
| | MII-2 |
| | MII-3 |
| Goat´s milk (MIII) | MIII-1 |
| | MIII-2 |
| | MIII-3 |
| Goat´s milk (MIV) | MIV-1 |
| | MIV-2 |
| | MIV-3 |

## 2.2 Biochemical Characterization of *Enterococcus* strains

MRS agar contains selective components which inhibit the growth of the most of microorganisms, permitting the selective growth of lactobacilli and enterococci after isolation of the selected strains, they were biochemically characterized, including growth on bile esculin agar, highly selective for these microorganisms; other considered tests were catalase test, 6.5% of NaCl medium and bile salts.

## 2.3 Phytopathogenic Bacteria

Phytopathogenic microorganisms used in this project were *Erwinia carotovora, Clavibacter michiganensis sp. michiganensis* and *Xanthomonas axonopodis*. The pathogenic strains were obtained from the Culture Collection of the Centre for Applied Microbiology, Greencorp Biorganiks de Mexico SA de CV. Saltillo, Coahuila, Mexico. These bacteria were activated in 250 ml Erlenmeyer flasks with 100 ml of nutrient broth supplemented with potato infusion at 30ºC for 24h.

## 2.4 Growth Conditions of Enterococci

For production of cell free extracts, two culture media were compared. Potato broth which was formulated with glycerol (10g), potassium dibasic phosphate (1.5 g), magnesium sulfate (1.5 g) enriched with potato 20g, and MRS Broth which is a commercial medium. Cell concentration was assessed by counting in a Neubauer chamber after 24 h of incubation at

37ºC. Influence of initial pH (6.0, 6.5 and 7.0) and temperature (30, 37 and 39ºC) on Enterococci culture were determined.

## 2.5 Preparation of Cell-Free Extract

Selected strains of *Enterococcus* sp. were cultivated at appropriate conditions according to results of the analysis described above. After obtaining the cultures, samples were centrifuged in conical tubes at 4467 *g* for 15 min at 4ºC. Supernatant was carefully separated, to completely remove the cells. Extracts were filtered through 0.2 μm sterile nylon membranes (Millipore®). The filtered extracts were used to assess their potential for plant-pathogenic bacterial growth inhibition.

## 2.6 Potential Inhibition of Cell-Free Extract

Nutritive agar plates were completely inoculated with each phytopathogenic bacterium. Four small holes of 5 mm diameter were made on the surface of agar plates and filled with 60 μL of each cell-free extracts and then, plates were incubated at 28ºC for 24h. After that, the formed inhibition zones were measured with a vernier.

## 2.7 Experimental Design and Data Analysis

Two complete randomized experimental designs with factorial fix were used, the first design to evaluate the inhibition potential of free-cell extracts against phytopathogenic bacteria; and the second for determination of sensitive of phytopathogenic bacteria against free-cell extracts. In each case, a Tukey-Kramer test ($P$ = 0.05) was carried out for mean comparisons.

## 3. RESULTS AND DISCUSSION

In this document we describe the isolation of *Enterococcus* strains, and the evaluation of the growth inhibition potential of their cell-free extracts on three important phytopathogenic bacteria (*Clavibacter michiganensis, Erwinia carotovora* and *Xanthomona axonopodis)*.

## 3.1 Isolation and Identification of *Enterococcus* spp

Enterococci strains were isolated using MRS agar supplemented with sodium acetate for growth inhibition of other bacteria promoting the development of lactic acid bacteria [15]. Three strains were isolated from each sample (Table 1) and the results of the Gram staining corresponded to typical morphology expected for enterococci (Table 2); the Fig. 1 shows Gram-positive cocci (MII-1 strain) that often occur in pairs (diplococci) or short chains. *Enterococcus spp.* is microorganism negative catalase, which grew in 6.5 % NaCl and bile salts; the results are shown in the Table 2. All the strains were negative catalase; the three strains of whey and the third strain of buttermilk do not shown grow in NaCl 6.5 % medium and bile salts, therefore these strains were discarded as enterococci.

## 3.2 Growth Conditions of *Enterococcus* sp.

*Enterococcus* family includes 28 species with facultative anaerobic metabolism unable to develop spores or any visible capsule [16]. The cultivation of the these microorganisms is easy because they can grow on basic substrates used in microbiological laboratories at 10–

45ºC, with the optimum temperature of about 37ºC. The microorganism can tolerate increased concentrations of NaCl (up to 6.5%) and bile salts (up to 40%), as well as higher substrate pH values (up to pH 9.6). The microorganism can withstand heating to 60ºC for 30 minutes. Most enterococci proliferate under common aerobic conditions. In the present study, growth conditions of enterococci were determinant in the selection of two strains, because only these strains showed inhibition potential against the phytopathogenic bacteria. Comparison was conducted primarily in potato broth (described above) and MRS broth, showing the strains greater growth on the last culture medium. Strains showed better growth at 37ºC and pH = 7.0.

**Table 2. Results to the biochemical tests performed for Enterococcical identification**

| Strains (codes) | Biochemical tests | | | |
|---|---|---|---|---|
| | Morphology | Catalase | Growth in 6.5 % NaCl médium | Growth in bile salts |
| MI-1 | Cocci, Gram (+) | (-) | (-) | (-) |
| MI-2 | Cocci, Gram (+) | (-) | (-) | (-) |
| MI-3 | Cocci, Gram (+) | (-) | (-) | (-) |
| MII-1 | Cocci, Gram (+) | (-) | (+) | (+) |
| MII-2 | Cocci, Gram (+) | (-) | (+) | (+) |
| MII-3 | Cocci, Gram (+) | (-) | (-) | (-) |
| MIII-1 | Cocci, Gram (+) | (-) | (+) | (+) |
| MIII-2 | Cocci, Gram (+) | (-) | (+) | (+) |
| MIII-3 | Cocci, Gram (+) | (-) | (+) | (+) |
| MIV-1 | Cocci, Gram (+) | (-) | (+) | (+) |
| MIV-2 | Cocci, Gram (+) | (-) | (+) | (+) |
| MIV-3 | Cocci, Gram (+) | (-) | (+) | (+) |

*(+), positive result, (-) negative result*

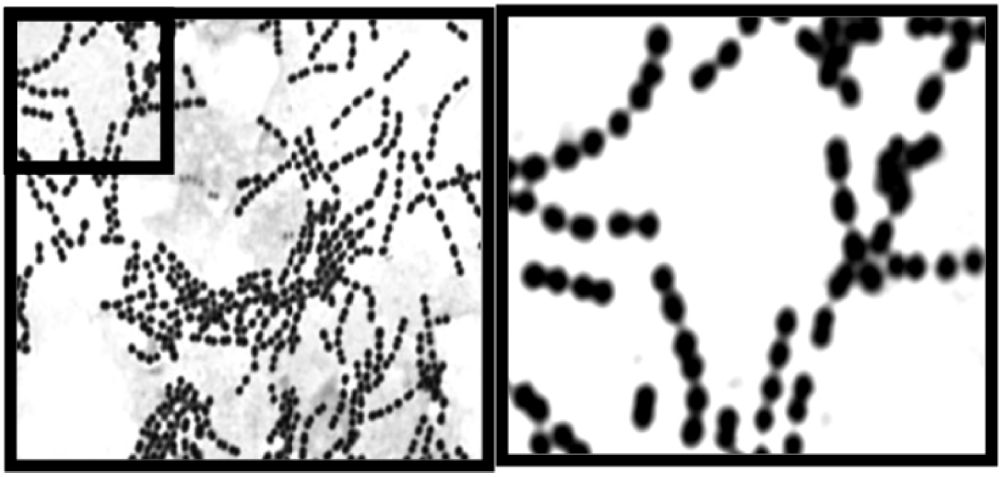

**Fig. 1. Morphological evaluation from US M.O.S microscope of a gram stain of *Enterocoocus spp.* MII-1 strain**

## 3.3 Potential Inhibition of Cell-Free Extract

Enterococci are part of enteric commensal microbiota with a widespread occurrence in the different ecosystems. They have the ability to produce the antimicrobial substances with an antagonistic effect against the various bacteria, spoilage species including. Many of them also exert beneficial (probiotic) influence on host organisms [17]. To evaluate the inhibition capacity of cell-free extracts from *Enterococcus spp.* strains. Inhibition zones were measured and only two *Enterococcus* strains (MII-1 and MIV-2) showed inhibition potential against three plant pathogenic bacteria (Table 3). A 2x3 factorial treatment arrangement was used with two factors strains with two levels (MII-1 and MIV-2 strains) and plant pathogenic bacteria with three levels (*Clavibacter michiganensis sp. michiganensis, Erwinia carotovora* and *Xanthomona axonopodis*). The results showed that MIV-2 strain inhibition potential against plant-pathogenic bacteria is greater than MII-1 strain (Table 4). Furthermore, the phytopathogenic bacteria showed no significant difference among themselves, as regards sensitivity to each treatment (Table 5).

**Table 3. Inhibition halos of phytopathogenic bacteria by cell-free extracts from two *Enterococcus spp***

| Strain | *vs* | Inhibition halo (mm) | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | Mean value |
| MIV-2 | *Xanthomonas axonopodis* | 3.5 | 3.0 | 2.5 | 3.5 | 3.1 |
| | *Clavibacter michiganensis* | 5.0 | 4.0 | 3.0 | 3.0 | 3.8 |
| | *Erwinia carotovora* | 2.0 | 2.0 | 2.5 | 4.0 | 2.6 |
| MII-1 | *Xanthomonas axonopodis* | 3.0 | 2.5 | 3.5 | 3.5 | 3.1 |
| | *Clavibacter michiganensis* | 2.0 | 2.0 | 1.5 | 2.0 | 1.9 |
| | *Erwinia carotovora* | 2.0 | 2.0 | 2.5 | 2.0 | 2.1 |

**Table 4. Inhibition potential by cell-free extracts from two Enterococci against phytophathogenic bacteria**

| Strain | Inhibition (mm) | Group |
|---|---|---|
| MII-1 | 2.38 | 1 |
| MIV-2 | 3.17 | 2 |

**Table 5. Inhibition of phytopathogenic bacteria by cell-free extracts from two enterococci**

| Phytopathogenic bacteria | Sensitivity (mm) | Difference among groups |
|---|---|---|
| *Clavibacter michiganensis* | 2.375 | 1 |
| *Erwinia carotovora* | 2.813 | 1 |
| *Xanthomonas Axonopodis* | 3.125 | 1 |

There are reports that show the ability of certain lactic bacteria like *Enterococcus* spp to produce final fermentation products and these has been characterized as microbial growth inhibitory substances, among them are bacteriocins which represent a topic of strong research [18-19]. Studies with lactic acid bacteria have been demonstrated to produce substances called bacteriocins with antimicrobial activity, and these are used in the food industry for control of microorganisms potentially pathogens for humans. On the other hand, bacteriocins do not modify food properties [20].

Stromptová V, Lauková [21] determined bacteriocin production in *Enterococcus spp.* isolates of chicken origin and *in vitro* properties of bacteriocin- producing strain *E. faecium* EF55 that could be promising as a probiotic. Moreover, reduction effect of EF55 strains towards poultry *Eimeria* spp. Oocysts was achieved. Currently, more attention has also attracted bacteriocins as the possible antimicrobial agents for reduction or elimination of certain pathogens [17,22-24]. Although in this study was observed the inhibition capacity of the enterococci after fermentation, it is important to note that would be very interesting research the chemical analysis of the products formed by enterococci to determinate what is inhibiting the phytopathogenic bacteria. Moreover, identify and characterize the products into the extracts would be a great complement for this work.

## 4. CONCLUSION

Cell-free extracts of *Enterococcus spp.* MIV-2 and MII-1 strains isolated from goat´s milk and buttermilk, respectively; show inhibition potential against phytopathogenic bacteria evaluated, promising to be a great alternative to prevent various diseases in horticultural crops, which can be useful to avoiding economic losses in this sector. Further research is needed in order to improve and optimize the culture conditions to obtain extracts with high activity for the inhibition of the phytopathogenic bacteria.

## ACKNOWLEDGEMENTS

Authors thank CONACYT (Mexican Council of Science and Technology) for the financial support through Project 2009-INNOVAPYME-109692. Also, authors thank the technical facilities given by Green Corp Biorganiks de Mexico SA de CV.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Wayne SC. Crop Production. Evolution, History and Technology. John Wiley y Sons, Inc. USA. 1995;57-127.
2. Villaseñor MEH .Importancia del trigo. *In:* El Trigo de Temporal en México (eds). INIFAP, CIR-CENTRO, México. 2000;7-24.
3. Ávila DJD, Santoyo C, Schwentesius R, Palacio, M. El Mercado del Trigo en México ante el TLCAN. Centro de Investigaciones Económicas, Sociales y Tecnologías de la Agroindustria y la Agricultura Mundial, Universidad Autónoma Chapingo. Chapingo, Estado de México. 2001;132.
4. Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Dazak P. Emerging infectious diseases of plants: pathogen pollution, climate change and agro technology drivers. Trends n Ecology and Evolution. 2004;19:535-44.
5. Duveiller E, Bragard C, Rudolph K, Fucikovsky Z. General concepts and methods for the identification of pathogenic bacteria of wheat. *In*: The Bacterial Diseases of Wheat. Concepts and Methods of Disease Management. (eds). CIMMYT. México: 1997; 5-23.foundation seed health program. Plant Disease. 1998;72:935-38.
6. Hernández LA, Villaseñor MHE, Barrera EG, Rosas RM. Efecto de las enfermedades foliares sobre la calidad y micoflora en la semilla de Trigo. Revista. Fitotecnia Mexicana. 1998;21:25-35.

7. Espitia RE, Villaseñor MHE. El rendimiento de grano en relación a la morfología, desarrollo y fisiología en trigo. *In*: El trigo de Temporal en México. INIFAP, CIR-CENTRO, México. 2000;53-83.
8. Forster RL, Schad NW. Control of black chaff of wheat with seed treatment and a
9. Mehta YR .Management of *Xanthomonas campestris pv. Undulosa* and *hordei* through cereal seed testing. Seed Science and Technology. 1990;18:467-476.
10. Toben H, Mavridis A, Rudolph KWE. On the occurrence of basal glume root wheat and barley caused by *Pseudomonas syringae* pv. *atrofaciens* in west Germany Journal of Plant Disease and Protection. 1991;98:225-235.
11. Agrios G. Fitopatología. Editorial Limusa. 1996;838.
12. Valero S. Introducción a las Actividades Agropecuarias, Barinas, Ediciones de la Universidad Ezequiel Zamora; 2000.
13. De Costa DM, Erabaduptiya HRUT. An integrated method to control postharvest diseases of banana using a member of *Burkholderia cepacia* complex. Postharvest and Biology Technology. 2005;36:31-39.
14. Wisniewski ME, Wilson CL. Biological control of postharvest diseases of fruits and vegetables: Recent advances. HortScience 1992;27:94-98.
15. EDM Chemicals, MRS Agar; 2002.
16. Cermak P, Landfeld A, Mericka P, Houska M. *Enterococcus faecium* growth model. Czech Journal of Food Science. 2009;27:361-371.
17. Lauková A, Marciñakova M, Strompfova V, Ouwehand AC. Probiotic potential of enterococci isolated from canine feed. Folia Microbiologica. 2008;53:84-88.
18. Elisa CMM. Bacteriocinas producidas por cepas de *Enterococcus* ,2003. Accesed 24 Agust 2012. Available: http://www.universia.com.ar/contenidos/.../642.htm.
19. Arqués J, Rodríguez E, Tomillo J, Gaya P, Núñez M, Medina M. Tratamientos combinados de altas presiones y bacterias lácticas productoras de bacteriocinas en la inactivación de patógenos. En: Bacteriocinas de bacterias lácticas en la mejora de la calidad de los alimentos, 2003;181-192.
20. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology. 2001;71(1):1-20.
21. Stromptová V, Lauková A. *In vitro* study on bacteriocin production of *Enteroccci* associated with chickens. Microbial host interactions. 2007;13:228-237.
22. Strompfová V, Lauková A, Mudronová D. Effect of bacteriocin-like substance produced by *Enterococcus faecium* EF55 on the composition of avian gastrointestinal microflora. Acta Veterinaria Brno. 2003;72:559-564.
23. Lauková A, Guba P, Nemcová R, Vasilková Z. Reduction of Salmonella in gnotobiotic Japanese quails caused by enterocin A-producing EK 13 strain of *Enterococcus faecium.* Veterinary Research Communcations. 2003;27:275-280.
24. Lauková A, Guba P, Nemcová R, Mareková M. Inhibition of salmonella entérica serovar Düsseldorf by enterocin A in gnotobiotic Japonese quails. Journal Veterinarni Medicina. 2004;49:47-51.




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