Screening Food Microbiota for Novel Antimicrobial Compounds suitable for Food Preservation

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Abstract

The excessive use of antimicrobials has led to the decrease of their effectiveness against foodborne pathogens and spoilage bacteria. Therefore, innovations are needed for discovery of new potent antimicrobials to be used in food. In this study, twenty one samples of fermented food products were screened for beneficial microorganisms that show potent antimicrobial activity. Out of 1500 tested isolate, an *Enterococcus durans* OSY-EGY strain, isolated from an Egyptian cheese, was found to produce an antimicrobial compound at pH 4.6 and a concentration of 800 arbitrary units/ml. This compound is most active against *Listeria* sp., *Lactobacillus* sp., *Pediococcus* sp., and *Lactococcus lactis*. The antimicrobial compound retained its activity after storage at 4°C for 12 months, heating at 100°C for 80 minutes and 120°C for 25 minutes, treatment with alpha-chymotrypsin, trypsin, carboxypeptidase, aminopeptidase, papain enzymes and over a pH range from 1.5 to 13.5. The compound was purified by cationic exchange resin and C18silica cartridges with elution by dimethyl sulfoxide which indicated the compound's cationic and amphipathic nature. Polyacrylamide gel electrophoresis showed the purified compound as a single band and the matrix-assisted laser desorption ionization showed that the molecular mass of the compound is 5200 kDa. In conclusion, the investigated antimicrobial compound is showing unique physico-chemical characteristics and stability to proteolytic enzymes, heat, acidic and alkaline pH; these traits make it a good candidate as a food preservative.

Introduction

The excessive use of antimicrobials in hospitals and in animal feed led to the decrease of their effectiveness against foodborne pathogens. Antimicrobial resistance has been detected in many zoonotic food-transmitted pathogens, including Methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella* Sp., *Campylobacter* Sp., *Escherichia coli* O157:H7, *Listeria* Sp., and *Yersinia* Sp. The microbial Food spoilage is also a problem as it causes economic losses due to the food quality deterioration. Therefore, innovations are needed for discovery of new potent antimicrobials to be used in food. The natural environment is considered an important source for beneficial microorganisms that are capable of producing effective antimicrobials (Clardy et al.2006). Bacteriocins are antimicrobial peptides or proteins produced by bacteria to kill other bacteria from related (narrow spectrum) or unrelated (broad spectrum) genera. The competition on the nutrients and space enhance the production of the bacteriocins as a bacterial defense weapon. The antimicrobial compound Nisin that is produced by *Lactococcus lactis* is the most studied antimicrobial agent used in food applications for several years due to its desirable characteristics: (1) Nisin have a potent and wide antimicrobial spectrum against many Gram-positive foodborne spoilage and pathogenic bacteria, (2) It is generally recognized as safe (GRAS). However, the disadvantage of nisin is its limited stability at neutral and alkaline pH, so it is necessary to discover novel bacteriocins that have better biochemical characters. In order to discover alternatives, a high number of bacteriocins produced by members of lactic acid bacteria group have been discovered and their application as bio-preservatives in foods has been studied (Cintas et al.2001). Lactic acid bacteria are gram-positive, non-spore-forming bacteria that ferment carbohydrates and produce lactic acid as the end product so they are called fermentative bacteria (Stiles and Holzapfel 1997). These bacteria are able to produce different antimicrobial factors that include lactic acid, hydrogen

peroxide and bactericidal peptides called bacteriocins that are able to kill spoilage or pathogenic microorganisms that enhance food safety and prolong the shelf life. The most common bacteriocin-producing lactic acid bacteria genera are *Lactococci, lactobacilli, Pediococococci, Leuconostoc, and Enterococci.* The spectrum of inhibitory activity by bacteriocins can be either narrow or wide. Some of the bacteriocins that are produced by LAB are applied for use as food bio-preservatives. *Enterococcus* which is a member of lactic acid bacteria group is found in the gastrointestinal tract of warm blooded animals, (Franz et al. 1999). *Enterococci* survive harsh environmental conditions, such as 60oC for 30 min, 6.5% NaCl, or pH of 9.6, (Sherman 1937). *Enterococci* could also be found as natural microbiota in different foods such as milk, dairy products like cheeses and vegetables (Giraffa 2002).They also can be found in various fermented food products. The *Enterococci* could be found in cheese as starter culture because their lipolytic and proteolytic activity in addition to its citrate utilization function in the development of flavor, aroma, texture and ripening, (Foulquie-Moreno et al. 2006). In addition the *Enterococci* could be found in cheese due to poor hygienic quality as fecal contamination that can lead to some defects in the finalized product, (Lopez Diaz et al. 1995). The most common used strains of *Enterococci* as starter culture for cheese production and as probiotics in some foods and dietary supplements are *Enterococcus faecalis* and *E. faecium* (Holzapfel et al. 1998). Bacteriocins produced by *enterococci* are called enterocins. Enterocins have different characteristics, such as mode of action, molecular weights, inhibitory activity spectrum and biochemical structure. They are mostly placed under the category of class II bacteriocin. Recently, enterocins have received much attention because they inhibit food-borne pathogens, such as *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp. and *L. monocytogenes*. The purpose of this study is Searching for a potent antimicrobial compound from beneficial bacteria, purifying this compound and determine its antimicrobial spectrum, physico-

chemical properties and primary structure and investigating its effectiveness and safety for use as a food preservative.

Materials and Methods

Food samples and sample preparation:

Samples were collected and screened for beneficial microorganisms that produce potent antimicrobial agents. Food samples were purchased from local food stores (Columbus, OH); these included vegetables (Turkish and Egyptian-style pickled vegetables), milk, raw milk cheeses, mold ripened cheese, dressing and yogurt samples. Samples were suspended in saline solution or sodium citrate solution and homogenized using stomacher. The homogenates were 10 fold serially diluted and 100 Microliters of each dilution were cultured on MRS agar plates and the plates were incubated at 30°C for 48 h.

Strain screening for antimicrobial activity (Guo *et al*. 2012):

The grown colonies were transferred to new MRS agar plates and incubated at 30°C for 48 hrs. The incubated plates were overlaid with soft-agar medium seeded with *Listeria innocua* as Gram positive indicator or *Escherichia coli* K12 as Gram negative indicators . The plates were incubated at 37°C overnight and checked for evidence of antimicrobial activities against the indicator bacteria by observing inhibition zones surrounding tested colonies. Nisin producer *Lactococcus lactis* was used as control positive to compare the inhibition zones. The isolate that show the strongest inhibition was selected for further analysis.

Antimicrobial bioassay using the spot-on-lawn method (He et al. 2007):

The isolate that show the strongest inhibition to the indicator bacteria was inoculated in MRS broth and incubated at 30°C for 48 hrs. The fermentate was centrifuged and filter sterilized to obtain the

cell free supernatant (CFS). Because of the bacterial ability to produce different antimicrobial factors other than bacteriocins, it was necessary to exclude these factors. For organic acid exclusion, the pH of the CFS was adjusted to 7 using NaoH followed by 2 hrs incubation at 25°C. For H_2O_2 exclusion the CFS supernatant was treated with catalase enzyme and incubated for 2 hrs incubation at 25°C. For phage exclusion the agar in the inhibition zone was cut and overlaid with the sensitive indicator followed by incubation. The antimicrobial activity was retested after the three experiments. For the bioassay of antimicrobial activity, CFS was double fold serially diluted and 10 Microliters of each dilution was spotted on a lawn of sensitive indicator microorganisms. The spotted plates were incubated at 37°C overnight and checked for the inhibition zones. Antimicrobial activity was expressed in arbitrary units (AU)/ml; this value is the reciprocal of the highest dilution displaying a zone of inhibition.

Determination of antimicrobial inhibition spectrum

The spectrum of the antimicrobial activity of the investigated compound was detected through spotting the cell free supernatant of culture on a lawn of selected indicator microorganisms obtained from the ATCC, the antimicrobial activity was tested against *L.innocua*, *Pediococcus* Sp., *Lactococcus lactis* and *Lactobacillus* Sp.

Identification of the antimicrobial compound producer isolate:

The morphological characteristic of the isolate was investigated by Gram staining and examination under microscope. The isolate identity was determined by 16S rRNA gene amplification and sequencing (Drancourt et al. 2000). The producer isolate genomic DNA was extracted using (DNeasy blood and tissue kit), the targeted gene was amplified in a thermocycler using Universal primers for 16S rRNA gene (Weisburg et al. 1991). The Amplified genes was purified , sequenced and the resulted DNA sequence was compared to known sequences in the National Center for

Biotechnology Information database (NCBI GenBank) using the Basic Local Alignment Search Tool (BLAST) algorithm.

Isolation and purification of the antimicrobial agents with different resins:

The investigated compound was purified from the cell free supernatant by using the following resins:

A-XAD 16N: The isolate was culture and the cell free supernatant was partially purified by 10% XAD16N and incubated for18 hr at 25°C followed by washing with HPLC water and elution with 500 ml 95% ethanol with concentration of the eluted fraction using Speed-Vac concentration. The concentrated pellet was suspended and tested for the antimicrobial activity.

B- Cationic exchange resin: 500 mL of cell free supernatant were incubated with a cationic exchange column (Macro-Prep High S support; Bio-Rad, Hercules, CA, USA) for 4 hours. The compound was retained on the resin and eluted with 2.0 M NaCl.

C- C_{18} silica cartridges: Cell free supernatant was subjected to solid phase extraction using C_{18} silica cartridges (Sep-Pak; Waters Corporation, Milford, MA, USA). The column was washed with HPLC water followed by washing with 0%, 25%, 50%, 75% and 100% acetonitrile and elution with 100 % dimethyl sulphoxide.

Verification of molecular mass of antimicrobial compound:

The molecular mass of the antimicrobial compound was identified by analyzing the 100% DMSO eluted active fraction through polyacrylamide gel electrophoresis using Tris-Tricine peptide precast gel system. After electrophoresis, one-half of the gel was stained with Coomassie blue, while the other was washed and then overlaid with MRS soft agar seeded with the indicator sensitive bacteria, incubated and examined for antimicrobial activity. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) was used to determine the molecular mass of

the investigated compound by analyzing the semi-purified compound obtained from purification using the XAD 16N resin and Cationic exchange chromatography**.** The semi-purified fraction was mixed in a ratio of 1:5 (vol/vol) with cyano-4-hydroxycinnamic acid matrix dissolved in 50% acetonitrile with 0.1% TFA in water. MALDI-TOF MS analysis was conducted on a Bruker UltrafleXtreme MALDI-TOF/TOF operated in reflection positive-ion mode and accelerated at a voltage of 28 kV and a nitrogen laser.

Testing stability to temperature, pH and enzymes:

For the thermal stability test, the CFS was exposed to 55°C, 80°C and 100°C up to 80 minutes and 120°C up to 25 minutes. For the pH stability test, the PH of CFS was adjusted from 4.6 to pH 1.5, 7.0, 9.0 and 13.5 using concentrated NaOH or HCL, followed by incubation for 2 hrs at 25°C and re-neutralization of the CFS to pH 7. CFS was tested for sensitivity to selected enzymes, including neutral protease, Alpha-Chymotrypsin, Trypsin, Carboxypeptidase, Aminopeptidase, Neutral and alkaline phosphatase and papain. The enzymes were prepared at 1.0 mg/ml and equal volumes of each enzyme solution and CFS were mixed and incubated at 37°C for 2hrs. The antimicrobial activity was retested after the three experiments.

Results and Discussion

Bacteriocins are naturally produced bacterial antimicrobial compounds that have the ability to inhibit the growth or kill other bacteria. *Enterococcus,* which is a member of Lactic Acid Bacteria, presents in dairy and meat products as natural or added culture to enhance aroma and flavours due to its lipolytic and proteolytic activity. Some *Enterococcus* isolates were found to have antimicrobial activity due to the production of the antimicrobial peptides enterocins. In this study, Out of approximately 1500 tested isolate, a Gram positive bacterial strain isolated from Egyptian hard cheese, was identified by 16S rRNA gene sequencing technique and designated as

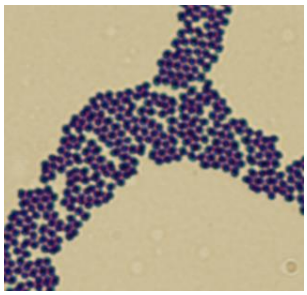
Enterococcus durans OSY-EGY. The *Enterococcus durans* OSY-EGY was found to produce an antimicrobial compound at pH 4.6 with concentration of 800 arbitrary units/ml. It showed strong antimicrobial activity against other bacteria including *listeria innocua, lactobacillus plantarum, lactococcus lactis and Pediococcus*. Further work should be done to investigate its antimicrobial spectrum against other spoilage and pathogenic bacteria. Stability to heat change, pH change, and degradative enzymes treatment was tested and the produced antimicrobial compound retained its activity after storage at 4°C for 12 months, heating at 100°C for 80 minutes and 120°C for 25 minutes, treatment with alpha-chymotrypsin, trypsin, carboxypeptidase, aminopeptidase, papain enzymes and over a pH range from 1.5 to 13.3. While most of the bacteriocins produced by *Enterococcus* are sensitive to the digestion by proteases enzymes such as Alpha-Chymotrypsin, Trypsin, Carboxypeptidase, Aminopeptidase, Neutral and alkaline phosphatase and papain enzyme that confirm their pretentious nature, this antimicrobial compound showed resistance against the previously mentioned proteases enzymes which indicates it may had a modification in its structure. This protease resistance is similar to the resistance shown by the cyclic antibacterial peptide enterocin AS-48 produced by *Enterococcus* (Grande Burgos *et al* 2014). The binding of the antimicrobial compound to cationic exchange resin and elution with 2.0 M NaCl indicates its cationic nature, which means it is positively charged. While the molecular mass of the compound was determined to be 5200 KD using MALDI-TOF and PAGE, further confirmation needs to be done on a pure compound since the sample used was a semi-purified fraction. The strong binding of this antimicrobial compound to C_{18} silica cartridges and its elution with DMSO solvent indicates the hydrophobic nature of the compound. Future work such as whole genome sequencing will be done on the producer isolate to identify the genetic information, HPLC purification and structural elucidation of the investigated antimicrobial compound.

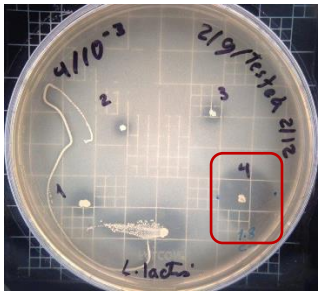
Table 1. Antimicrobial activity of the compound produced by *Enterococcus durans* OSY-EGY against sensitive bacteria.

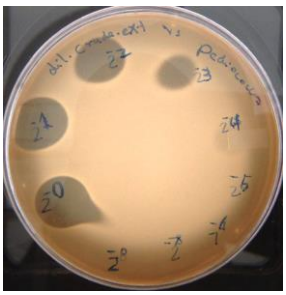
Tested bacteria	**Activity**
Listeria innocua ATCC 33090	+
Pediococcus pentosaceus	+
Lactococcus lactis ATCC 11454	+
Lactobacillus plantarum ATCC 8014	+
Lactobacillus fermentum	+
Lactobacillus cellubiosis OSU 919	+
Lactobacillus brevis OSU	+
Lactobacillus cuvatus OSY-HJC6	+/-
Leuconostoc mesentroids ATCC 14935	+/-

Table 2. Treatments that did not eliminate the antimicrobial activity of the compound produced by *Enterococcus durans* OSY-EGY

Factor	**Treatment**
Refrigerated storage	12 months at 4°C
Heat	100°C for 80 minutes
	120°C for 25 minutes
Enzymes	Alpha-chymotrypsin
	Trypsin
	Carboxypeptidase
	Aminopeptidase
	Papain
	Neutral/ alkaline phosphatase
	Catalase
pH	1.5-13.5







(A) (B) (C)

Fig. 1. Gram staining of *Enterococcus durans* OSY-EYG (A), the inhibition activity of *Enterococcus durans* OSY-EYG against the indicator *L.innocua* (B) and the bioassay of antimicrobial activity using spot on lawn method.

Fig. 2. Coomassie blue stained gel of a protein standard and the purified compound (A) and the overlaid gel with sensitive bacteria for antimicrobial activity confirmation of the purified compound (B).

Conclusion

In conclusion, the investigated antimicrobial compound is showing unique physico-chemical characteristics and stability to proteolytic enzymes, heat, and extreme acidic or alkaline pH. These traits make it a promising compound as a food preservative. More studies are needed to determine the feasibility of its use in food preservation as an alternative to the chemical preservatives.

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