COMBITORIAL STUDY OF BACTERIOCIN FROM *ACINETOBACTER VARIABILIS* WITH ESSENTIAL OILS AGAINST FOOD BORNE PATHOGENS

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ABSTRACT

Bacteriocins are a kind of ribosomal synthesized antimicrobial peptides produced by bacteria, which can kill or inhibit bacterial strains closely-related or non-related to the producing bacteria, but will not harm the bacteria themselves by specific immunity proteins. The application of combined preservative factors is very effective in controlling the growth of food spoilage and foodborne pathogenic bacteria. Antimicrobial activity of the bacteriocin extract from *Acinetobacter variabilis* and in combination with some natural organic essential oils (carvacrol, eugenol and thymol) on the growth of pathogenic bacteria *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhi* were investigated. All the organic compounds tested did not exhibit any antimicrobial activity against the microorganisms by Kirby-Bauer disc diffusion. Investigation of the interaction between the organic compounds and nisin against the test organisms revealed different patterns, varying from synergistic to antagonistic. Combinations of nisin with carvacrol, eugenol, or thymol resulted in synergistic action against the indicator organisms. The activity of bacteriocin with eugenol showed highest activity at lowed acidic pH against the indicator organisms wherein synergistic activity was not observed with carvacrol and thymol. This study highlights the potential of the combination of these organic compounds with bacteriocin to inhibit pathogen growth in food.

Key words: Antimicrobial activity, Bacteriocins, Essential oils, Kirby-Bauer disc diffusion, Synergistic action.

INTRODUCTION

Bacteriocins are a kind of ribosomal synthesized antimicrobial peptides produced by bacteria, which can kill or inhibit bacterial strains closely-related or non-related to the producing bacteria, but will not harm the bacteria themselves by specific immunity proteins. Bacteriocins become one of the weapons against microorganisms due to the specific characteristics of large diversity of structure and function, natural resource, and being stable to heat. Many recent studies have purified and identified bacteriocins for application in food technology, which aims to extend food preservation time, treat pathogen disease and cancer therapy, and maintain human health. Therefore, bacteriocins may become a potential drug candidate for replacing antibiotics in order to treat multiple drugs resistance pathogens in the future (Yang *et al*. 2014).

A high diversity of various bacteriocins is produced by many lactic acid bacteria (LAB) and is found in numerous fermented and non-fermented foods. Several bacteriocins from LAB extend potential applications in food preservation, thus help foods to be naturally preserved and richer in organoleptic and nutritional properties (Ramu *et al*. 2015). Though chemical preservatives for the preservation of food are successful to some extent, their quality is not as satisfying as fresh food. Hence, an alternative is required and bacteriocins serve the purpose. Nisin is currently the only bacteriocin widely used as a food preservative. Nisin is a ribosomally synthesized peptide that has broad-spectrum antibacterial activity, including activity against many bacteria that are food-spoilage pathogens. Nisin possesses anti-microbial activity against a wide range of Gram-positive bacteria, particularly those that produce spores. It inhibits certain strains of the food pathogen, such as *Clostridium botulinum, Staphylococcus aureus, Streptococcus haemolyticus, Listeria monocytogenes, Bacillus stearothermophilus, Bacillus subtilis* and some others.

Nisin remains the most commercially important bacteriocin, although other bacteriocins have been characterized and developed for possible approval and use. Numerous bacteriocins have been characterized chemically, biochemically, genetically and also at the molecular level to understand their basic mode of action.

Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of "natural" and "traditional" foods, processed without any addition of chemical preservatives, are becoming more attractive. Thus, because of recent consumer demand for higher quality and natural foods, as well as of strict government requirements to guarantee food safety, food producers have faced conflicting challenges (Franz *et al*., 2010). Chemical additives have generally been used to combat specific microorganisms. The application of bacteriocins as biopreservatives for vegetable food matrices started approximately 25 years ago. In these years, a lot of studies have focused on the inhibition of spoilage and/or human pathogens associated with vegetable foods and beverages by bacteriocins, and their application appeared as a good alternative to chemical compounds and antibiotics. When deliberately added or produced in situ, bacteriocins have been found to play a fundamental role in the control of pathogenic and undesirable flora, as well as in the establishment of beneficial bacterial populations (Collins *et al*., 2010).

There are many antibacterial substances produced by animals, plants, insects, and bacteria, such as hydrogen peroxide, fatty acids, organic acids, ethanol, antibiotics, and bacteriocins. Antimicrobial peptides (AMPs) or proteins produced by bacteria are categorized as bacteriocins. Scant nutrients in the environment trigger microbial production of a variety of bacteriocins for competition of space and resources. Bacteriocins are abundant, have large diversity, and the genes encode ribosomally synthesized antimicrobial peptides or proteins, which kill other related (narrow spectrum) or non-related (broad spectrum) microbiotas as one of the inherent defense system weapons of bacteria (Cotter *et al*., 2005). More than 99% of bacteria can produce at least one bacteriocin, most of which are not identified (Riley and Wertz, 2002). The killing ability of bacteriocins is considered a successful strategy for maintaining population and reducing the numbers of competitors to obtain more nutrients and living space in environments. Unlike most antibiotics, which are secondary metabolites, bacteriocins are ribosomally synthesized and sensitive to proteases while generally harmless to the human body and surrounding environment.

Sensitivity to pH

pH is known to exhibit a dramatic effect on the production of the bacteriocin. Bacteriocins differ greatly with respect to their sensitivity to different pH. Studies on the effect of a range of pH from 1-12 show that optimal activity is attained at pH 4 and 5 (Jack *et al*, 1995). Most bacteriocins produced by lactobacilli are considered to be more tolerant of acid than alkaline pH (Tagg *et al* 1976). Bacteriocin sensitivity to pH is because of the fact that in acidic medium protein molecule get degraded which result in zero activity of bacteriocin. Both bacteriocin production and degradation rates are higher at pH 5.0 and 5.5, resulting in sharper activity peaks than at pH 6.0 or 6.5. In food industries, bacteriocins produced in alkaline conditions are now gaining more attention because pH of many food products is between neutral to alkaline. In acidic pH such as at pH 3 bacteriocin shows poor activity, exhibiting no zone of inhibition at this pH. Bacteriocin is more stable and shows good antimicrobial activity at optimum pH 6.5 or 7.

Essential oils

Eugenol

Eugenol is a component of clove oil and other essential oils. Eugenol plays a prominent role in dental and oral hygiene preparations. Eugenol is used as flavor, irritant, sensitizer and can be used to produce local anesthetics. Eugenol-producing dental materials are used in clinical dentistry (Markowitz K *et al., 1992*). Direct application of eugenol to pulp tissue may result in extensive tissue damage Low concentrations of eugenol exert anti-inflammatory and local anesthetic effects on dental pulp. Eugenol and the essential oils have also been observed to possess membrane stabilizing properties on synaptosomes, erythrocytes and mast cells. Eugenol can cause significant suppression of lipid peroxidation and low density lipoprotein oxidation. Eugenol has some interesting properties. Its specific gravity is slightly more than 1.06 at room temperature, making it heavier than water (Sarvana Kumar Jaganathan *et al.*, 2010). **Carvacrol and thymol**

Carvacrol, or cymophenol, $C_6H_3CH_3(OH)(C_3H_7)$, is a monoterpenoid phenol. It has a characteristic pungent, warm odor of oregano. Carvacrol is present in the essential oil of *Origanum vulgare* (oregano), oil of thyme, oil obtained from pepperwort, and wild bergamot. The essential oil of Thyme subspecies contains between 5% and 75% of carvacrol, while Satureja (savory) subspecies have content between 1% and 45%. Origanum majorana (marjoram) and Dittany of Crete are rich in carvacrol, 50% and 60-80% respectively.

The interaction between EO compounds and bacteriocins can produce four possible types of effects: indifferent, additive, antagonistic and synergistic effects. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects while the absence of interaction is defined as indifference.

Nisin with essential oils

The principle behind the antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons.

Nisin Z and thymol (essential oil) have been tested, alone and in combination, for antibacterial activity against *Listeria monocytogenes* ATCC 7644 and *Bacillus subtilis* ATCC 33712. The antibacterial effect of nisin Z, produced by *Lactococcus lactis* KE3 isolated from the traditional Moroccan fermented milk, was greatly potentiated by sub-inhibitory concentrations of thymol in both bacterial strains (Ettayebi e*t al*, 2000*)*. Data shows that the concentration of nisin required for effective control of food-borne pathogenic bacteria could be considerably lowered by the use of thymol in combination. The use of low concentrations of nisin could lead to a less favourable condition for the occurrence of nisin-resistant bacteria sub-populations.

MATERIALS AND METHODS

Culturing of the bacterium for bacteriocin production:

- Bacteria was previously isolated and characterized from food sample.
- The bacteria was identified as *Acinetobacter variabilis* by 16s RNA sequencing.
- The bacteria were grown in optimized media incubated at 30 °C for 48hrs in shaking condition at 120 rpm for the bacteriocin production.
- The cultures were centrifuged at 10000 rpm for 5 min and the supernatant was filtered in Whatman No.1 filter paper and cell free supernatant was collected.

Combinatorial action of bacteriocin with essential oils at different pH range:

1.1. Bacteriocin sensitivity to different pH ranges

- The bacteriocin extract was subjected to check the resistance to change in pH from 3-9 by conducting the antagonistic assay.
- 1ml of extract and 1ml of each buffer of pH ranging from 3-9 were combined and kept for 4hrs. Residual activity was determined by Kirby-Bauer disc diffusion method.

1.2. Kirby-Bauer disc diffusion assay

- Indicator organisms were selected from the major food borne pathogens viz., *Escherichia coli, Listeria monocytogenes* and *Salmonella typhi*.
- 24 hrs fresh culture of indicator bacteria were prepared and the cell density was adjusted to A=1.0 at 625nm according to Mc Farland standard.
- A volume of 100 µl of inoculum of each indicator bacteria was swabbed on prepoured sterilized nutrient agar plates using sterilized cotton bud.
- The sterile discs of 5 mm in diameter were prepared using Whatmann no 1 filter paper and were placed on bacteria lawn swabbed in nutrient agar plates and 10 µl of culture supernatant was dispensed in each disc.
- The plates were incubated at 37°C for 24 hrs and the clear zones formed around the disc were measured in millimeter including the disc diameter.

1.3. Synergistic activity of bacteriocin with essential oils at different pH range.

- Essential oils –eugenol, carvacrol and thymol were separately subjected for antagonistic activity against the indicator organisms.
- The combination at different concentration ratios (1:1, 1:1.5, 1:2, 1:2.5, 1:3) of bacteriocin extract: oils and vice versa were taken at pH range of 3-7.
- The extract and oil were mixed vigorously and 50µl buffer of pH 3-7 were added separately and allowed to settle for 20minutes.
- The mixtures were then assayed for Kerby-Bauer disc diffusion method.

RESULTS AND DISCUSSION

Synergistic action of bacteriocin extract with essential oil

Eugenol oil

The antibacterial activity against *E.coli* was checked at constant oil concentration and varied extract concentration and the results are presented in **Table 1**. Antibacterial activity against *E.coli* was checked at constant extract concentration and varied oil concentration and the results are presented in **Table 2**.

The antibacterial activity against *L.monocytogenes* was checked at constant oil concentration and varied extract concentration and the results are presented in **Table 3**. Antibacterial activity against *L.monocytogenes* was checked at constant extract concentration and varied oil concentration and the results are presented in **Table 4**

The antibacterial activity against *S.typhi* was checked at constant extract concentration and varied oil concentration and the results are presented in **Table 5**. Antibacterial activity against *S.typhi* was checked at constant extract concentration and varied oil concentration and the results are presented in **Table 6**.

It is evident from the tables that an appropriate and higher concentration of extract and lower concentration of oil ensures a proper resistant against the change in pH. This also helps in absolute antagonistic activity against the pathogenic indicator organisms.

The effect of pH on the antagonistic activity of the supernatant revealed that the protein was affected at pH 3-6 and pH 8-9 and the optimum active pH was found to be 7.

Oil alone did not show any antagonistic activity but supernatant showed a zone of 14mm for *E.coli*, 11mm for *L.monocytogenes* and 13mm for *S.typhi*.

Carvacrol and Thymol oil

The extract was immiscible with Carvacrol and Thymol oil which resulted in the non-mixing of the extract and oil and we were unable to conduct the antagonistic activity.

CONCLUSION

The literature clearly indicates the combinatorial / synergistic action of nisin with natural organic compounds like Eugenol, Carvacrol, Thymol and Cinnamic acid at 1:1 ratio concentration against the indicator organisms like *Bacillus* and *Listeria* . Highest activity was observed with nisin combined with Eugenol and Cinnamic acid but lowest with Carvacrol and Thymol. (Khalil *et al*., 2000; Olasupo *et al*., 2003). The literature showed the increased activity of nisin when it is synergistically combined with organic compounds.

The antimicrobial activity against *M. luteus* of Nisin-citric acid complex and Nisin- AuNP complex (gold nano particle) at range of pH 3-8 revealed a very good activity of 60IU/ml. the activity was sustained for seven days of storage (Adhikari, *et al.*, 2012).

With these literatures as reference, the extracts which were sensitive to change in pH which was observed by decreased or no antagonistic activity against the indicator organisms experimented to combine the extract with the essential oil and observed at different range of acidic pH of 3-6 at different concentration range of oil and extract. The results, observed revealed that the oils bind to the extract and resists the effect of pH and increases the antagonistic activity against major food borne pathogens used. The experiments was successful only with Eugenol but not with Carvacrol and Thymol.

TABLES and FIGURES

Table 1.

pH	Zone of inhibition (mm)				
	1:1	1:1.5	1:2	1:2.5	1:3
3	6	6	6	**12.7**	12.0
4	6	7	5.5	**10**	8
5	7.5	7	6	**10**	10.5
6	6.7	9.7	9.5	**12.5**	10.5
7	8.5	8	13.5	**14.5**	14

Antagonistic activity of the eugenol with the synergistic action at different concentrations of extract against *E.coli*.

Table 2.

pH	Zone of inhibition (mm)				
	1:1	**1:1.5**	1:2	1:2.5	1:3
3	6	**6**	5	5	5
4	6	**6**	6	5	5
5	6.5	**6.7**	6.2	5.1	6.5
6	6.7	**10.3**	8.2	9.5	10
7	8.5	**10.7**	9.5	9.2	7.2

Antagonistic activity of the extract with the synergistic action with eugenol at different concentrations against *E.coli*.

pH	Zone of inhibition (mm)				
	1:1	1:1.5	1:2	**1:2.5**	1:3
3	6.2	5	5	**11**	11
4	13	5	5	**13.2**	11.7
5	11.7	7	8	**13.5**	12.7
6	8.2	12.8	10.2	**14**	11.7
7	8	11	10.5	**14.2**	11.7

Table 3. Antagonistic activity of the eugenol with the synergistic action at different concentrations of extract against *L. monocytogenes*.

pH	Zone of inhibition (mm)				
	1:1	**1:1.5**	1:2	1:2.5	1:3
3	6.2	**6.5**	6	5	5
4	13	**13**	5	5	5
5	11.7	**11**	11	10.5	11.2
6	8	**8.3**	6	8	7
7	8	**8.2**	7.5	6.2	5.2

Table 4. Antagonistic activity of the extract with the synergistic action with eugenol at different concentrations against *L. monocytogenes*.

pH	Zone of inhibition (mm)				
	1:1	1:1.5	**1:2**	1:2.5	1:3
3	5	5	**10**	10	10
4	5.1	5.1	**11**	11	10
5	6	8	**13**	9.5	10.5
6	5.5	8	**10.7**	10	10.5
7	6.3	10.5	**12**	10	11

Table 5. Antagonistic activity of the eugenol with the synergistic action at different concentrations of extract against *S. typhi*.

pH	Zone of inhibition (mm)				
	1:1	**1:1.5**	1:2	1:2.5	1:3
3	5	**5**	5	5	5
4	5.1	**5.5**	5	5	5
5	6	**9**	6.5	8.5	5.1
6	5.5	**8.5**	8	8.2	7.2
7	6.3	**6.5**	6.4	6	6.5

Table 6. Antagonistic activity of the extract with the synergistic action with eugenol at different concentrations against *S. typhi*.

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