BACTERIOCINS: A GREEN ANTIMICROBIAL PESTICIDE

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- This technology has been dully tested at an industrial level from the time it was developed in 2008. Also a US Patent Application was filed on March 23/2011.
- This technology is considered to participate for the Small Business Award.
- The EPA award focus area corresponds to the design of greener chemicals.
- Rights on the Patent are been assigned to VH BIOTECHNOLOGY, Inc. a US Company dully registered in the State of Texas.

ABSTRACT

Anti-microbial pesticides are used for a variety of applications, e.g. food production, cleaning of medical devices, animal husbandry, fuels, oil extraction, cooling water systems, process water systems and wastewater treatment. Those pesticides are directed against living organisms, but frequently not restricted to “target organisms”. This implies that they inevitably can also harm the health of non-target organisms such as humans or animals. Pesticides contain highly hazardous substances which are potentially harmful for humans, particularly for children and pregnant woman. Also, current investigations indicate that the improper or indiscriminate use of antimicrobial compounds has increased in concert with a growing frequency of antimicrobial resistance in bacteria, which drives to the development of even more toxic antimicrobials, yet the microbes adapt again. Consequently, there is a particular interest in technologies that substitute chemical plaguicides used as microbicides.

Trough evolution, bacteria have acquired the ability to produce a heterogeneous array of molecules with inhibitory properties against other microorganisms. One of those groups of molecules corresponds to bacteriocins. Bacteriocins can be defined as gene-encoded, ribosomally synthesized antimicrobial peptides often small in size (20 to 60 amino acid residues), cationic and amphiphilic molecules whose cytotoxic action is related with permeabilization of the target-cell membrane.

Bacteriocins are not toxic to eukaryote organism and are generally recognized as safe substances. Moreover, they are currently considered for therapeutic applications such as the development of new vaccines against pathogenic multidrug-resistant bacteria and as cytotoxic agents against cancerous cells in humans.

Recently a bacteriocin composition was developed to be used as a microbicide agent in various commercial applications. The bacteriocins are obtained by standard fermentation methods with lactic acid bacteria. The novel green antimicrobial agent has been used in pulp and paper mills, in fuels, in biofuels, in cooling water systems and in poultry farms. Relevant data on those applications showing the efficacy of bacteriocins as an antimicrobial agent in industrial applications and animal husbandry is provided.

THE PROBLEM

Pesticides are used worldwide to protect crops and structures, manage pests, and prevent the spread of disease. They can be categorized with basis on target organisms, e.g. as acaricides, algicides, bactericides, fungicides, herbicides, insecticides, molluscicides, nematicides, piscicides, slimicides and termiticides.

Pesticides are intended to be toxic, but only to the target organisms to be controlled. However, due to their intrinsic properties and while beneficial to society, these pesticides can pose human health and environmental risks. The risk from a pesticide is determined from its hazardous properties and the likely exposure of humans and the environment throughout its life cycle.

A growing body of scientific evidence demonstrates links between exposures to hazardous pesticides and potentially serious adverse impacts on human health and the environment. Currently licensed pesticides include known and suspected carcinogens, endocrine disruptors, allergens, neurotoxins and reproductive toxicants. Infants, children, pregnant women and the developing foetus are particularly vulnerable to pesticides.

Indirect pesticide toxicity is related to persistence in the environment through the mechanisms of bioconcentration – a tendency for a compound to accumulate in an organism, e.g. preferentially in fat tissues for lipophilic organochlorines; and biomagnification – an increase in concentration up the food chain.

In the industrial sector, microorganisms may interfere with manufacturing processes by causing rot or slimes to form, blocking valves and pipes, reducing heat transfer efficiency at cooling systems or corroding steel, plastic and rubber, thus microbial pesticides are used to control undesired microbial growth. However, current investigations indicate that the use of antimicrobial compounds has increased in concert with a growing frequency of antimicrobial resistance in bacteria. Microbial resistance, due to improper or over use of antimicrobial biocides drives the development of even more toxic antimicrobials, yet the microbes adapt.

Until now it seems that the only commercial bacteriocin is nisin and its applicability for industrial purposes is almost inexistent. Currently, there are no applications nor any patent concerning the use of bacteriocins as natural commercial biocides for elimination and/or prevention of microbial growth and/or microbial biofilm of industrial process-water systems.

This document describes application cases showing that bacteriocins - produced by standard fermentation methods using lactic acid bacteria strains - are an efficient green substitute for chemically synthetized microbicides applied for example in cooling water systems, pulp and paper mills, metal working fluids, in fuels, and in fracture and secondary recovery fluids at oil extraction operations. As a replacement for ‘chemical’ biocides - between several possibilities - the bacteriocins may be used in poultry farms in order to overcome problems associated with sanitation of animal drinking water with hypochlorite.

GENERAL DESCRIPTION OF BACTERIOCINS

Through evolution, bacteria have acquired the ability to produce a heterogeneous array of molecules with inhibitory properties against other microorganisms. These molecules include the followings (Jack et al. 1995): (1) toxins (e.g., diphtheria, tetanus, and cholera toxins); (2) bacteriolytic enzymes such as lysozyme and phospholipase A; (3) bacteriophages and defective bacteriophages; (4) by-products of primary metabolic pathways such as organic acids, ammonia, and hydrogen peroxide, and various other secondary metabolites (idiolytes) produced by bacteria that have demonstrable antibacterial activity; (5) antibiotic substances like gramicidin, valinomycin, and bacitracin that are synthesized by multienzyme complexes; and (6) bacteriocins.

Bacteriocins are defined as gene-encoded, ribosomally synthesized proteins, which have inhibitory properties against some bacteria but are not active against the cells which produce them. They vary greatly in size and sequence homology but tend to be small cationic peptides of between 20 and 60 amino acids with amphipathic characteristics and high isoelectric points (Kaiser and Montville, 1996).

In general, they tend to be active against a wide range of gram-positive bacteria. However, many of the bacteriocins produced by gram-positive bacteria kill species other than those that are likely to have the same ecological niche. In fact, some bacteriocins have been reported to inhibit gram-negative species.

Bacteriocins are classified largely based on their molecular weight differences. The major classes of bacteriocins include: (I) lantibiotics, (II) small heat stable peptides, (III) large heat labile proteins, and (IV) complex proteins whose activity requires the association of carbohydrate or lipid moieties (Abee et al. 1995).

The antimicrobial activity of bacteriocins is based on a transmembrane pore-forming mechanism in which the bacteriocin combines with negatively charged surface constituents of target bacteria, thus disrupting important cell function and resulting in an efflux of intracellular compounds, ion leakage, energy depletion, loss of proton motive force, and, ultimately, cell death (Brodgen 2005; Montville and Bruno 1994).

Bacteriocins are attracted to bacterial surfaces by an electrostatic bonding mechanism with structures on the bacterial surface. Bacteriocins being in general cationic (i.e., they contain an excess of lysyl and arginyl residues), are attracted to the net negative charges that exist on the outer envelope of Gram-negative bacteria — for example, anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) — and to the teichoic acids on the surface of Gram-positive bacteria.

Once close to the bacterial surface, bacteriocins must traverse capsular polysaccharides before they can interact with the outer membrane, which contains LPS in Gram-negative bacteria, and traverse capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in Gram-positive bacteria. Membrane permeability can be described as a three step process: (I) initially the bacteriocin is bound parallel to the cell surface, (II) the bacteriocin peptide begin to orientate perpendicular to the membrane, and (III) the bacteriocion molecule is orientated perpendicularly and inserts into the membrane. Thus the membrane pore is essentially a protein channel. Mainly two models for pore-formation have been proposed for bacteriocins: the 'carpet-like' model and the 'barrel-stave" model. For example, in the 'barrel-stave model', peptide helices form a bundle in the membrane with a central lumen, much like a barrel composed of helical peptides as the staves (Moll et al. 1999; Kaiser and Montville 1996).

Combinations of bacteriocins have also been tested in order to increase their antimicrobial activities. The simultaneous use of two or more bacteriocins is useful not only to lower the added bacteriocin doses, but also to avoid regrowth of bacteriocin-resistant/adapted cells (Mulet-Powell et al. 1998).

BACTERIOCINS FROM LACTIC ACID BACTERIA

Bacteriocins produced by lactic acid bacteria (LAB) are of particular interest since LAB possesses the status of GRAS (Generally Recognized as Safe). LAB are regarded as safe microorganisms for human consumption because they and their metabolites have been consumed in fermented foods for innumerable generations without adverse effects in the population. In fact from the earliest days after birth, LAB constitute the natural microbiota of the human digestive tract (Ruiz-Larrea et al. 2009). Furthermore, some LAB metabolites are considered anti-inflammatory, immunomodulatory and anti-cancer agents with strong anti-proliferative effects on tumor cells in the colon (Licciardi et al. 2010).

Baceriocins from LAB offer various appropriate characteristics for general use: (i) are generally recognized as safe substances, (ii) are not active and nontoxic on eukaryotic cells, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Galves et al. 2007).

BACTERIOCIN TOXICITY

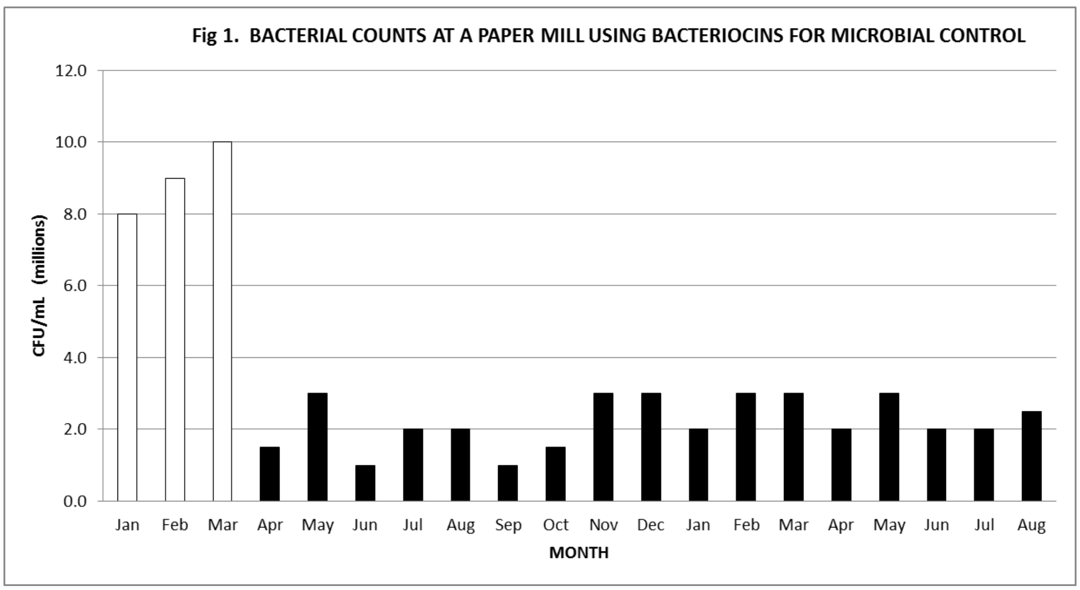
Bacteriocins are not toxic to eukaryote organism. Moreover, they are currently considered for therapeutic applications in humans: Carroll and O' Mahoney (2011) and Millette et al. (2008) consider the use of bacteriocins for the development of new vaccines against pathogenic multidrug-resistant bacterial species. Patent WO 2006053445 describes the use of bacteriocins as a cytotoxic agent against cancerous cells in humans administered by a way that can be intravenous, oral, transdermal, subcutaneous, mucosal, intramuscular, intranasal, intrapulmonary, parenteral, intrarectal, intratumoral and topical.

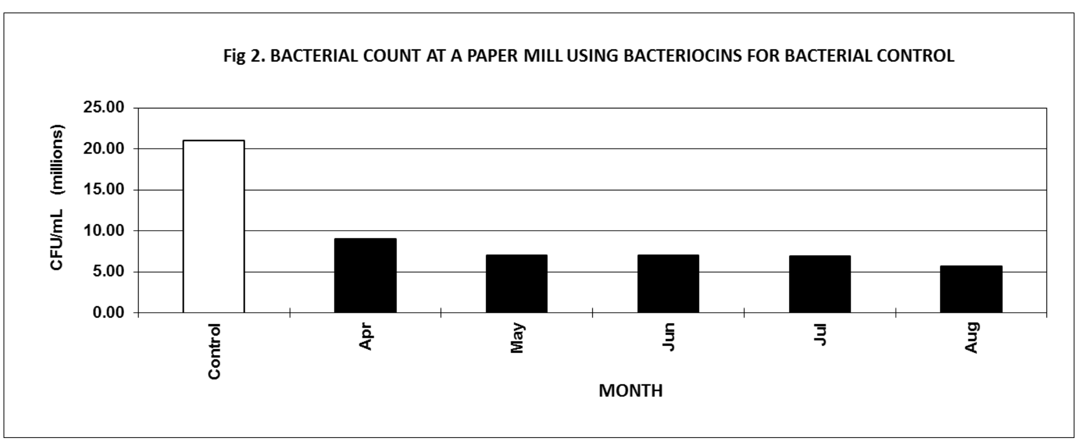
EXAMPLES OF BACTERIOCINS APPLICATION AS A BIOCIDE

EXAMPLE 1. USE OF BACTERIOCINS FOR MICROBIAL CONTROL IN PULP AND PAPER MILLS

The tendency in pulp and paper mills is to close the circulation of process waters, to operate closer to neutral pH and to increase use of recycled fiber. Those trends favor the growth of microorganisms. Excessive microbial contamination in pulp and paper mills lead to frequent cleaning, increases maintenance costs, lower productivity due to breaks during production and lower quality of the paper due to holes, spots and bad smell.

Following data was taken in two paper mills using bacteriocins for microbial control. Figure No. 1 shows the average monthly values of Colony Forming Units (CFU) on samples taken at the paper machine wire pit in a mill producing tissue grades. Figure No. 2 shows average monthly CFU counts at the head box in a paper mill producing cardboard. In both cases, white bars indicate control values i.e. when the mill was not using bacteriocins.





EXAMPLE 2 BACTERIOCINS FOR MICROBIAL CONTROL IN FUELS

Uncontrolled microbial contamination in fuels and fuel systems remains a largely unrecognized but costly problem at all stages of the petroleum industry from crude oil production through fleet operations and consumer use. Biofuels are also affected in higher proportions by microbial contamination.

Two samples of standard commercial fuel (gasoline and diesel) were treated with bacteriocins in order to reduce microbial contamination. Table No. 1 shows the test results

Table No. 1 MICROBIAL CONTROL OF FUEL SAMPLES WITH BACTERIOCINS			
Sample	Control	Treated	% Reduction
Diesel Fuel	480,000 CFU/g	260 CFU/g	99.95%
Gasoline Fuel	800 CFU/g	90 CFU/g	89 %

EXAMPLE 3 BACTERIOCINS VERSUS HIGH-TEST HYPOCHLORITE

High-test hypochlorite (HTH) is the preferred method to sanitize the drinking water at poultry farms. However, chlorine is unable to remove bacterial bio-films, it increases the pH of the water; but also it will be inactivated by organic matter (as all halogens do). Furthermore, overdose of chlorine affects negatively body weight gain of birds and eggshell quality. The bacteriocin composition was tested in a poultry farm in order to replace HTH dosage. Two tanks with drinking water were used for this test. One was treated with standard HTH and the other one with the bacteriocin composition. Table 2 shows the lab report for untreated and treated samples. Results show that bacteriocins are as effective as HTH to sanitize drinking water at poultry farms.

Table No. 2 SANITIZATION OF WATER WITH BACTERIOCINS IN A POULTRY FARM		
	SAMPLE 1	**SAMPLE 2**
SAMPLE TREATMENT	With Bacteriocins	With HTH
ORIGIN OF SAMPLE	Raw drinking water	Raw drinking water
DOSAGE	100 gr/M3 water	12 gr chlorine/ M3 water
REACTION (retention) TIME	14 hours	14 hours
PRE-TREATMENT CONDITIONS		
Mesophilic Microorganisms Count	34 CFU/gr	40 CFU/gr
Coliform Bacteria Count	> 3 Microorganisms /100 cm3	> 2 Microorganisms/100 cm3
POST-TREATMENT CONDITIONS		
Mesophilic Microorganisms Count	0 CFU/gr	0 CFU/gr
Coliform Bacteria Count	0 Microorganisms /100 cm3	0 Microorganisms/100 cm3

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