BioProtect™ Technical Summary

DISCLAIMER:

This report has been compiled as a technical summary and presented for educational purposes only and is not a marketing document pertaining to the "BioProtect™" range of products. Global BioProtect makes no claims that the product discussed throughout this report is a "microbicide" or a "pesticide" due to U.S. EPA regulations against making such claims.

The report contains abstracts and extracts from scientific papers, third party test data, field trials data, observations and hypothesis of the writer.

The source documents have not been listed, a lot of the information contained has come from Wikipedia, which itself has sourced the information from other scientific papers. There is a lot of information available about surfactin but information about Fengycin and Iturin is scarce, except to say that they are lipid peptides, display similar characteristics to surfactin and that there are synergies between the three lipidpeptides.

BioProtect™

Global BioProtect produces a microbial based product that is a combination of the bacterium species Bacillus subtilis and the Lipidpeptides that it produces. The product has a multiple of uses including: odor control, insect control, microbial control, fungal control and the breakdown of organic material across multiple business sectors ranging from wastewater, waste management, and service industries to agriculture.

The product consists of three key lipidpeptides: Surfactin, Iturin and Fengycin, with surfactin being the most powerful and prolific, bacteria in spore plus a naturally derived surfactant. This combination of components is 99.7% organically derived and displays many unique properties in an easy to use liquid form.

In laymen's terms, all three lipidpeptides display antimicrobial and antifungal properties, are super emulsifiers, are very powerful bio detergents and when combined with a very small percentage of Sodium dodecyl benzenesulfonates, (a common surfactant used predominately in laundry detergent), synergize to create a unique product, add to this, Bacillus subtilis in spore and you have a product with unlimited potential.

Mode of Action

It can be hypothesized that when BioProtect is applied to an environment the lipidpeptides "alter the environment" by "knocking down" or "suppressing" other microorganisms, many of which cause odor. The lipidpeptides disrupt the cell membrane of other microorganisms and organic material causing the cells to "break open" allowing the Bacillus subtilis to come out of spore and break down or consume organic material rapidly.

Most, if not all microbial based products on the market rely on bacteria spores and/or enzymes to break down organic material and the consensus is that the higher the spore count, the better the product works and this may be the case in "traditional" microbial products, but not with BioProtect.

The spore counts in competing products are upwards 100 million cfu/mL where BioProtect is around 2 million cfu/mL, however as discussed the lipidpeptides in BioProtect "knock down" other microorganisms leaving the Bacillus subtilis unopposed to multiply and consume organic material without the need to compete with other organisms, thus the 2 million cfu/mLl is more than sufficient to outperform other microbial products on the market. A recent upgrade of the manufacturing process has enabled consistency in spore numbers.

Surfactin

Surfactin is a very powerful surfactant commonly used as an antibiotic. It is a bacterial cyclic lipidpeptide, largely prominent for its exceptional surfactant power. Its amphiphilic properties help this substance to survive in both hydrophilic and hydrophobic environments. It is an

antibiotic produced by the Gram-positive endospore-forming bacteria Bacillus subtilis. In the course of various studies of its properties, surfactin was found to exhibit effective characteristics like antibacterial, antiviral, antifungal, anti-mycoplasma hemolytic activities.

Surface Tension

Surfactin, like other surfactants, affects the surface tension of liquids in which it is dissolved. It can lower the water's surface tension from 72 mN/m to 27 mN/m at a concentration as low as 20 ppm. Surfactin accomplishes this effect as it occupies the intermolecular space between water molecules, decreasing the attractive forces between adjacent water molecules, mainly hydrogen bonds, creating a more fluid solution that can go into tighter regions of space increasing water's wetting ability. Overall, this property is significant not only for surfactin but for surfactants as they are primarily used as detergents and soaps.

Molecular Mechanisms

There are several prevailing hypotheses for how surfactin works. These are described below.

Cation-Carrier Effect

The cation-carrier effect is characterized by surfactin's ability to drive monovalent and divalent cations an organic barrier. The two acidic residues aspartate and glutamate form a "claw" of sorts which easily stabilizes divalent cations. Calcium ions make for the best-fitting cations stabilizing the surfactin conformation and functioning as an assembly template for the formation of micelles. When surfactin penetrates the outer sheet, its fatty acid chain interacts with the acyl chains of the phospholipids, with its head group in proximity to the phospholipids polar heads. Attachment of a cation causes the complex to cross the bilipidic layer undergoing a flip-flop. The head group aligns itself with the phospholipids of the inner sheet and the fatty acid chain interacts with the phospholipids acyl chains. The cation is then delivered into the intracellular medium.

Pore-Forming Effect

The pore-forming (ion channel) effect is characterized by the formation of cationic channels. It would require surfactin to self-associate inside the membrane, since it cannot span across the cellular membrane. Supramolecular-like structures by successive self-association could then form a channel. This hypothesis for the most part applies only to uncharged membranes where there is a minimal energy barrier between outer and inner membrane leaflets.

Foaming Properties

Foaming properties of surfactin were investigated and compared to those of sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA). Foams were formed by a bubbling technique. Evolution of the foam volume and the liquid in the foam was monitored with optical and conductimetric methods to characterize foam formation and stability. Excellent foaming properties of surfactin were shown by its higher ability to form and stabilize the foam at a concentration as low as 0.05 ppm, in comparison with SDS and BSA. Surfactin produced a foam with intermediate maximum density and stabilized the liquid in foam, as well as BSA.

Surfactin is a lipopeptide produced by various strains of Bacillus subtilis and is a very powerful surfactant. Here we present the first report on the interfacial behavior of surfactin. The adsorption of surfactin at the interface of diluted solutions ($5 \times 10^{-8}$to 5×10^{-7}M) is around 3×10^{18} molecule m^{-2}, a value indicating that surfactin molecules are in a very packed situation. Surfactin spreads readily at the (air/water) interface: the equilibrium spreading pressure $\pi e \approx 30$ mN m^{-1} reaches 45 mN m^{-1} when electrolytes (KCl or $CaCl_2$) are dissolved in the alkaline subphase. We have plotted the compression isotherm curves and determined surface parameters. These parameters vary only a little with temperature but are very affected by the pH of the subphase. Plotting the transition pressure value π_t as a function of pH results in a titration curve from which one can deduce the pK value of surfactin at the interface. This value, $pK_s \approx 6$ is around 2 pH units higher than the pK of surfactin in solution. The addition of electrolytes (I = 0.15 M) in the alkaline subphase leads to the neutralization of the surfactin monolayer (protonation of the acidic residues L Glu[1] and L Asp[5] of the peptide cycle). This neutralization is complete in the case of Ca^{++} ions but not in the case of monovalent cations (Na^+ or K^+). When surfactin monolayers are subjected to successive compression—expansion cycles we observe a reproducible hysteresis loop. The surface parameters of surfactin are compared to those of iturins, lipopeptides also extracted from B. subtilis, the structure and properties of which are very similar.

Detergent Effect

The detergent effect draws on surfactin's ability to insert its fatty acid chain into the bilipidic layer causing disorganization leading to membrane permeability. Insertion of several surfactin molecules into the membrane can lead to the formation of mixed micelles by self-association and bilayer influenced by fatty chain hydrophobicity ultimately leading to bilayer solubilization.

Biological Properties

Surfactin has a nonspecific mode of action, which results in both advantages and disadvantages. It's advantageous in the sense that surfactin can act on many kinds of cell membranes, both Gram-positive and Gram-negative. Its non-specificity also has bearing on its tendency to ***not produce resistant strains of bacteria***. Consequently, the negative is that this efficient mode of cell destruction is indiscriminate, and attacks red blood cells with deadly efficiency.

Antibacterial Properties

Surfactin, true to its antibiotic nature, has a very significant antibacterial property, as it can penetrate the cell membranes of all types of bacteria. There are two main types of bacteria and they are Gram-negative and Gram-positive. The two bacteria types differ in the composition of their membrane. The Gram-negative bacteria have an outer lipopolysaccharide membrane and a thin peptidoglycan layer followed by a phospholipids bilayer, whereas the Gram-positive bacteria lack the outer membrane and carry a thicker peptidoglycan layer as well as a phospholipids bilayer. This is an essential factor that contributes to surfactin's detergent-like activity as it can create a permeable environment for the lipid bilayer and causes disruption that solubilizes the membrane.

For surfactin to carry out its antibacterial property successfully, the bacterium needs to be treated with a high concentration. In fact, surfactin needs to be in concentrations between 12–50 ppm for it to carry out minimal antibacterial effects. This is also known as the minimum inhibitory concentration (MIC).

Antifungal Properties

The aim of the study was to evaluate antifungal and antibacterial properties of surfactin isolated from Bacillus subtilis growing on molasses. Molasses replaced the traditional microbiological media to culture the B. subtilis. 10 phytopathogens and 30 E. coli strains were used in the study. The results demonstrated the ability of surfactin produced by Bacillus sp. growing on molasses to inhibit mycelial growth of the 4 fungi from 10 tested and all E. coli strains measured by agar plate inhibition assays. Fungi inhibited to the greatest degree as measured by the inhibition zones were Botrytis cinerea A 258 (~50% of inhibition), Sclerotinia sclerotiorum K 2291 (~50% of inhibition), Colletotrichum gloeosporioides A 259 (~40% of inhibition), Phoma complanata A 233 (~38% of inhibition), Phoma exigua var. exigua A 175 (~20% of inhibition). Among the E. coli, high inhibition growth was noted in 76% of the isolates. Application of natural products such as bio surfactant may be a new approach to biological control therefore reducing the need for synthetic chemical compounds.

Surface-active properties including dynamic adsorption, monolayer stability, micelle forming capacity, and foaming aptitudes of surfactin-C_{15}/iturin A-C_{15} mixtures were studied. Surfactin-$C_{15}$and iturin A-C_{15} molecules interact in synergism on the most surface-active properties evaluated at 20 °C at the air–water interface and in aqueous solution (pH 8.0). The synergism is positive on the adsorption effect, monolayer stability, foam density, and liquid stability in foam.

Fengycin:

Fengycin is a biologically active lipopeptide produced by several Bacillus subtilis strains. The structure is composed of a β-hydroxy fatty acid linked to a peptide part comprising 10 amino acids, where 8 of them are organized in a cyclic structure. This lipopeptide is known to develop antifungal activity against filamentous fungi and to have hemolytic activity 40-fold lower than that of surfactin another lipopeptide produced by B. subtilis. Like most the natural antimicrobial peptides, fengycin likely acts by making the plasma membrane of the target cell more permeable. The molecular mechanism underlying this membrane perturbation is not yet fully understood. Due to the complexity of biological systems, the study of interactions between antimicrobial peptides and living cells provides mainly global information about this phenomenon. It is therefore necessary to investigate the interaction of bioactive peptides with different types of model membranes, such as lipid mono- or bilayers, to obtain more precise information about the mechanisms involved.

Only a few studies have been devoted to the characterization of lipid-fengycin interactions. Recently, a monolayer study has demonstrated a concentration-dependent perturbing effect of fengycin on the structural and morphological characteristics of DPPC monolayers. Another study has shown that fengycin organization within a ceramide monolayer is strongly dependent on the environmental conditions (pH, temperature) and fengycin concentration. The major aim of this work is, therefore, to reveal fengycin's mechanism of membrane perturbation via complementary biophysical approaches:

1. The Langmuir trough technique in combination with Brewster angle microscopy is used to obtain information about the penetration properties of fengycin into a lipid monolayer, thus allowing us to directly visualize changes in the monolayer morphology in situ.
2. Ellipsometry is used to investigate the adsorption of fengycin onto supported lipid bilayers. This technique provides time-resolved measurements of changes in the thicknesses and refractive indexes of adsorbed layers and thereby information on the amount on the surface together with structural information.
3. Differential scanning calorimetry (DSC) is used to measure the thermotropic properties of lipid bilayer in the presence of fengycin and therefore indirectly provides insight into the organization of molecules within the bilayer.
4. Finally, the cryogenic transmission electron microscopy (cryo-TEM) provides information on the structural organization of lipid/surfactant systems. The use of cryo-TEM offers unique possibilities for direct observation of microstructures in terms of both internal structure and morphology. The specimen is prepared with flash freezing in such a way that artifacts due to conventional drying and staining procedures are avoided. This ensures that the structures are maintained in their original state.

Membrane models used in this work differ in their complexity (monolayers, supported bilayers, or vesicles) as well as in their nature (dipalmitoylphosphatidylcholine (DPPC) and dioleylphosphatidylcholine (DOPC)) and physical state (fluid or rigid state of the acyl chains). The simplest membrane model is a monomolecular layer of phospholipid spread at the air/water interface. Although monolayers do not reflect the complexity of biological membrane structure, many studies have demonstrated that the monolayer technique is powerful in membrane insertion analysis. This system can be considered half of the membrane bilayer. It

offers the possibility to simulate what happens when an active molecule, soluble in the extracellular medium, interacts with the membrane surface of target cells. However, the absence of a second membrane leaflet means the reduction of van der Waals interactions, which may be important in natural membranes.

Flat bilayers are useful membrane models due to their physical properties and simple geometry. In this study, a supported model membrane is formed via the adsorption of a phospholipid-surfactant mixture onto a silica-water interface, as described. This method has been shown to be a flexible and reproducible route to build membrane models with controlled composition. The most complex membrane models used in this study are large unilamellar vesicles (LUV)—bilayer vesicles whose dimension and curvature are of the same order of magnitude as that in natural membranes. The combination of the different lipid membrane models and biophysical techniques provides a comprehensive and detailed analysis of the mechanism of membrane perturbation by fengycin. The results are discussed in relation to the antimicrobial properties of fengycin.

Synergy Surfactin/Fengycin

The surface-active properties of two lipopeptide classes from Bacillus subtilis S499 (surfactins and fengycins A) were examined at liquid/liquid interfaces and in model emulsions. The study of structure-function relationships was carried out. For the surfactins, the interfacial organization and conformation were also investigated. The dynamic adsorption properties of lipopeptides at water/apolar solvent interface are close to those of low molecular weight surfactants. Their monomolecular films formed at the water/n-dodecan interface have a dilatational elasticity higher than the classical surfactants and close to the flexible proteins. However, at the macroscopic level, the emulsifying activity and the short-term stabilizing properties of lipopeptides are not more effective than those of the sodium dodecylsulfate. The behavior of lipopeptides at liquid/liquid interface is mainly related to the nature of the peptide ring. A more hydrophobic character (like surfactins) is efficient to reduce the meso-equilibrium interfacial tension, to form smaller droplets and to better stabilize the emulsions in the short term. The lipid chain structure influences slightly the adsorption properties. Lipopeptides with a shorter chain adsorb faster at the interface but those with a longer chain reduce the meso-equilibrium interfacial tension to a lower value. At the macroscopic level, the influence of the lipid chain is not pronounced. These properties are strongly influenced by the NaCl presence. The AFM, the XPS, the ATR-IR, the film balance and the molecular modelization techniques were used to examine the conformation of the surfactins at interfaces. Most of them show that for low compression conditions the peptide ring of the surfactin is lying at the interface and that the lipid chain has a disorganized structure. Per the molecular modelization, the ring forms a bowl structure with the two acidic residues protruded in the hydrophilic medium. In the models, the most energetic stable conformation presents a folding of the lipid chain on the peptide ring. The ATR-IR technique shows the existence of a beta-turn in the peptide ring. In the models, the orientation of the three homologous molecules at the interface is similar. However, per the ATR-IR spectra, the secondary structure of the peptide ring is different between the C13 and the C14 and C15 homologous.

Iturin

Iturin exhibits strong antifungal activity against pathogenic yeast and fungi. It interacts with the cytoplasmic membrane of the target cell forming ion conducting pores. Its mode of action could be attributed to its interaction with sterols and phospholipids.

Synergy Surfactin/Iturin

Surface-active properties including dynamic adsorption, monolayer stability, micelle forming capacity, and foaming aptitudes of surfactin-C_{15}/iturin A-C_{15} mixtures were studied. Surfactin-$C_{15}$and iturin A-C_{15} molecules interact in synergism on the most surface-active properties evaluated at 20 °C at the air–water interface and in aqueous solution (pH 8.0). The synergism is positive on the adsorption effect, monolayer stability, foam density, and liquid stability in foam.

Sodium DDBSA

Sodium dodecyl benzenesulfonates are organic compounds with the formula $C_{12}H_{25}C_6H_4SO_3Na$. They are colorless salts with useful properties as surfactants. They are usually produced as a mixture of related sulfonates. They are major components of laundry detergent as well as displaying some antimicrobial characteristic.

Synergy Surfactin/DDBSA

Microbial testing has shown that a synergy exists between Surfactin and DDBSA, whereas both components were tested individually for log reduction against Escherichia Coli, Salmonella Enterila, Listeria Monocytogenes and Campylobacter Jejuni. Both showed reduction results, however when combined the reduction were markedly different. Similar results are displayed in the cleaning and surfactant qualities of the components when combined.

Test and Trial Data

Toxicity

Global BioProtect carried out acute toxicity testing to demonstrate the safety of the product to both humans and animals and a set of environmental toxicity testing, with the following results:

Note: *Category 1 is the most toxic, category 5 the least toxic and unclassified, the toxicity is so low that it can't be classified*

- OEDC 402 Acute Dermal Toxicity Study – ***Category 5***
- OEDC 403 Acute Inhalation Toxicity Study – ***Unclassified***
- OEDC 404 Acute Irritation/Corrosion Study – ***Unclassified, "non-irritant"***
- OEDC 405 Acute Eye Irritation/Corrosion Study – ***Unclassified "not irritating to eyes"***
- OEDC 425 Acute Oral Toxicity Study – ***Category 5***
- OEDC 406 Skin Sensitization Study – ***Weak sensitizer***

- OEDC 201 Fresh Water Algal Growth Inhibition Test with *Desmodesmus Subspicatus* – ***No significant effects***
- OEDC 202 Acute Toxicity to *Daphnia Magna* - ***No significant effects***
- OEDC 203 96-Hour Acute Toxicity to *Danio Rerio* - ***No mortality observed.***

Third Party Laboratory Testing:

BioProtect has been tested against some of the most common pathogens including *Escherichia Coli, Salmonella Enterila, Listeria Monocytogenes* and *Campylobacter Jejuni*.

Benchmarks Environmental Laboratories Inc.

Columbus, Ohio, USA

- *Listeria Monocytogenes* - Reduction **5,700 cfu/mL > 0 cfu/mL**
- *Campylobacter Jejuni* – Reduction **3,700 cfu/mL >10 cfu/mL**
- *Escherichia coli* – Reduction **11,000 cfu/mL > 28 cfu/mL**
- *Salmonella Enterila* – Reduction **10,000 cfu/mL > 14 cfu/mL**

Snell Scientifics LLC.

Meansville, Georgia, USA

This test was conducted to demonstrates reduction in fruit flies and fruit fly larvae when BioProtect was sprayed directly onto the insect and prolonged activity in stopping the insects from returning. The test showed a 98% reduction when sprayed twice and no regrowth from larvae.

B. Michaels Group Inc.

Palatka, Florida, USA

Comparison testing was conducted comparing BioProtect against 12 leading drain cleaner product available and the results are as follows:

- Plug test penetration against organic material – BioProtect 2nd quickest, Liquid Plumber was the only product to perform faster.
- Biodegradation of organic matter in a drain over a 6-hour period, BioProtect 95% in 6 hours, the best performing of all comparison products.

- Corrosive activity testing, BioProtect and two others were shown to be the least corrosive and on par with each other, whilst products such as Clorox bleach and Liquid Plumber were the most corrosive.
- Overall, BioProtect outperformed the completion as the best overall drain cleaner for organic material.

Field Trials:

Brisbane, Australia

The grease interceptor at a regional shopping center was treated with BioProtect to reduce the build-up of suspended solids within the system.

The average monthly pump out costs (calculated on solid loads in the interceptor) prior to the application of BioProtect were $935. These costs reduced to $320 the first month and $76 in the second month, showing an overall reduction in pump out costs of 92%.

Los Angeles, California, USA

A large seafood processing plant was being charged an annual levy of approximately $80,000 to discharge to the Municipal Waste Water System.

A trial was conducted adding BioProtect into all the drains in the plant over a 7-day period and discharge water samples were taken and tested by an independent laboratory with the following results.

  - COD @ 903 mg/L down from baseline of 1848 mg/L
  - TSS @ 153 mg/L down from baseline of 463 mg/L
- This represents approximately a 45% reduction of COD levels and a 67% reduction in TSS levels against the baseline data.
- This reading would eliminate the annual Wastewater Levy.

Auckland, NZ

The Hydrogen Sulfide levels in a sewerage pump station wet well were monitored for 12 consecutive days with an average reading of 4 ppm of H2S recorded, BioProtect was then applied to the walls of the pump station wet well for the next 6 consecutive days, the H2S levels were undetectable.

Columbus, Ohio, USA

Field testing over 35 days where the "litter" in two control sheds were treated with a competitor's product, and two sheds were treated with BioProtect. Results in the BioProtect treated sheds showed:

- An average increase in bird weight of 5%
- An average reduction in morbidity rates of 0.5%
- Reduced ammonia levels < 25 ppm, reducing need for extraction fans (energy savings)
- Reduced environmental odor and flies
- Reduction in foot pad and breast burns on the birds

Note: All test and trial data available upon request.

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