Fungicides from the soil

Bacillus micro-organisms living in the very soil where crops are grown were put to the test and proofed to produce ingenious weapons for the effective control of diseases and mould on fruit.

THE PERISHABLE PRODUCE industry experiences considerable post-harvest crop losses every year. It is estimated that between 25% and 40% of the fruit and vegetables harvested worldwide are lost to post-harvest diseases. Much of this is due to decay caused by microorganisms (fungal, bacterial and viral phytopathogens) that produce diseases that occur during the transport, storage and sale of fresh produce.

Synthetic chemical fungicides that control post-harvest disease are increasingly associated with adverse environmental impacts and health issues, and are therefore falling out of favour due to the chemical residues they leave behind in the food chain.

In addition to some of the most effective fungicides having been deregistered, certain pathogenic strains have developed resistance to the fungicides. Internationally, consumers are demanding that natural alternative control methods be found.

The search for innovative and acceptable controls has led the agricultural sector to green chemistry as a way to manage and eliminate fungal and bacterial post-harvest diseases.

Unlocking potential

In a previous Hortgro Science and PHI Programme-funded research project, Prof. Kim Clarke, from the Department of Process Engineering at Stellenbosch University, identified the potential value of the metabolic bioproducts of *Bacillus amyloliquefaciens* to be a green fungicide against *Botrytis cinerea* in table grapes. She found that her chosen biocandidate acts as a natural microscopic factory of lipopeptides that demonstrate the ability to act as fungicides across a broad spectrum. She furthermore established that the bioproducts, even in crude, unpurified

1 Prof. Kim Clarke, project leader.
2 To upscale the production process from shake flask (500ml) to the level of a bioreactor with a capacity of two litres, is a necessary task.
3 There is a clear absence of fungal growth around wells (1 to 3) containing anti-fungal lipopeptides (ie. iturin and fengycin) in crude form. Fungi grew around, towards and over the control well ("C") containing water.
4 Dr Vivek Rangarajan, post-doctoral researcher.

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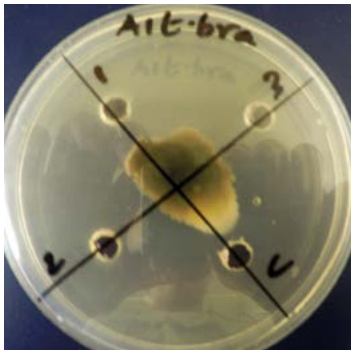
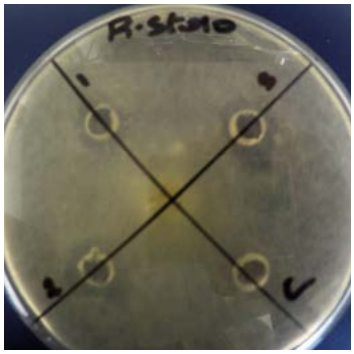
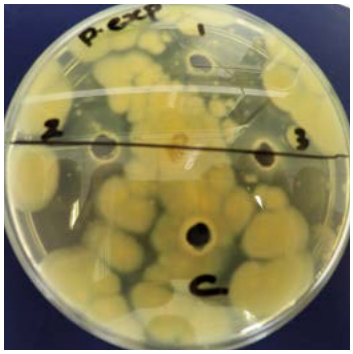
Botrytis cinerea



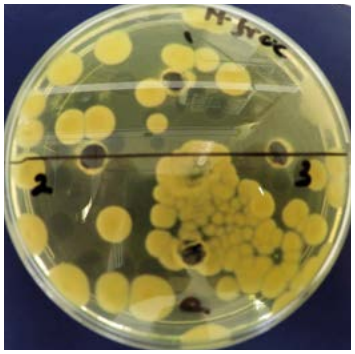
Alternaria brassicicola



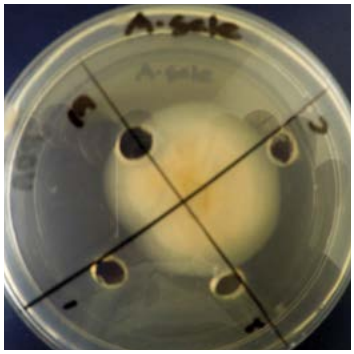
Rhizopus stolonifer



Penicillium expansum



Monilinia fructigena



Aspergillus sclerotiorum

form, are effective in any physical and chemical environment.

Building on her previous work, Prof. Clarke designed a follow-up project focusing on pathogens that eat away at the profits of stone fruit producers.

Working with her on this two-year study (also funded by the PHI Programme and Hortgro Science) was a uniquely competent, multi-disciplinary team of specialists with expertise in the fields of chemical engineering, biochemistry and microbiology. They were Dr Vivek Rangarajan and two post-graduate students, Sebenzile Mazibuko and Willem Herbst.

"Our research focused on optimising the biological production and purification of the lipopeptide molecules to produce a standardised and consistent biofungicide on a laboratory scale, which would be effective in a wide range of physical and chemical environments," says Prof. Clarke.

The study set out to:

- identify and quantify the key performance indicators for optimal antifungal lipopeptide production;

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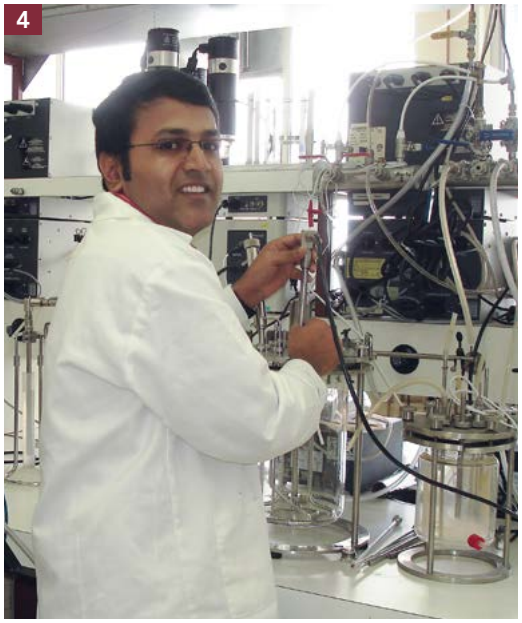

AWARD WINNING

At the 18th International Conference on Bioprocess Systems Engineering held in November 2016, Dr Vivek Rangarajan's paper on the purification of lipopeptides won the best presentation award.

Sebenzile Mazibuko won the best poster award at the 2016 PHI Programme Symposium.

PROJECT TITLE
Production of antimicrobial lipopeptides by *Bacillus* spp. for biological control of post-harvest phytopathogens in the perishable fruit industry

PRINCIPAL INVESTIGATOR
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DURATION
Two years

PHI PROGRAMME & INDUSTRY CONTRIBUTIONS
R829 216 & R179 216

LEAD INSTITUTION
Stellenbosch University (Department of Process Engineering)

BENEFICIARY
The entire fresh fruit industry

FOCUS AREA
Post-harvest disease control

HUMAN CAPITAL DEVELOPMENT
One post-PhD student and two MSc students

PUBLICATIONS AND PAPERS
Three published, one under review

PRESENTATIONS
Six completed, one pending

1 *Bacillus* spp. culture in shake flasks.
2 Supernatant after centrifugation.
3 Precipitation of lipopeptides caused by adding acid.
4 Lipopeptide precipitate to be sent for separation and drying.

- identify and quantify appropriate antifungal lipopeptide concentration and purification procedures; and
- evaluate the effectiveness of different lipopeptides in various grades of purity on phytopathogens targeting the fruit industry.

Method

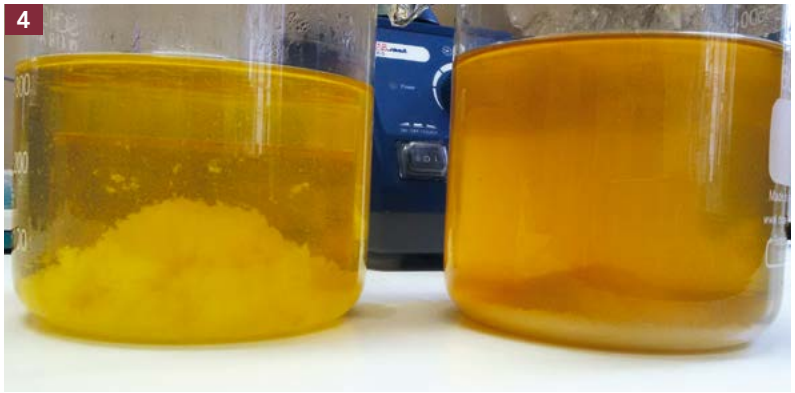
The team applied chemical engineering and life science principles to produce, extract, purify and fractionate a cocktail from *Bacillus* cultures that contained approximately 100 homologues of the lipopeptide families surfactin, iturin and fengycin.

1. Identify and quantify the key performance indicators for optimal antifungal lipopeptide production:
Firstly, a kinetic analysis was conducted to determine the parameters for producing the antifungal lipopeptides surfactin, iturin and fengycin from *B. amyloliquefaciens* under the most efficient conditions in both shake flasks and laboratory scale bioreactors.

The lipopeptide concentration, yield, productivity and selectivity with changes in oxygen, and nitrate and ammonia ratios and concentrations, were closely monitored.

Findings: The team determined the nitrate and ammonia ratio and concentration for optimal kinetics, in terms of lipopeptide concentration, yield, productivity and selectivity, during the production process. They also found that lipopeptide production is enhanced in the presence of oxygen.

2. Identify and quantify appropriate antifungal lipopeptide concentration and purification procedures:
Having determined how to produce the



LEARNING ABOUT LIPOPEPTIDES

Antimicrobial lipopeptides are natural antifungal agents found widely in nature. They kill various types of bacteria, fungi and viruses by drilling a hole in the membrane of the microbe and then inactivating and destroying the organism by disrupting transport across the membrane.

Because they primarily disrupt the cell membrane, they are effective against a broad spectrum of pathogenic microorganisms, with less probability of resistance.

The antibiotic compounds released by *Bacillus* spp. are considered one of the most effective biological control mechanisms for plant pathogens. However, when the entire bacterial culture – which is a living organism – is applied directly, the successful release of the antibiotic compounds depends on near-perfect climatic conditions. Such dependency limits use on a commercial scale and prompted research into ways to extract the compounds and make them available without Nature's intervention.

maximum yield, the team's focus shifted to finding a way to improve the efficacy of the lipopeptide cocktail by concentrating and purifying the product through acid precipitation and solvent extraction.

To reduce the pH of the supernatant, acid (four pH levels) was added to the filtered *Bacillus* culture to trigger the precipitation of the lipopeptides. Eleven different solvents were also added to the filtered *Bacillus* culture to affect concentration and purification.

Findings: The team ascertained that precipitation of the cocktail at a pH of between one and two resulted in maximum recovery of the antifungal lipopeptides (78% for fengycin and 67% for iturin); it was marginally higher than at pH 3. However, optimal purity was obtained at pH 3 (64% for fengycin, and 3% for iturin), thus establishing pH 3 as the preferred condition.

They found that among all the organic solvents that could remove lipid and protein impurities from the antifungal lipopeptides, methanol proved to be most efficient, showing 100% extraction and the highest percentage purity (74%) of antifungal lipopeptides.

"Even if the lipopeptide cocktail is not pure, it can still be very effective," says Prof. Clarke. "Cocktails of varying purities will have different applications. A less pure product could be applied to agricultural crops in general, while a purer product could be required for specific phytopathogens affecting fruit in post-harvest storage."

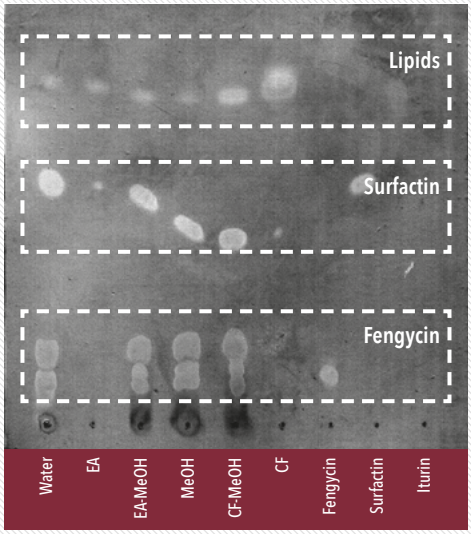
3. Evaluate the effectiveness of different lipopeptides in various grades of purity on target plant phytopathogens targeting the fruit industry:

The team tested the efficacy of the partially purified and purified lipopeptide cocktails against various fungal phytopathogens.

Findings: Some of the most aggressive and destructive plant pathogens, *Botrytis cinerea*, *Alternaria brassicicola* and *Rhizopus stolonifer*, *Monilinia fructigena*, *Penicillium expansum* and *Aspergillus sclerotiorum* affecting post-harvest fruits were used to assess the efficacy of antifungal lipopeptides. The antifungal lipopeptides from *B. amyloliquefaciens* as a 10x concentrate were effective against all tested phytopathogens, except *A. sclerotiorum*.

A LITTLE TLC GOES A LONG WAY

Dr Vivek Rangarajan adapted the method of thin layer chromatography (TLC) to the measurement of the lipopeptides fengycin, iturin and surfactin. Compared to high performance liquid chromatography (HLCP) that takes two hours to process one sample, Vivek's TLC method processes 20 samples in only 45 minutes. It saves time and money, and delivers the desired indicative results.

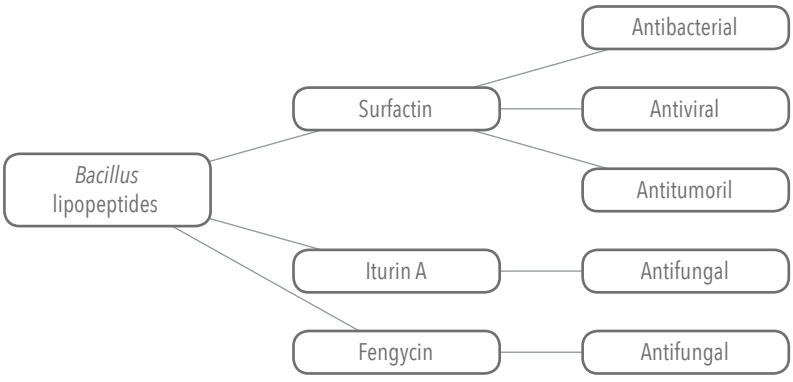


> "Increasingly, the integration of engineering and life science disciplines is recognised as the key that unlocks the potential of new bioproducts to progress from the initial research stage to successful production and implementation."
>
> Prof. Kim Clarke

Conclusion

The bacterially produced bioproducts

Figure 1: Bioproducts effective against pathogens



> Our research is novel in that it seeks to examine the application of the active biofungicide products themselves, rather than using the entire *Bacillus* culture.
>
> Prof. Kim Clarke

have considerable potential as an effective and environmentally friendly alternative for the post-harvest control of fungal diseases in crops, either as a multi-product cocktail or as individual fractions, tailor-made for specific plant diseases. The fact that the producing organism has GRAS (generally regarded as safe) status, is a considerable advantage.

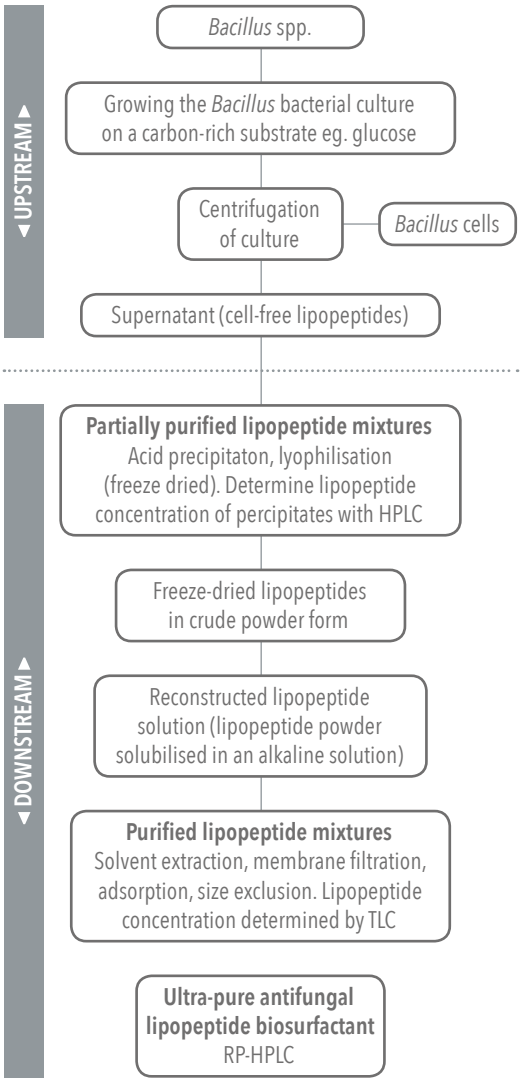
"We envisage that it is possible to manipulate the lipopeptide homologues to tailor-make a sniper-like fungicide that targets a specific phytopathogen," says Prof. Clarke. "Another advantage is that the lipopeptides' mechanism of action is the non-specific disruption of the cell membrane, suggesting that they will be effective against a wide spectrum of phytopathogens, including resistant strains."

Future research

To improve product purity, the next step will be the application of a more selective process. Called macroporous resin adoption, it is a process where the lipopeptides are absorbed into the resin surface under optimal conditions of pH, temperature and solvent composition.

For commercial application, the process has to be adapted to produce the lipopeptide in higher volumes of a standardised quality and purity, delivering a product of low to medium value that is cost-competitive.

Figure 2: Diagram showing the upstream production and downstream recovery, isolation and purification of lipopeptides



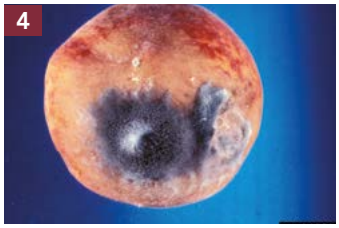
WHAT IS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)?

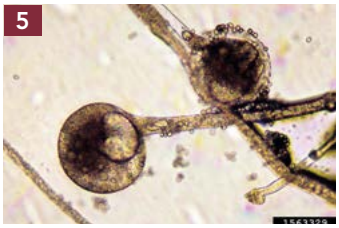
This is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurised liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

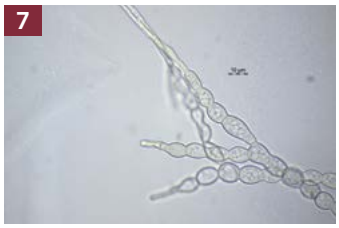
HPLC has been used for manufacturing (eg. during the production process of pharmaceutical and biological products), legal (eg. detecting performance enhancement drugs in urine), research (eg. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (eg. detecting vitamin D levels in blood serum) purposes.

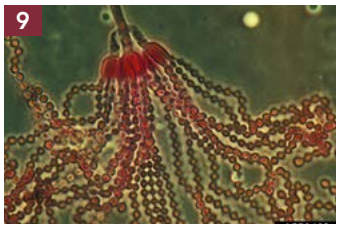
(Source: Wikipedia)

10 MEng students, Sebenzile Mazibuko (right) and Willem Herbst.









Phytopathogens affecting post-harvest fruits: (**1**) Asexual *Botrytis cinerea* spores, also known as grey mould. (Courtesy of Paul Bachi, University of Kentucky Research and Education Center*); (**2**) *Alternaria brassicicola* on cabbage; (**3**) Spores of the *Alternaria* fungus. (Courtesy Bruce Watt); (**4**) Filamentous growth of *Rhizopus stolonifer*, also known as bread mould, on a peach. (Courtesy of University of Georgia Plant Pathology, University of Georgia*); (**5**) Spores of *Rhizopus stolonifer*, also known as bread mould. (Courtesy of Charles Averre, North Carolina State University*); (**6**) *Monilinia* fructigen, or brown rot on a nectarine; (**7**) Spores of *Monilinia* fructicola. (Courtesy of Tom Creswell, Purdue University*); (**8**) Symptoms of *Penicillium expansum*, also known as blue mould, expressed on an apple. (Courtesy of Gerald Holmes, California Polytechnic State University at San Luis Obispo*); (**9**) Spores of the *Penicillium* spp. fungi. (Courtesy of Gerald Holmes, California Polytechnic State University at San Luis Obispo*)

** Bugwood.org*