Biorenewables Development Centre

Plants • Processes • Products

Report developed for:

KET4CP ekolive s.r.o.

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Assessment of the antimicrobial potential of Ekolive solutions.

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1. Introduction

Ekolive uses an innovative bioremediation-based process for recovery of metals from mining sites called bioleaching, aided by heterotrophic bacteria. During the process, a by-product, referred to from here on as leachate, is produced, which the company believes may have antimicrobial properties.

The company wanted to investigate the antimicrobial potential of the leachate against known plant pathogens. The Biorenewables Development Centre (BDC) undertook an anti-microbial challenge study on their behalf, in order to assess the effectiveness of the material against the following:

- *Erwinia amylovora* NCPPB No. 100: Bacterial fruit tree pathogen, typically apple and pear trees.
- *Pectobacterium carotovorum subsp. Carotovorum* NCPPB No. 312: Bacteria which causes a disease known as soft rot which is common in a number of agricultural crops.
- *Botrytis cinerea* strain isolated by the University of York- Fungal species which causes disease in a number of fruits.

The methodology used is detailed in Appendices 1 and 2.

2. Results

The results of the analysis are given in Table 1.

Table 1: Antimicrobial testing results

Microorganism	CFUs per mL[a]					
	Control count	Blank	Concentrated leachate solution		Diluted leachate solution	
			100 %	50 %	100 %	50 %
Erwinia amylovora	1.83×10^6 (3.18×10^5)	2.20×10^4 (2.31×10^3)	0	0	0	0
Pectobacterium carotovorum	1.40×10^6 (3.00×10^5)	2.60×10^4 (2.60×10^4)	0	6.67×10^1 (6.67×10^1)	0	3.33×10^1 (3.33×10^1)
Botrytis cinerea	3.33×10^5 (1.47×10^5)	3.03×10^3 (2.43×10^3)	7.33×10^2 (3.19×10^2)	5.00×10^2 (2.83×10^2)	1.27×10^3 (3.27×10^2)	2.67×10^2 (1.08×10^2)

a) The standard error of the mean is given in brackets.

The control counts correspond to the number of colony forming units (CFUs) per mL, of each microorganism, added to the 9 mL leachate and blank test suspensions. The two log reductions recorded for the blank, compared to the control counts, was to be expected due to the dilutions associated with the initial inoculation and the neutralising steps detailed in appendices 1 and 2.

There were no CFUs present on any of the *E. amylovora* agar plates meaning that all of the leachate suspensions tested resulted in a four log (99.99%) reduction, in this particular bacterial strain.

The 100% test suspensions of the concentrated and diluted leachate also led to a four log (99.99%) reduction of *P. carotovorum*. The 50% suspensions also displayed antimicrobial activity yielding a three log (99.9%) reduction.

Some antifungal activity was observed against *B. cinerea*, (up to a one log/90% reduction), but the test suspensions were not as effective as they were against the bacterial strains tested.

3. Conclusion

The results of this antimicrobial study have demonstrated the antimicrobial properties of Ekolive's concentrated and diluted leachates. When tested against the plant pathogens E. amylovora and P. carotovorum a reduction of up to 99.99% was observed. The leachates also displayed some antifungal properties, however, this was low compared to its antibacterial potential as only a one log reduction was achieved.

4. Appendices

Appendix 1: Methodology for bacterial strains

DAY 1 -

- Streak out the bacteria onto nutrient agar and incubate at 26 °C to produce an 18-to-24-hour culture for use the next day.

DAY 2 –

- Formulate the test suspensions as follows:

  Test suspension 1: 100 % Concentrated leachate
  - Aseptically add 9 mL of concentrated leachate to three sterile 50 mL tubes.

  Test suspension 2 50 % Concentrated leachate
  - Aseptically add 4.5 mL of sterile deionised water (DI) to three sterile 50 mL centrifuge tubes followed by 4.5 mL of concentrated leachate. Gently invert to mix.

  Test suspension 3: 100 % Diluted leachate
  - Aseptically add 9 mL of diluted leachate to three sterile 50 mL tubes.

  Test suspension 4: 50 % Diluted leachate
  - Aseptically add 4.5 mL of sterile DI to three sterile 50 mL centrifuge tubes followed by 4.5 mL of diluted leachate. Gently invert to mix.

  Test suspension 5: Blank
  - Aseptically add 9 mL of sterile maximum recovery diluent (MRD) to three sterile 50 mL centrifuge tubes.

- Using MRD create a test inoculum to give a microbial suspension in the range of $1x10^5$ to $4x10^6$ CFU/mL.
- Add 1 mL of undiluted test inoculum each of the 50 mL tubes containing test suspensions 1 to 5. Vortex to mix and leave at room temperature for 60 minutes.
- After 60 minutes add 1 mL of each to 9 mL of D/E Neutralising Broth, vortex to mix, then make serial dilutions and plate out onto nutrient agar plates.
- Plate out the test inoculum onto nutrient agar plates.
- Once dry incubate all of the plates at 26 ^oC for 48 hours.
- After 48 hours count the colony forming units (CFUs) on the plates.

4.1. Appendix 2: Methodology for fungal strain

- Formulate the test suspensions as follows:

  Test suspension 1: 100 % Concentrated leachate
  - Aseptically add 9 mL of concentrated leachate to three sterile 50 mL tubes.

  Test suspension 2 50 % Concentrated leachate
  - Aseptically add 4.5 mL of sterile deionised water (DI) to three sterile 50 mL centrifuge tubes followed by 4.5 mL of concentrated leachate. Gently invert to mix.

  Test suspension 3: 100 % Diluted leachate
  - Aseptically add 9 mL of diluted leachate to three sterile 50 mL tubes.

  Test suspension 4: 50 % Diluted leachate
  - Aseptically add 4.5 mL of sterile DI to three sterile 50 mL centrifuge tubes followed by 4.5 mL of diluted leachate. Gently invert to mix.

  Test suspension 5: Blank
  - Aseptically add 9 mL of sterile maximum recovery diluent (MRD) to three sterile 50 mL centrifuge tubes.

- Using MRD create a test inoculum to give a spore suspension which equates to a concentration of $1x10^5$ to $4x10^6$ CFU/mL.

- Add 1 mL of undiluted test inoculum to each of the 50 mL tubes containing test suspensions 1 to 5. Vortex to mix and leave at room temperature for 60 minutes.

- After 60 minutes add 1 mL of each to 9 mL of D/E Neutralising Broth, vortex to mix, then make serial dilutions and plate out onto potato dextrose agar plates.

- Plate out the test inoculum onto potato dextrose agar plates.

- Once dry incubate all of the plates at 26 ^oC for 72 hours.

- After 72 hours count the colony forming units (CFUs) on the plates.

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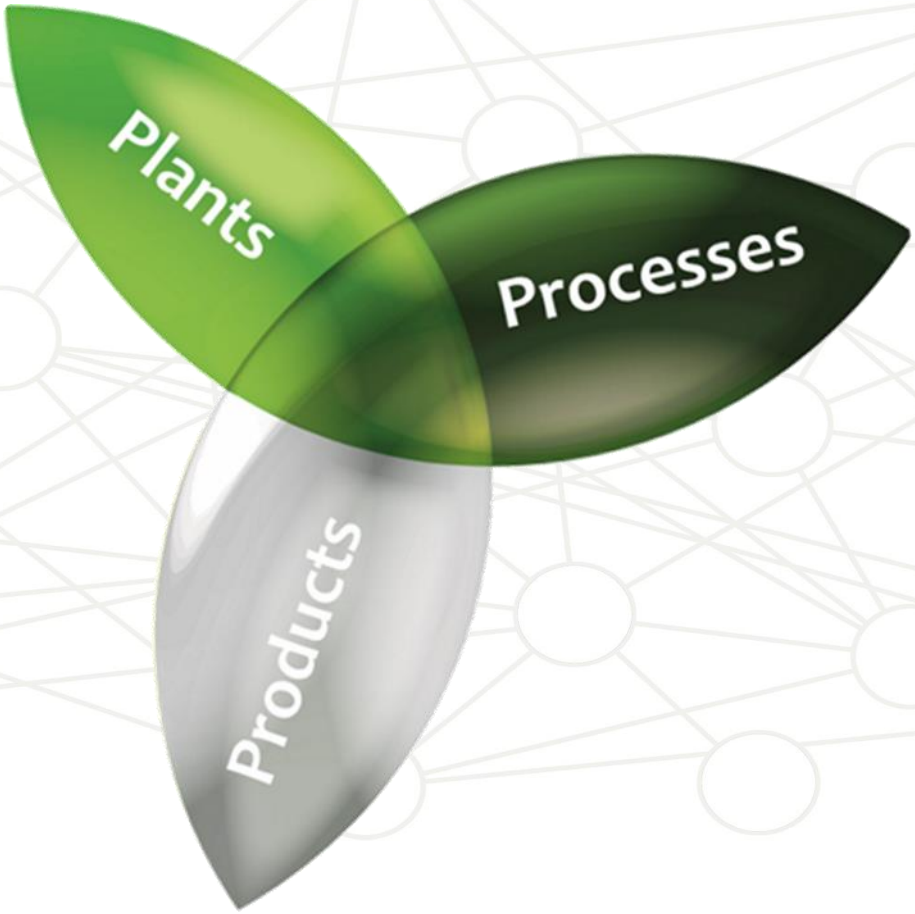

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