VALUABLE PRODUCTS FROM ORGANIC WASTES: ENGINEERING AN ANTIMICROBIAL YEAST AS INDUSTRIAL PLATFORM FOR NON-STERILE BIOPROCESSES

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Introduction

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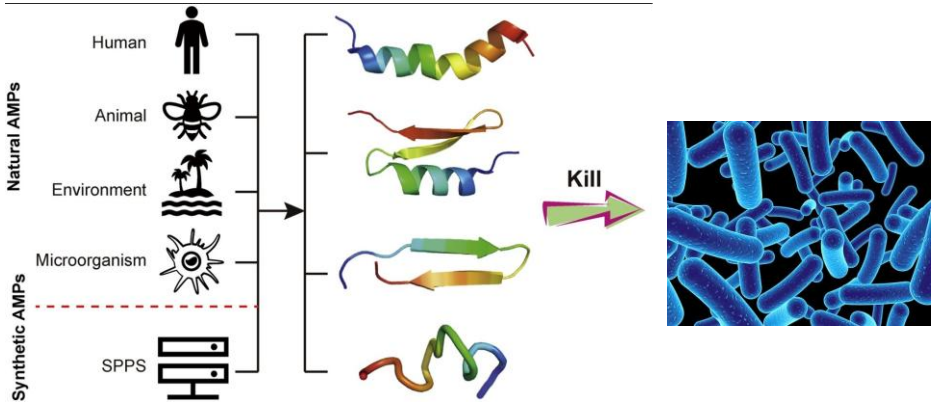
- South Africa annually generates over 9 million tonnes of food waste, at a cost > R61.5 billion
- This energy-rich waste exacerbates environmental issues when consigned to landfills, issues such as greenhouse gas emissions and water pollution
- Most organic wastes have a moisture content surpassing 70% by weight, exceeding the mandated threshold of 40%
- This scenario presents an opportunity to divert waste away from landfills, towards processes that utilize sugar- and starch-rich materials for bioethanol production
- However, in industrial ethanol fermentations, the yeast *Saccharomyces cerevisiae* is hindered by metabolites (lactic & acetic acid), produced by contaminating bacteria
- These bacteria compete for fermentable sugars & nutrients, reducing ethanol yield
- As pre-sterilization of feedstocks is economically unfeasible at large scale, antibiotic supplementation is used which has cost implications & may contribute to the emergence of drug-resistance

Introduction

- Antimicrobial peptides (AMPs) are promising antimicrobials with applications in clinical and agricultural settings
- Their reduced probability of acquired resistance compared to antibiotics makes them appealing
- We set out to develop a technology to consume the organic fraction of municipal solid waste without relying on traditional antibiotics and to engineer yeast strains capable of co-producing AMPs and/or starch-degrading enzymes:
  - facilitate bioethanol production from food waste without the need for pre-sterilization
  - require lower exogenous amylase addition



Aims & Objectives

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- Production of ethanol from food waste (FW) presents challenges due to the variability in feed composition and the potential for microbial contamination

- This study aimed to address these challenges by focusing on:
  - optimization of ethanol production from FW
  - development of antimicrobial yeast strains

Objectives:

1. Enhance ethanol concentrations, volumetric productivity, and yields from pre- and post-consumer FW substrates
   - high solid fermentation and fed-batch feeding techniques
   - compare the efficacy of a starch CBP yeast strain (*S. cerevisiae* ER T12) in minimizing enzyme dosage
2. Assess decontamination strategies (thermal treatment, chemical approaches, AMPs), minimize enzyme dosages, and utilize an enzyme-producing yeast
3. Engineer *S. cerevisiae* strains with antimicrobial properties to thrive in non-sterile environments.
   - Test 7 candidate AMPs for activity in yeast; create industrial AMP producing strain
   - Use CRISPR-Cas9 to create an industrial lysozyme producing strain

Main results – FW fermentations

- Pre-consumer waste: mixed potato, grain-based waste (corn, cereal, bread, etc.), and canned beans.
- Post-consumer food waste: mixture of different restaurant wastes
- Conventional cooked starch hydrolysis was used for the hydrolysis of the food waste using a thermostable α-amylase and a glucoamylase for gelatinization and saccharification.

Effects of liquefaction temperatures & decontamination strategies on ethanol fermentation of pre- and post-consumer FW.

	Pre consumer FW		Post consumer FW	
	Ethanol (g/L)	Yield %	Ethanol (g/L)	Yield %
Liquified at 55°C				
Negative control 55 °C[A]	21.2±1.6	48.2±3.2	26.9±1.1	67.3±2.2
Positive control 55 °C[B]	40.6±0.7	92.2±1.1	31. 2±2.4	77.8±5.1
$K_2S_2O_5$	23.3±0.4	52.6±0.9	27.2±5.0	67.9±13.0
Nisin A	20.8±1.5	47.2±2.5	30.4±1.6	75.8±2.7
Lysozyme	22.3±0.7	50.6±1.8	29.9±1.1	75.4±2.6
Nisaplin	23.4±1.1	52.9±2.5	28.9±0.4	72.5±1.0
Mix [C]	23.1±1.3	52.3±2.6	28.8±0.5	72.2±1.7
Liquified at 95°C				
Negative control 95 °C[A]	38.1±2.3	87.9±4.9	28.2±3.1	69.2±7.2
Positive control 95 °C[B]	40.5±0.5	93.74±0.3	29.4±4.2	71.1±9.2
$K_2S_2O_5$	38.7±2.8	89.65±6.4	28.59±3.42	70.06±7.0
Nisin A	36.5±0.9	84.2±2.2	29.0±2.7	70.9±4.4
Lysozyme	37.4±1.8	86.1±5.1	28.9±3.5	69.8±6.8
Nisaplin	36.6±1.6	85.2±3.7	28.4±3.2	70.6±6.9
Mix [C]	35.4±1.3	82.0±2.8	28.5±3.9	72.0±6.6

A. Negative control, liquefaction with thermal stable enzymes at specified temperature, no decontamination

B. Positive control, thermal sterilisation in an autoclave before liquefaction

C. Mixture of AMP nisin A (500 mg/kg) and lysozyme (500 mg/kg)

Main results – FW fermentations

- A significant difference was observed between the substrates
- The influence of the gelatinization temperature was greater with pre-consumer FW
- Increased ethanol yield due to adding AMPs was best seen with the post-consumer food waste at low gelatinization temperatures
  - The influence of the AMPs was more significant at lower gelatinization temperatures
- Thermal sterilisation was effective for post-consumer FW at a low liquefaction temperature of 55 °C with a significant ($p < 0.05$) increase in ethanol yield of 77.79% compared to 67.29% using unsterilised feed
- At a liquefaction temperature of 55 °C after thermal sterilisation of pre-consumer FW, an ethanol yield of 92.2% was obtained, compared to 48.2% in the unsterilised feedstock
- Thermal sterilisation effectively served simultaneously as a decontamination and gelatinisation strategy.

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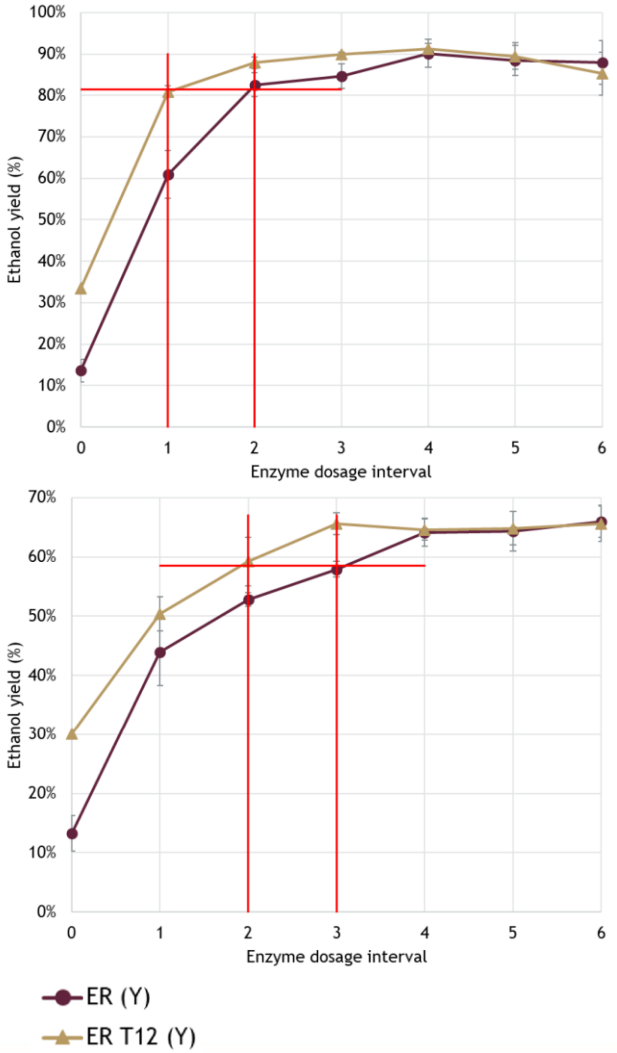
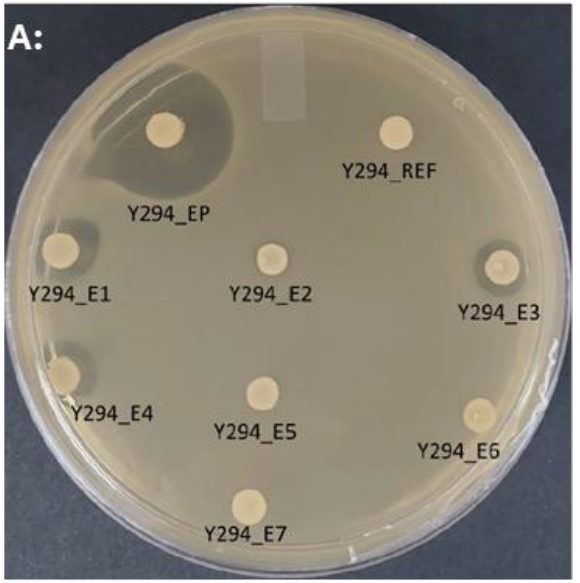
Main results – FW fermentations

- Yeast strains producing heterologous amylase activities can mitigate the cost of exogenous enzyme addition
- We observed substantial decreases in enzyme dosages of up to 33% by using the starch consolidated bioprocessing (CBP) yeast *S. cerevisiae* ER T12 without affecting the ethanol yield or productivity:
  - *S. cerevisiae* ER T12 yield = 80.87% ± 1.40; productivity = 1.51 g/L/h
  - Ethanol Red® : yield = 82.56% ± 2.81; productivity = 1.54 g/L/h
- The same enzyme reduction of 33% was observed for the post-consumer FW
- Ethanol concentration from the pre-and post-consumer FW increased to 74.11 ± 0.75 g/L and 48.52 ± 1.32 g/L, without a significant effect on the ethanol yield when the solids loading was increased to 20.6% and 21.06% w/v
  - *surpasses the minimum threshold for economic viability*
- This suggests that the developed process holds potential as an alternative to landfilling



Main results – AMP expression

- 7 synthetic AMP encoding genes were subcloned to yeast expression vectors and transformed into a laboratory yeast strain
- Transformants were subjected to antimicrobial activity testing against a range of microorganisms.
- Transformants engineered to express the AMPs Garvieacin Q, Carnobacteriocin BM1 and Piscicolin 126 respectively, showed antimicrobial activity against *Listeria spp.* and *Enterococcus spp.*
  - *First time these AMPs have been reported to show activity when produced in yeast*
- We attempted to create an antimicrobial industrial *S. cerevisiae* strain by integrating the Carnobacteriocin BM1 encoding gene into the genome of the industrial strain *S. cerevisiae* Ethanol Red®
- We confirmed the presence of the gene but, no antimicrobial activity was detected
  - This strain was created using the CRISPR-Cas9 method; we attempted traditional plasmid-based methods for genome integration, however, none of the transformants created showed antimicrobial activity



B:

Target organism	Pediocin PA-1 (+)	Garvieacin Q	Carnobacteriocin BM1	Piscicolin-126	Hiracin-JM79; Aureocin A53; Nisin-A or Pyrrhocoricin
L. monocytogenes Edge	✓	✓	✓		
L. monocytogenes ATCC 23074	✓	✓	✓	✓	
L. monocytogenes ATCC 7644	✓	✓	✓	✓	
L. monocytogenes ATCC 19114	✓	✓	✓		
L. innocua ATCC 33090	✓	✓			No Activity
E. faecalis ATCC 29212	✓	✓	✓		
L. pentosus DSM no 20314	✓				
L. pentosus DSM no 20223	✓				

Main results – AMP & hLYZ expression

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- As human lysozyme was previously reported to be active against a range of possible fermentation contaminants, a synthetic gene encoding this enzyme was also obtained
- Using Gibson assembly, the hLYZ gene was fused to the MF-α secretion signal
  - "repair fragment" for the a CRISPR-Cas9-based yeast transformation strategy
- The guide RNA gene cassette was successfully cloned and co-transformed with the repair fragment into the *S. cerevisiae* Ethanol Red® strain
- Three transformants were PCR-confirmed to have been transformed with the hLYZ gene
- Unfortunately, no lysozyme activity could be detected with against *Micrococcus luteus*, the standard indicator organism for lysozyme activity
  - addition of posttranslational modification genes are required
- Constructs were designed for expression of the PTM genes – not completed prior to the end of the project
- An *S. cerevisiae* Ethanol Red® strain producing the AMP Enterocin A was active against *L. monocytogenes* EDG-e (obtained from a collaborator)
  - In co-cultures this yeast strain partially suppressed the growth of *L. monocytogenes* EDG-e.
  - In fermentation co-culture conditions, the Enterocin A expressing strain produced 4% more ethanol compared to the wild type, a small but significant difference ($p < 0.05$)

Conclusions

- This project highlighted the feasibility of using food waste as raw material for bioethanol production, demonstrating a waste valorisation strategy that aligns with the principles of the circular economy
  - Applying thermal sterilisation led to a statistically significant increase in the ethanol yield of pre- and post-consumer FW fermentation at low liquefaction temperatures compared to an unsterilised control
  - Addition of exogenous AMPs, lysozyme and nisin A led to marginally significant increases in ethanol yield compared to the negative control, although using AMPs as a decontamination strategy requires further development
  - The low reliance on decontamination of the feedstock and the reduction of exogenous enzymes required due to the usage of an advanced yeast (ER T12) reduced the operational cost and improves the viability waste-based ethanol fermentation process
- This study was also able to show that an engineered antimicrobial industrial yeast strain grown under fermentation conditions with a bacterial contaminant, produced a higher ethanol yield compared to the wild type, proving one of the principles we sought to establish