BRIEFING NOTE

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

R. den Haan, E. van Rensburg, J.F. Görgens, G. van Lill, J. van Rooyen, K. Trollope
University of the Western Cape & Stellenbosch University, South Africa

KEY FINDINGS

This study investigated the feasibility of using unsterilised pre- and post-consumer starch rich waste as feedstock for producing bioethanol. Thermal treatment and subsequent liquefaction at 55°C, boosted ethanol production from food waste compared to fermentation without such treatment. This approach was more effective than chemical decontamination and the application of exogenous antimicrobial peptides (AMPs). The application of a recombinant amylase producing yeast strain led to a reduction of ~33% in the dosage of starch degrading enzymes required for fermentation. Ethanol concentrations of 74.1 g/L and 48.5 g/L were obtained from pre-and post-consumer waste, surpassing the minimum threshold for economic viability. CRISPR-based construction of an *S. cerevisiae* Ethanol Red® strain containing a lysozyme encoding gene was completed, however, no lytic activity was found. Screening of seven novel AMP encoding genes in yeast revealed three AMPs that were not previously shown to be active in this host. We were able to demonstrate that an AMP producing yeast strain partially suppressed the growth of a contaminating bacterial strain during fermentation, leading to a marginal increase in ethanol production. However, broad inhibition toward a likely group of industrial fermentation contaminants was not attained.

INTRODUCTION

South Africa annually generates over 9 million tonnes of food waste, incurring a staggering cost upwards of R61.5 billion. This energy-rich waste, when consigned to landfills, exacerbates environmental issues such as greenhouse gas emissions and water pollution. The government mandates that landfill material should maintain a moisture content of less than 40% by weight. However, most organic wastes exceed this threshold, with a moisture content often surpassing 70% by weight, posing significant challenges for waste producers.

This scenario presents an opportunity to divert waste away from landfills and towards processes that utilize sugar- and starch-rich materials for bioethanol production. However, in ethanol fermentations, the yeast *Saccharomyces cerevisiae* yeast plays a crucial role that is hindered by metabolites like lactic acid and acetic acid, produced by bacteria present in the waste feedstocks. These bacteria rapidly proliferate, competing with yeast for fermentable sugars and nutrients, thereby reducing ethanol yield. At large production scales, pre-sterilization of feedstocks is economically unfeasible, commonly leading to antibiotic supplementation to limit bacterial growth. However, aside from the cost implications, there is mounting evidence suggesting that non-clinical antibiotic use contributes to the emergence of drug-resistant microbes. Hence, our objective was to develop a technology that tackles the problematic organic fraction of municipal solid waste without relying on traditional antibiotics. Moreover, we aimed to engineer yeast strains capable of co-producing antimicrobial peptides (AMPs) and/or starch-degrading enzymes to facilitate bioethanol production from food waste without the need for pre-sterilization while requiring lower exogenous amylase addition. AMPs offer promise as novel antimicrobials with applications in both clinical and agricultural settings, and their reduced probability of acquired resistance compared to antibiotics makes them particularly appealing.

METHODOLOGY

The 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 two key objectives namely

optimization of ethanol production and development of antimicrobial yeast strains. First, we sought to enhance ethanol concentrations, volumetric productivity, and yields from both pre- and post-consumer FW substrates. This involved assessing various decontamination strategies, minimizing enzyme dosages, and utilizing an enzyme-producing yeast. Additionally, we aimed to maximize ethanol concentration through high solid fermentation and fed-batch feeding techniques. Decontamination methods, including thermal treatment, chemical approaches, and antimicrobial peptides (AMPs), were evaluated for their impact on ethanol production metrics such as rate, yield, and concentration. Furthermore, we compared the efficacy of the starch consolidated bioprocessing (CBP) yeast strain *S. cerevisiae* ER T12 in minimizing enzyme dosage while maintaining ethanol yield, concentration, and productivity against the industrial standard, *S. cerevisiae* Ethanol Red®.

The second aspect of this study involved engineering *S. cerevisiae* strains with antimicrobial properties to thrive in non-sterile environments. Initially, we identified seven candidate antimicrobial peptides from online databases and synthesized codon-optimized genes for expression in the laboratory strain *S. cerevisiae* Y294. Promising candidate genes were then integrated into the genome of *S. cerevisiae* Ethanol Red®. Subsequently, we conducted laboratory-scale evaluations of these engineered yeast strains in co-culture with representative contaminant bacteria. Additionally, we optimized and introduced the human lysozyme encoding gene, encoding an effective antibacterial protein, into yeast using CRISPR-Cas9 tools tailored for this purpose.

MAIN RESULTS

Three waste groups were selected to be mixed namely potatoes, grain-based waste (corn, cereal, bread, etc.), and canned beans. This selection was made based on the composition of wastes processed at Interwaste on a yearly average weight and was considered as pre-consumer waste. Post-consumer food waste consisted of a 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, respectively. The effects of liquefaction temperatures and decontamination strategies on ethanol fermentation parameters of pre- and post-consumer FW were determined in fermentation with 10% solids loading (Table 1).

Table 1. Effects of liquefaction temperatures and decontamination strategies on ethanol fermentation parameters of pre- and post-consumer FW. Fermentations were carried out for 72 h at 10% (w/v) solids loading with an α-amylase and glucoamylase dosage of 1.345 µL/g and 2.084 µL/g substrate, using *S. cerevisiae* Ethanol Red®.

	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±6.45	28.59±3.42	70.06±7.07
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)

We observed a significant difference between the different substrates, and it was also seen that the influence of the gelatinization temperature was much greater with pre-consumer food waste than with post-consumer food waste. Increasing the ethanol yield due to adding AMPs was best seen with the post-consumer food waste at low gelatinization temperatures. Thus, the influence of the AMPs was more significant at lower gelatinization temperatures, though substrate conversion was less efficient at these temperatures. Thermal sterilisation proved 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% recorded after fermentation using an unsterilised feed. Similarly, at a liquefaction temperature of 55 °C after thermal sterilisation of pre-consumer FW, an ethanol yield of 92.2% was obtained, substantially higher than the 48.2% yield of the control where the feedstock remained unsterilised. This data suggested that thermal sterilisation effectively served simultaneously as a decontamination and gelatinisation strategy.

Yeast strains producing heterologous amylase activities have been engineered to mitigate the cost of exogenous enzyme addition in bioconversion processes. Substantial decreases in enzyme dosages of up to 33% were achieved by using the starch CBP yeast *S. cerevisiae* ER T12 without affecting the ethanol yield of 80.87% ± 1.40 and productivity 1.51 g/L/h compared to 82.56% ± 2.81 and 1.54 g/L/h when using *S cerevisiae* strain Ethanol Red® as benchmark (Figure 1). The same enzyme reduction of 33% was observed for the post-consumer FW. The ethanol concentration of the pre-and post-consumer FW was increased by 96% to 74.11 ± 0.75 g/L and 85% to 48.52 ± 1.32 g/L, surpassing the minimum threshold for economic viability, without a significant effect on the ethanol yield when the solids loading was increased to 20.6% and 21.06% w/v. This suggests that the developed process holds potential as a viable alternative to landfilling.

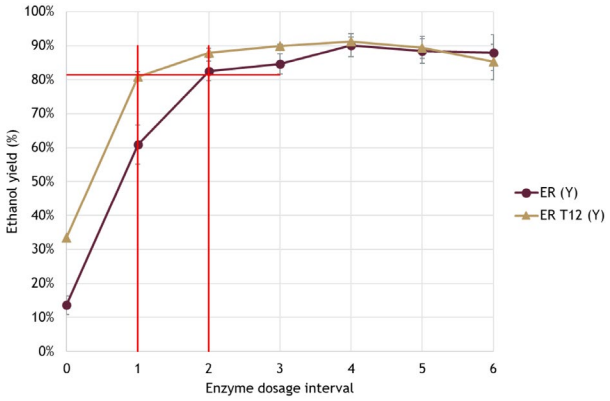
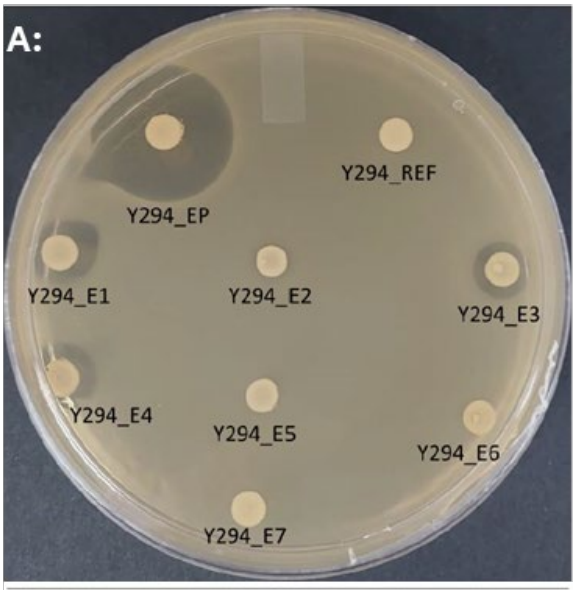


Figure 1. The impact of enzyme dosages on ethanol yield (as a percentage of the theoretical maximum) using pre-consumer FW. A comparison between the yeast strains Ethanol Red® and ER T12 demonstrated that ER T12 had superior performance at lower enzyme dosages. The data points are based on 10% solids loading.

For the second part of the project, synthetic AMP encoding genes were subcloned to yeast expression vectors under control of the *ENO1* gene promoter and terminator and the constructs were transformed into the laboratory yeast strain *S. cerevisiae* Y294. These transformants were subjected to antimicrobial activity testing against a range of microorganisms. We found that the transformants engineered to express the AMPs Garvieacin Q, Carnobacteriocin BM1 and Piscicolin 126 respectively, showed antimicrobial activity against *Listeria spp.* and *Enterococcus spp.* (Figure 2). We subsequently attempted to create an antimicrobial industrial *S. cerevisiae* strain. We integrated the Carnobacteriocin BM1 encoding gene into the genome of the industrial strain *S. cerevisiae* Ethanol Red®,



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	✓				

Figure 2. Antimicrobial activity screening of episomally expressed AMP encoding genes in recombinant S. cerevisiae Y294_E(1-7) strains. (A) Agar-overlay assays of the AMP producing recombinant yeast strains against L. monocytogenes ATCC 23074. (B) Antimicrobial activity range of the recombinant Y294_E(1-7) strains: Soft agar-overlay method; ✓ indicates a visible zone of inhibition. The negative and positive controls were Y294_REF and Y294_EP (Pediocin PA1), respectively. E1 to E7 refer to the seven AMPs selected for the study namely Garvieacin Q, Hiracin JM79, Carnobacteriocin BM1, Piscicolin 126, Aureocin A53, Nisin A and Pyrrhocoricin.

targeting either δ-sequences or the intergenic regions of chromosome 11. Even though we confirmed the presence of the gene, no antimicrobial activity was displayed by these strains or an alternative strain *S. cerevisiae* YI13. These strains were created using the CRISPR-Cas9 method for gene integration. We next attempted traditional plasmid-based methods for genome integration, which required the subcloning of the AMP genes. We integrated the Garvieacin Q and Carnobacteriocin BM1 encoding genes, respectively

into both *S. cerevisiae* Ethanol Red® and *S. cerevisiae* Y294. However, none of the mentioned clones created showed antimicrobial activity, even though the presence of the genes were confirmed in each case.

A synthetic gene encoding human lysozyme (hLYZ) was also obtained. Using restriction enzyme- and *in vitro* homologous recombination (Gibson assembly) multi-step cloning strategies, the hLYZ gene was fused to the MF-α secretion signal to create an expression cassette ($HXT7_P$-MFα-hLYZ-$ADH1_T$). This "repair fragment" was then used in a CRISPR-Cas9-based yeast transformation strategy to repair double stranded breaks introduced by Cas9, integrating the cassette. The guide RNA gene cassette was successfully cloned and co-transformed with the repair fragment into the *S. cerevisiae* Ethanol Red® strain. After multiple rounds of re-streaking, three transformants were tested with PCR and confirmed to have been successfully transformed with the hLYZ gene. Unfortunately, no lysozyme activity could be detected with against *Micrococcus luteus*, the standard indicator organism for assaying lysozyme activity. Given literature reports on lysozyme expression in yeast, these results are not unexpected as the addition of posttranslational modification genes are required. The plasmid pMarkerFreeUXI-3_PGK_P-PDI_AmdSym was constructed but not transformed. Unfortunately, successful construction of the pMarkerFree_XI-3_$ENO1_P$-ERO1-$ENO1_T$_AmdSym plasmid could not be achieved during this project. We hope to pursue the successful production of lysozyme in yeast in a follow up project.

We obtained an *S. cerevisiae* Ethanol Red® strain expressing the AMP Enterocin A gene from a collaborator. When screened for antimicrobial activity, this strain was active against *L. monocytogenes* EDG-e. We co-cultured this yeast strain with *L. monocytogenes* EDG-e and measured the growth of both the yeast and bacteria using a plate count method. We found that that the Enterocin A producing yeast partially suppressed the growth of the bacterial contaminant *L. monocytogenes* EDG-e. In anaerobic fermentation co-culture conditions, the Enterocin A expressing strain produced 4% more ethanol compared to the wild type ($p < 0.05$). This study thus was able to show that an engineered antimicrobial industrial *S. cerevisiae* 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 in this project.

This project highlighted the feasibility of using FW as an effective 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 the negative control, where no sterilisation was applied. The AMPs lysozyme and nisin A had a marginally significant increase in the 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.

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