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Additional glucoamylase genes increase ethanol productivity on rice and potato waste streams by a recombinant amylolytic yeast

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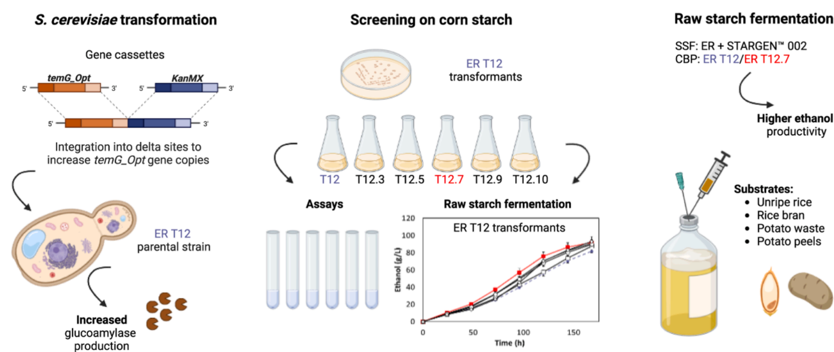
HIGHLIGHTS

- Rice-based substrates had noteworthy concentrations of free fermentable sugars.
- The ER T12.7 transformant displayed improved raw corn starch fermentation.
- Additional glucoamylase gene copies improved ethanol productivity on potato starch.
- Rice and potato-based substrates are attractive feedstocks to produce bioethanol.
- Ethanol concentrations of 88.76 g/L were obtained during unripe rice fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

The implementation of consolidated bioprocessing for converting starch to ethanol relies on a robust yeast that produces enough amylases for rapid starch hydrolysis. Furthermore, using low-cost substrates will assist with competitive ethanol prices and support a bioeconomy, especially in developing countries. This paper addresses both challenges with the expression of additional glucoamylase gene copies in an efficient amylolytic strain (*Saccharomyces cerevisiae* ER T12) derived from the industrial yeast, Ethanol Red™. Recombinant ER T12 was used as a host to increase ethanol productivity during raw starch fermentation; the ER T12.7 variant, selected from various transformants, displayed enhanced raw starch conversion and a 36% higher ethanol concentration than the parental strain after 120 h. Unripe rice, rice bran, potato waste and potato peels were evaluated as alternative starchy substrates to test ER T12.7's fermenting ability. ER T12.7 produced high ethanol yields at significantly improved ethanol productivity, key criteria for its industrial application.

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1. Introduction

Consolidated bioprocessing (CBP) is a proposed alternative for starch-to-ethanol conversion of various raw starch-based substrates. The combined hydrolysis and fermentation by a recombinant yeast in a single step simplifies the design and set-up of the starch conversion, thus reducing operation costs (Van Zyl et al., 2012). Raw starch-hydrolyzing amylases from various sources have been expressed in laboratory and industrial *Saccharomyces cerevisiae* strains to convert starch to ethanol (reviewed by Cripwell et al., 2020). One of the main bottlenecks in implementing CBP of starchy biomass is the relatively low amylolytic activity at the onset and within the first 24–48 h of fermentation (Görgens et al., 2015; Cripwell et al., 2020). To overcome this "lag phase" during raw starch fermentations, enzyme-supplemented CBP processes with laboratory (Sakwa et al., 2018) and industrial amylolytic *S. cerevisiae* strains have been investigated (Cripwell et al., 2020). Results demonstrated that enzyme supplementation with as little as 10% of the recommended STARGEN™ 002 loading decreased the lag phase, thus shortening the raw starch fermentation time for corn starch (Cripwell et al., 2019a) and broken rice (Myburgh et al., 2019) to approximately 96 h; while Malherbe et al. (2023) demonstrated that the lag phase could be eliminated and the fermentation time decreased to 48 h on raw sorghum flour. However, cost-effective ethanol production requires none or less than a 10% commercial enzyme loading, which is only achievable if the CBP strain can rapidly hydrolyze starch within the first 24 h to provide sufficient glucose for yeast cell growth. Cripwell et al. (2019b) investigated various fungal amylases for raw starch hydrolysis and evaluated codon optimization and different secretion signals for improved hydrolysis of corn starch. Furthermore, Myburgh et al. (2020) used intron-mediated enhancement to improve well-known yeast promoters to achieve higher levels of α-amylase activity in *S. cerevisiae*.

The current biofuel industry relies on starch-based substrates, with corn being the preferred feedstock for large-scale bioethanol production (Li et al., 2022). However, concerns regarding food security have necessitated the evaluation of alternative starchy substrates for bioethanol production, especially in developing countries. Ethanol production costs can also be reduced with the use of cheaper substrates that would contribute to developing a starch bioeconomy; therefore, to achieve this, agro-industrial and lignocellulosic-based substrates have been evaluated for bioethanol production as they do not compromise food security (Chohan et al., 2020; Gupte et al., 2022). Despite the attractive nature of lignocellulosic material as an alternative feedstock, processing technology is still under development, whereas the starch-to-ethanol route has established procedures for industrial-scale ethanol production (Cripwell et al., 2020). However, one of the main bottlenecks in the starch-to-ethanol industry remains the cost of the feedstock (mostly corn). Therefore, the potential of alternative, abundant low-cost starchy waste streams should be explored. Although the conversion of potato waste to valuable products is a noteworthy topic in biofuels and waste utilization, reports are limited to laboratory-based studies (Li et al. (2022).

Agricultural waste material rich in polysaccharides has several advantages as feedstock for second-generation biofuel production. Some options include potato waste (Arapoglou et al., 2010; Izmirlioglu and Demirci, 2015), sweet potato residues (Wang et al., 2016), rice by-products (Favaro et al., 2017) and food waste (Hashem et al., 2019). Feedstock material from the potato and rice industries has considerable starch (Brojanigo et al., 2020; Ebrahimian et al., 2022) and represents attractive options to explore a CBP approach (Brojanigo et al., 2022). Furthermore, potatoes represent the third-largest global food crop, and their production has increased significantly in recent years due to their drought-resistant and nutritional value. It is estimated that two-thirds of the world's population consumes potatoes (Dongyu, 2022), but this rise in demand will also result in a growing waste stream that can be harnessed as a carbon-rich feedstock alternative. The potato processing industry produces several different types of waste, such as 15–40% peels, mash, pulp and potato processing wastewater (Arapoglou et al., 2010; Singh et al., 2022), which can represent alternative materials to produce bioethanol and other desirable products (Ebrahimian et al., 2022; Izmirlioglu and Demirci, 2015). Minimizing land use and lowering feedstock costs are among the driving forces for using these substrates, but process optimization is required to effectively convert these waste materials to fermentable sugars.

The enzymatic hydrolysis of starch depends on its structure and composition; A-type crystalline starches in rice are hydrolyzed by *endo*-corrosion, while potato granules have B-type crystalline structures that are *exo*-corroded (Kowsik and Mazumder, 2018). Cereal starches, such as rice, have pores on the surface of the granule that allow for enzyme access and more efficient penetration of the amylases. In contrast, potato granules lack surface pores, and the granules are digested at a slower rate (from the outside in). When starchy materials are considered a substrate for ethanol fermentation, it is essential to understand how they will interact with the amylases. The amylose:amylopectin ratio, granule size and surface characteristics are thus key factors (Wang et al., 2022).

Several studies showed that a higher glucoamylase activity relative to α-amylase activity might improve the rate of starch hydrolysis (Görgens et al., 2015). In previous work, industrial *S. cerevisiae* strains were constructed to co-express an α-amylase (*temA*) and codon-optimized glucoamylase (*temG_Opt*) gene from *Talaromyces emersonii* (Cripwell et al., 2019a). The recombinant Ethanol Red™ transformant, ER T12, displayed the highest amylolytic activity and contained an estimated four α-amylase and seven glucoamylase copies. Gronchi et al. (2022) compared the hydrolytic performance of strains containing different amylase ratios, and highlighted the role of glucoamylase in a baseline α-amylase/glucoamylase ratio of 1:2.5 for raw starch conversion. Therefore, this study aimed to improve the hydrolytic performance of the previously constructed amylolytic ER T12 strain by increasing the number of integrated codon-optimized *T. emersonii* glucoamylase *(temG_Opt)* copies using delta-integration at random sites. Several low-cost waste materials, namely unripe rice, rice bran, potato waste and potato peels, were evaluated as feedstocks for ethanol production. To our knowledge, this is the first report on using industrial amylolytic *S. cerevisiae* strains for CBP of potato waste and potato peels.

2. Methods and materials

2.1. Media and cultivation conditions

The *S. cerevisiae* parental strain was cultured in YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or maintained on YPD agar plates (including 15 g/L agar). The *S. cerevisiae* ER T12 transformants were selected for and maintained on SC plates containing 2% corn starch to visualize hydrolysis zones (Sakwa et al., 2018). The *S. cerevisiae* transformants were cultivated aerobically in 20 mL double-strength SC (2 × SC) medium as described by Cripwell et al. (2019a).

2.2. Substrate preparation and analysis

Organic waste streams from the industrial processing of rice (*Oryza sativa* L.) and potato (*Solanum tuberosum* L.) were evaluated as feedstocks, with raw corn starch (Sigma-Aldrich) serving as the benchmark. Unripe rice and rice bran were obtained from La Pila (Isola della Scala, Italy) as representatives of the range of rice waste streams (Favaro et al., 2017). Unripe rice has a relatively high starch content, whereas rice bran contains little starch. Potato peels and waste were sourced from Gruppo Napoleon (Verona, Italy). The substrates were dried for 48 h in a forced-air oven at 55 °C (freeze-dried for potato residues), milled and sieved through a 1.25 mm screen. Feedstocks were stored and characterized as described by Favaro et al. (2017).

2.3. Strains and plasmids

The strains and plasmids constructed or used in this study are summarized in Table 1.

2.4. DNA manipulations and yeast strain construction

To engineer the ER T12 recombinant strain, the codon-optimized *T. emersonii* glucoamylase (*ENO1*$_P$-*temG_Opt-ENO1*$_T$) and geneticin marker (*TEF1*$_P$-*kanMX-TEF1*$_T$) cassettes were amplified from plasmids yBBH1-TemG_Opt and pBKD2 (Table1), respectively, using primers described by Gronchi et al. (2022). The *S. cerevisiae* ER T12 strain was grown in 50 mL YPD broth (A_{600} of 0.7 – 0.9); competent cells were prepared and transformed as described by Cripwell et al. (2019a). The cultures were incubated at 30 °C for 3 h before plating onto YPD plates supplemented with 100, 200, or 300 μg/mL G418 disulfate salt (Merck, Germany). The plates were incubated at 30 °C for 1–2 days, and colonies were streaked out three times on YPD plates containing the respective G418 disulfate salt concentration. Five transformants, namely ER T12.3, ER T12.5, ER T12.7, ER T12.9 and ER T12.10 were selected after the initial screening (soluble corn starch, liquid assays) for further evaluation based on their outstanding amylolytic activity.

2.5. Protein analysis and enzymatic assays

The *S. cerevisiae* strains were inoculated in 125 mL Erlenmeyer flasks containing 20 mL 2 × SC and incubated at 30 °C for 48 h. Protein species in the extracellular crude supernatant were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Proteins were visualized with the silver staining method (O'Connell and Stults, 1997) using the Page Ruler Prestained SM0671 Protein Ladder (Fermentas, Shenzhen, China) as a molecular mass marker. Enzyme activity assays for quantitative analysis were performed as described by Cripwell et al. (2019a). All assays were conducted in triplicate, and activities were reported as units per volume (U/mL), where one unit is defined as the amount of enzyme required to release 1 μmol of glucose per minute.

2.6. Genomic DNA extraction and sequencing analysis

Genomic DNA was extracted from ER T12.7 cells grown overnight (Cripwell et al., 2019a). Paired-end sequencing with 150-base pair reads was performed using an Illumina NovaSeq sequencer by Novogene Company Limited (Hong Kong), with a sample size of 1 Gb. To increase the assembly quality, DNA was processed at the NGS facility of the Biology Department (University of Padova, Italy); libraries were prepared using the SQK-RBK004 rapid sequencing kit (Basile et al., 2021) and sequenced using the FLO-MIN106 R9 flow cell by Oxford Nanopore MinION single molecule sequencing, which provides long reads. The quality of the long reads was evaluated using NanoPlot (De Coster et al., 2018). The data processing and assembly used a hybrid approach that combined long and short reads as described in Gronchi et al. (2022), with Canu software (v2.1) (Koren et al., 2017) used for the correction and assembly of the long reads, and Pilon (v1.24) to refine the assembly with the short reads. The quality of the *de novo* assembled genome was assessed using QUAST (Gurevich et al., 2013). Copy numbers for the integrated genes (*temA* and *temG_Opt*) were estimated by considering only the short reads, and the results were validated with BLAST, as described by Cripwell et al. (2019a). The genome assembly of *S. cerevisiae* ER T12.7 was deposited with Genbank under the BioProject accession number PRJNA976209.

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Genotype	Reference/ Source
***S. cerevisiae* strains**		
ER[1]	*MATa/α* prototroph	Fermentis, Lesaffre, France
ER T12	δ-integration of *ENO1*$_P$-*temG_Opt-ENO1*$_T$; *ENO1*$_P$-*temA-ENO1*$_T$	Cripwell et al., 2019a
ER T12.3, ER T12.5, ER T12.7, ER T12.9, ER T12.10[2]	δ-integration of *ENO1*$_P$-*temG_Opt-ENO1*$_T$; *ENO1*$_P$-*temA-ENO1*$_T$; *TEF1*$_P$-*kanMX-TEF1*$_T$	This study
Plasmids		
yBBH1-TemG_Opt	*bla URA3 ENO1*$_P$-*temG_Opt-ENO1*$_T$	Cripwell et al., 2019b
pBKD2	δ-*ENO1*$_P$-*ENO1*$_T$-*TEF1*$_P$-*kanMX-TEF1*$_T$-δ	McBride et al., 2008

[1] Ethanol Red™ Version 1, referred to as ER.
[2] Amylolytic transformants with additional integrated copies of the *ENO1*$_P$–*temG_Opt-ENO1*$_T$ gene cassette; the number indicates the transformant number during the screening process.

2.7. Fermentations

For the initial screening, raw corn starch fermentations were performed at 30 °C in YPD broth with 5 g/L glucose and 20% (w/v) raw corn starch (flour) (Cripwell et al. 2019a). The best recombinant strain, *S. cerevisiae* ER T12.7, was further evaluated on alternative starchy substrates together with the ER and ER T12 strains in simultaneous saccharification and fermentation (SSF) and CBP configurations, respectively. A substrate load of 20% (w/v) was used for unripe rice and rice bran, but only 10% (w/v) was used for the potato waste streams with very high viscosity (Izmirlioglu and Demirci, 2012). During the SSF approach, the ER strain was supplemented with STARGEN™ 002 (Genencor International Inc., Palo Alto, CA, USA) at 1.42 μL/g solids, corresponding to 100% of the recommended enzyme loading. An additional control included the ER strain (no amylase production), to determine the amount of ethanol produced from free sugars within each agricultural substrate. Ampicillin (100 mg/L) and streptomycin (50 mg/L) were added to inhibit bacterial contamination. Samples were taken every 24 h and stored at −20 °C. All the fermentations were performed in triplicate.

2.8. Analytical methods, calculations and statistical analysis

For HPLC analyses, supernatant from samples was diluted 2-fold and passed through 0.22 μm nylon syringe filters. Ethanol, maltose, glucose, acetic acid and glycerol were quantified as described by Gronchi et al. (2019). Theoretical CO_2 yields were calculated based on ethanol production (Cripwell et al., 2019a). The ethanol yield (as % of the theoretical yield) was calculated using starch content and initial glucose in the fermentation broth; it was expressed as the amount of ethanol produced per gram of available glucose equivalent. This ethanol yield explicitly omits any additional ethanol produced by the fermentation of free sugars in the agricultural residues. To account for this experimentally, ethanol concentrations obtained by the untransformed *S. cerevisiae* ER strain were always subtracted. The data were analyzed using the Student's *t*-test.

3. Results and discussion

Exogenous enzyme addition accounts for around 8% of the total cost of producing ethanol from corn starch (Görgens et al., 2015). Amylolytic *S. cerevisiae* strains can potentially decrease production costs in the starch-to-ethanol industry by alleviating or reducing the enzyme dosage required for starch hydrolysis (Wang et al., 2021). In previous studies, the *S. cerevisiae* ER T12 strain displayed superior amylase activity and efficiently hydrolyzed raw starch from corn flour, broken rice, triticale and sorghum under CBP conditions (Cripwell et al., 2019a; Malherbe et al., 2023; Myburgh et al., 2019). However, a 24–48 h lag in ethanol

production is often observed during CBP fermentations compared to the SSF approach. In this study, the ER T12 strain was engineered for higher glucoamylase activity to increase ethanol productivity and improve its potential as a CBP yeast for commercial starch fermentations.

3.1. Transformation of ER T12 with additional copies of temG_Opt

The ER T12 strain was transformed with the *temG_Opt* glucoamylase gene cassette (Cripwell et al., 2019a) using delta-integration (Favaro et al., 2010) to enhance its amylolytic activity, as well as with the *kanMX* marker cassette to allow selection on agar plates containing different G418 disulfate salt concentrations. For the initial screening, thirteen stable transformants were selected (all from the selection plates containing 200 μg/mL G418 disulfate salt) and compared with ER T12 for relative glucoamylase activity with liquid assays using soluble starch (see supplementary material). Transformants displaying the highest glucoamylase activity were selected for further characterization, i.e. *S. cerevisiae* ER T12.3, T12.5, T12.7, T12.9 and T12.10.

3.2. Screening ER T12 transformants

Starch plates provided visual confirmation that the selected transformants could hydrolyze soluble starch (see supplementary material). SDS-PAGE analysis confirmed that the size of the recombinant TemA and TemG_Opt protein species were similar to those reported by Cripwell et al. (2019b) (see supplementary material). Recombinant amylases expressed in *S. cerevisiae* displayed signs of glycosylation, with calculated molecular weights of 66.29 kDa and 63.57 kDa for the TemA and TemG_Opt proteins, respectively.

3.3. Quantitative analysis of ER T12 transformants

The supernatant from strains ER T12.3, T12.5, T12.7, T12.9 and T12.10 was evaluated with liquid assays at 30 °C (the typical fermentation temperature) to compare and quantify the total extracellular amylase activity, as well as glucoamylase activity on raw and soluble starch (Table 2). Soluble starch has a higher degree of solubility and a larger accessible surface area than raw starch, facilitating improved substrate:enzyme interactions and resulting in better activity. In contrast, raw starch has a higher degree of crystallinity, and the starch granules are more tightly packed. This makes it more difficult for the amylases to attach to the substrate. Therefore, when performing raw starch assays, the substrate concentration must be increased to 2% (w/v) to allow better susceptibility to enzymatic attack.

Raw and soluble corn starch were included as assay substrates for comparative purposes since the *T. emersonii* amylases (TemA and TemG_Opt) contain a raw starch-binding domain. Although activity on soluble starch is higher and more reproducible, it allows for the standardization of assay conditions (Oliveira et al., 2019); however, it is also important to demonstrate amylase activity on raw (untreated) starch. The *S. cerevisiae* ER T12.7 strain displayed superior hydrolyzing capabilities with a total amylase activity of 10.70 U/mL and glucoamylase activity of 9.14 U/mL on soluble starch after 72 h (Table 2), which was 45% and 68% higher than the ER T12 strain, respectively. Since these amylases work synergistically, the *temA*:*temG_Opt* gene ratio in ER T12.7 seems more suitable for soluble starch than the other transformants. The T12.9 strain displayed slightly higher glucoamylase activity than ER T12.7 on raw starch (1.91 *vs* 1.88 U/mL), and comparable total amylase activity, but ER T12.7 was the better candidate when both raw and soluble starch are considered.

Table 2
Amylase activities (U/mL) after 72 h on raw and soluble corn starch for the industrial *S. cerevisiae* ERT12 transformants expressing additional *temG_Opt* glucoamylase copies.

Strain	2 % (w/v) raw starch		0.2 % (w/v) soluble starch	
	Reducing sugar[a]	Released glucose[b]	Reducing sugar[a]	Released glucose[b]
T12 (control)	1.41 ± 0.08[c]	0.27 ± 0.04	7.40 ± 0.91	5.43 ± 0.38
T12.3	1.67 ± 0.17	0.31 ± 0.03	9.36 ± 0.90	6.91 ± 0.62
T12.5	1.72 ± 0.22	0.35 ± 0.05	9.71 ± 1.42	8.36 ± 0.65
T12.7	1.88 ± 0.10	0.37 ± 0.05	10.70 ± 1.16	9.14 ± 0.65
T12.9	1.91 ± 0.08	0.36 ± 0.05	9.11 ± 0.80	8.34 ± 0.88
T12.10	1.34 ± 0.24	0.30 ± 0.02	7.29 ± 1.14	6.44 ± 0.16

[a] Reducing sugar assay detects all reducing sugars (monosaccharides and oligosaccharides) to quantify total extracellular activity.
[b] Glucose assay detects only glucose and indicates glucoamylase activity.
[c] Values represent the mean of three repeats and standard deviaton is reported.

3.4. Performance of transformants in corn starch fermentations

The five *S. cerevisiae* transformants were subsequently evaluated on raw corn starch under CBP conditions at 30 °C (Fig. 1) and compared to the ER T12 "parental" strain. Additional glucoamylase production by the transformants facilitated an increased ethanol production rate from 20% (w/v) corn starch, with ER T12.7 producing 19.96 g/L more ethanol than ER T12 after 120 h (Table 3). Although ER T12.7 was not as effective under CBP conditions as the SSF control (ER + 100% STARGEN™ 002 dosage), it performed significantly better than the other transformants. The ER T12.9 strain showed promising results with the enzymatic assays (Table 2), but its ethanol productivity was less than ER T12.7 under fermentation conditions (0.57 *vs* 0.63 g/L/h) (Table 3). After 120 h, the ethanol productively of T12.7 almost caught up with the SSF control (0.70 *vs* 0.63 g/L/h); it was significantly higher than the other transformants and 34% higher than the parental ER T12 strain. The estimated carbon conversion displayed by ER T12.7 was 19% higher than ER T12, demonstrating that the starch was hydrolyzed more efficiently and produced more ethanol at this time point. ER T12.7 was thus selected for further evaluation on rice- and potato-based substrates.

3.5. Fermentation of rice and potato by-products

When considering industrial applications, feedstock composition, especially free amino nitrogen, plays an important role in optimizing fermentation conditions (Gomes et al., 2021). Analytical-grade corn flour assists with screening recombinant strains as it yields results with good reproducibility, but its composition differs significantly from agricultural waste and industrial substrates. Furthermore, it lacks the endogenous protein component that plays an essential role in yeast

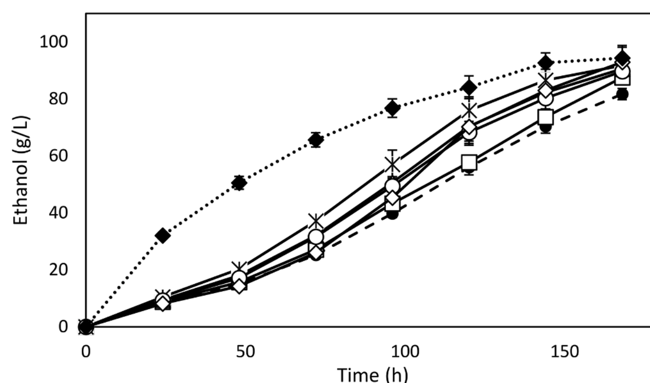

Fig. 1. Raw corn starch fermentation at 30 °C. Ethanol concentrations produced after 168 h by ER T12 (●, broken line), T12.3 (□), T12.5 (Δ), T12.7 (×) T12.9 (○) and T12.10 (◇), as well as ER + 100% STARGEN™ 002 (◆, dotted line). The fermentation was performed in YPD with 5 g/L glucose and 20% (w/v) raw corn starch. The experiments were performed in triplicate and error bars represent the standard deviation from the mean of the replicates.

Table 3
Products released by the *S. cerevisiae* ER T12 transformants after 120 h of fermentation at 30 °C in YPD broth containing 5 g/L glucose and 20% (w/v) raw corn starch.

S. cerevisiae ER strains	ER + 100% SG[1]	T12	T12.3	T12.5	T12.7	T12.9	T12.10
Substrate (g/L)							
Raw starch (dry weight)	185	185	185	185	185	185	185
Glucose equivalent	208.5	208.5	208.5	208.5	208.5	208.5	208.5
Products (g/L)							
Glucose	0.51	0.00	0.35	0.00	0.00	0.23	0.58
Glycerol	3.80	2.15	2.20	2.78	2.70	2.45	2.57
Maltose	0.50	0.47	0.36	0.27	0.29	0.34	0.31
Acetic acid	0.04	1.19	1.32	0.70	0.77	1.15	1.38
Ethanol	84.09	56.00	57.7	70.36	75.96	68.25	70.20
CO_2[2]	80.43	53.56	55.19	67.30	72.65	65.28	67.14
Total products (g/L)	169.37	113.37	117.12	141.41	152.37	137.7	142.18
Estimated carbon conversion (%)	81.21	54.36	56.16	67.80	73.06	66.03	68.17
Ethanol yield[3] (% of theoretical yield)	80.66	53.72	55.34	67.49	72.86	65.47	67.33
Ethanol productivity[4]	0.70	0.47	0.48	0.59	0.63	0.57	0.58

[1] SG = STARGEN™ 002.
[2] CO_2 concentration was deduced from the ethanol produced.
[3] Ethanol yield (% of the theoretical yield) was calculated as the amount of ethanol produced per gram of available glucose.
[4] Ethanol productivity was calculated based on ethanol concentrations produced per h (g/L/h).

fermentations (Gomes et al., 2021). Malherbe et al. (2023) demonstrated that the ER T12 strain could ferment raw triticale and sorghum (bran and flour factions) without any nitrogen supplementation, with ethanol yields exceeding 95% of the theoretical estimate.

To better evaluate the potential of ER T12.7 as a CBP strain on "real" substrates, starchy waste products from rice and potato processes were investigated. These substrates contain different starch levels and granule characteristics, and underwent different processing treatments that would affect enzymatic hydrolysis. Previous studies showed promising results for the SSF of broken rice (Schneider et al., 2018), and the CBP of rice-based substrates using amylolytic industrial strains (Favaro et al., 2017; Myburgh et al., 2019;), whereas potato waste and peels have not yet been evaluated under CBP conditions. The starch and protein content of the rice- and potato-based substrates differed significantly (Table 4); rice bran and potato peel contained less than 30% starch (w/w), substantially lower than unripe rice (68.6% starch). Rice bran had the highest protein content (13.7%), while unripe rice and potato peels contained 9.9% protein, followed by potato waste with 7.9%.

A similar trend in ethanol production was evident across all four substrates (Fig. 2): the SSF control (ER + 100% STARGEN™ 002) displayed slightly higher productivity during the first 24 h, and the ER T12.7 strain in general outperformed ER T12 under CBP conditions. After 120 h, the highest ethanol concentration was reached under CBP conditions on unripe rice (88.76 g/L), in contrast to 82.26 g/L ethanol produced by the SSF control. This was also the maximum ethanol level obtained across the four substrates. The recombinant ER T12 and T12.7 strains efficiently converted the raw starch in 20% (w/v) unripe rice, with 10% more ethanol produced by ER T12.7 than ER T12 after 48 h (Fig. 2**A**). On rice bran, the SSF and CBP approaches reached 43–44 g/L ethanol within 48 h (Fig. 2**B**), with ER T12.7 notably better than ER T12 at 24–48 h. The ER T12.7 strain also displayed enhanced hydrolysis of potato-based substrates. After 72 h, ER T12.7 produced significantly more ethanol than ER T12 on potato waste (34.34 and 29.45 g/L, respectively) (Fig. 2**C**).

The CBP process for potato peels required modifications in terms of substrate loading in the small-scale evaluations; 10% (w/v) resulted in adequate mixing, but 12.5, 15 and 20% w/v resulted in poor to no mixing that prevented yeast growth and substrate hydrolysis. The fermentation of 10% w/v potato peels yielded 18 g/L ethanol under both SSF and CBP conditions after 72 h (100% of the theoretical yield), with similar concentrations produced by ER T12.7 and the SSF control after 30 h (Fig. 2**D**).

Ethanol concentrations produced by ER T12.7 approached that of the SSF control only after 72 h on unripe rice, compared to 30 h for the other three feedstocks with lower starch content (Table 5). These time points were selected to compare the fermentation parameters of the different approaches (i.e. ethanol yield and productivity during SSF and CBP). After 72 h, the newly engineered ER T12.7 produced 54.17 g/L ethanol on unripe rice, a 28 and 38% improvement compared to the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains, respectively (Favaro et al., 2017). These results confirmed the improved hydrolytic ability of ER T12.7 relative to other reported industrial amylolytic strains, including ER T12.

Rice bran, an abundant by-product of rice production, is the grain coating removed during milling. It is an attractive feedstock for ethanol production due to a high protein content of 10–16% (Tiwari et al., 2015). Previous studies used rice bran as a substrate for amylase production under solid-state fermentation (Kumar and Duhan, 2011; Tiwari et al., 2015) and as a supplement during yeast fermentations (Moreira et al., 2019). Favaro et al. (2017) demonstrated the potential of rice bran as a CBP feedstock, although it has significantly less starch than unripe rice. This substrate had the lowest starch content (26.5%, Table 4), and the SSF control produced 37.19 g/L ethanol after 30 h under CBP conditions, compared to 34.55 g/L ethanol by ER T12.7 (91% of the theoretical, Table 5). The higher starch content of unripe rice contributed to the longer "lag phase" when evaluating ethanol production under SSF and CBP conditions, as more starch had to be hydrolyzed than in rice bran.

Table 4
Composition of the starchy substrate as determined through chemical analysis, expressed as % dry matter (DM). Values represent the mean of three technical repeats and standard deviaton is reported.

Substrate	DM (%)	Starch	Cellulose	Hemicellulose	Lignin	Protein	Ash
Unripe rice	87.9	68.6 ± 0.69	1.9 ± 0.13	3.9 ± 0.20	–	9.9 ± 0.75	1.6 ± 0.08
Rice bran	88.6	26.5 ± 0.30	4.6 ± 0.23	8.4 ± 0.41	2.6 ± 0.15	13.7 ± 0.89	8.2 ± 0.55
Potato peel	13.5	29.6 ± 0.33	5.0 ± 0.28	3.1 ± 0.22	2.1 ± 0.17	9.9 ± 0.68	4.5 ± 0.39
Potato waste	11.9	49.1 ± 0.58	2.2 ± 0.15	1.7 ± 0.09	0.8 ± 0.05	7.9 ± 0.59	4.4 ± 0.33
Raw corn starch	90.3	95.3 ± 1.09	ND	ND	ND	0.3 ± 0.04	ND

ND: not determined.

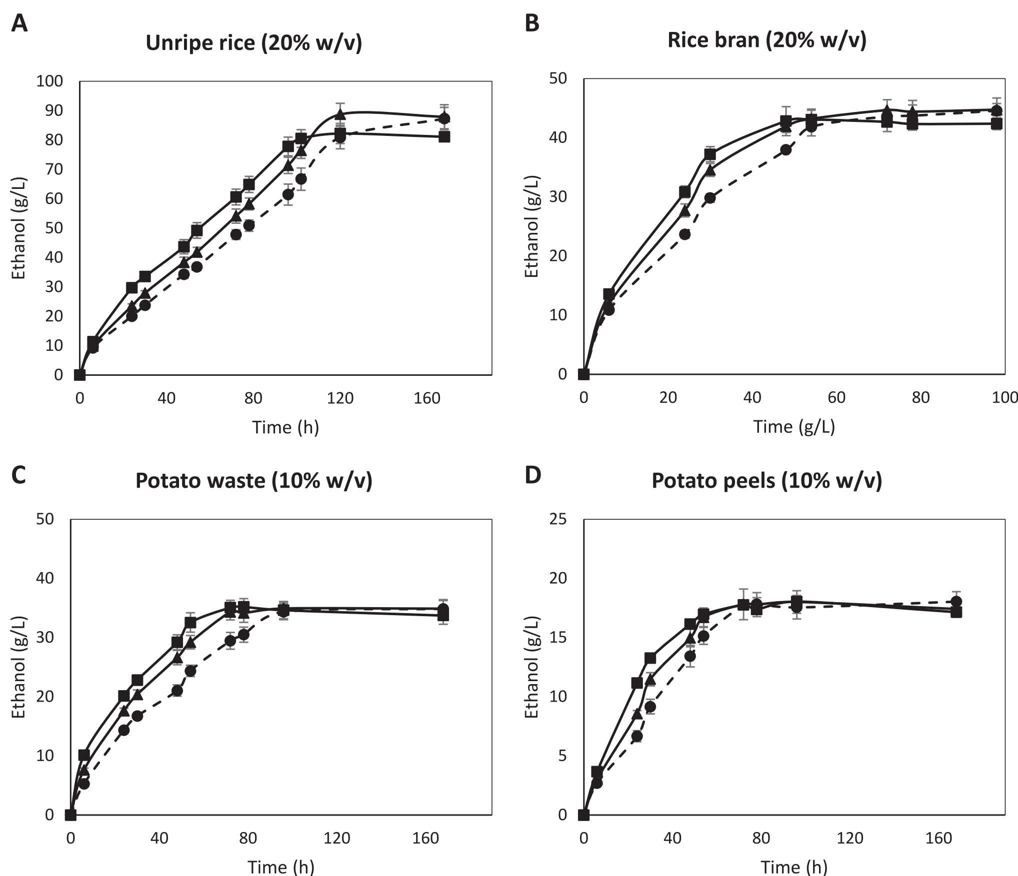

Fig. 2. Ethanol production from the fermentation of rice and potato by-products by *S. cerevisiae* strains: ER T12 (●), ER T12.7 (▲) and ER + 100% STARGEN™ (■). The experiments were performed in triplicate and error bars represent the standard deviation from the mean of the replicates.

Nitrogen, an essential additive during fermentation, is required by yeast for metabolite production and growth (Milessi et al., 2013). The natural/inherent high nitrogen content of rice bran could avoid the need for nutrient supplementation and thus lower the fermentation costs. Moreover, the bran fraction could be converted to fermentable sugars by cellulases, which would further increase the ethanol yields and thus enhance the attractiveness of rice bran as a biofuels feedstock (Cripwell et al., 2015).

Starch-rich by-products of the potato industry (e.g. potato waste and potato peels) are a zero-value waste material produced during potato processing and represent an attractive feedstock for bioethanol production (Arapoglou et al., 2010; Singh et al., 2022). In this study, 10% (w/v) potato waste (49.1% starch content) was efficiently converted to ethanol. After 30 h, ER T12.7 produced almost similar ethanol concentrations to the SSF control (20.37 *vs* 22.81 g/L, respectively, Table 5), and with a higher ethanol productivity than ER T12 (58 *vs* 48 g/L/h). Potato peels are traditionally incorporated into animal feed, but can also be used for the production of ethanol and lactic acid (Liang and McDonald, 2014) and bioplastics (Ebrahimian et al., 2022). The potato peels in this study contained 25% starch and 18% protein (Table 4), similar to that reported by Liang and McDonald (2014). Although rice bran had the lowest starch content, potato peels were the least attractive substrate as they contained only 32.86 g/L glucose equivalents. This significantly contributed to only 1.12 g/L ethanol obtained under fermentation conditions with only ER (no amylases), compared to 7.53 g/L ethanol from potato waste (Table 5).

Potato starch (Sigma) was previously investigated as a substrate for fermentations with *S. cerevisiae* (Kim et al., 2011), but this is the first study to use an industrial amylolytic strain for CBP of potato-based waste substrates. Izmirlioglu and Demirci (2015) adopted industrial potato waste (potato mash with 17–24% starch content), optimized media and commercial amylases for bioethanol production, producing 24.60 g/L ethanol after 48 h. Chohan et al. (2020) optimized the SSF of potato peel wastes to achieve 22.54 g/L ethanol from 12.25% w/v (pH of 5.78, 40 °C). The results presented here thus show significant advances in converting potato starch to ethanol.

3.5.0.1. The effect of starch type: Rice vs potato

Potato starch has a larger granule size, higher amylose content, and is more resistant to α-amylase than rice (Kowsik and Mazumder, 2018; Wang et al., 2022). Furthermore, the rice-based substrates in this study contained noteworthy concentrations of free fermentable sugars, mostly glucose and some maltose, as highlighted by the ethanol concentrations produced under fermentation conditions with the parental ER strain (Table 5). Although rice bran does not require extensive starch hydrolysis (26.5% starch), it can be used for ethanol production if the other carbohydrates can be hydrolyzed to fermentable sugars by cellulases or accessory enzymes. Unripe rice (68.6% starch) showed good potential as a starchy substrate for CBP, and its high protein content (13.2%) would provide additional nitrogen for fermentation (Favaro et al., 2017). Overall, the T12.7 strain greatly improved starch conversion with the lower starch-containing substrates, i.e. rice bran and potato materials. Despite these differences, more than 88% of the theoretical ethanol yield was achieved across all the substrates.

The secretion of recombinant amylases into the fermentation broth by strains ER T12 and ER T12.7 may significantly impact rice hydrolysis since it has a higher amylopectin content and is more easily digestible than potato granules (Kowsik and Mazumder, 2018). The STARGEN™ 002 amylase cocktail used in the SSF approach contains an *Aspergillus kawachii* α-amylase and *Trichoderma reesei* glucoamylase (Adams et al., 2012), while the recombinant amylases expressed by strains ER T12 and ER T12.7 originated from *T. emersonii*. The origin of the amylases and

Table 5
Conversion of rice and potato substrates to ethanol at 30 °C by amylolytic *S. cerevisiae* strains in YPD broth containing 5 g/L glucose.

Time (h)	Component	Glucose equivalent from starch (g/L)	ER	ER + 100% SG[1]	T12	T12.7
			S. cerevisiae strains			
	Unripe rice	152.29				
	Highest ethanol level (g/L)		11.73 ± 0.89	82.26 ± 3.41	87.29 ± 3.83	88.76 ± 3.85
	Ethanol yield[1] (% of theoretical yield)		–	89	94	96
72	Ethanol level		–	60.61 ± 2.69	47.85 ± 1.63	54.17 ± 2.43
	Ethanol productivity[2]		–	0.84	0.66	0.75
	Rice bran	58.83				
	Highest ethanol level (g/L)		15.11 ± 0.98	43.06 ± 1.77	44.58 ± 2.12	44.73 ± 1.04
	Ethanol yield[1] (% of theoretical yield)		–	86	91	91
30	Ethanol level		–	37.19 ± 1.28	29.81 ± 0.70	34.55 ± 1.09
	Ethanol productivity[2]		–	1.24	0.99	1.15
	Potato waste	54.50				
	Highest ethanol level (g/L)		7.53 ± 0.45	35.19 ± 1.41	34.85 ± 1.56	34.93 ± 1.06
	Ethanol yield[1] (% of theoretical yield)		–	91	90	90
30	Ethanol level		–	22.81 ± 0.85	16.73 ± 0.51	20.37 ± 0.78
	Ethanol productivity[2]		–	0.76	0.56	0.68
	Potato peel	32.86				
	Highest ethanol level (g/L)		1.12 ± 0.19	18.06 ± 0.77	18.05 ± 0.83	18.01 ± 0.95
	Ethanol yield[2] (% of theoretical yield)		–	88	88	88
30	Ethanol level		–	13.27 ± 0.10	9.16 ± 0.62	11.48 ± 0.56
	Ethanol productivity[3]		–	0.44	0.31	0.38

[1] SG = STARGEN™ 002.
[2] Ethanol yield (% of the theoretical yield) was calculated as the amount of ethanol produced per gram of available glucose equivalent from starch and the 5 g/L glucose added at the beginning of the fermentation.
[3] Ethanol productivity was calculated based on ethanol concentrations produced per h (g/L/h).

the potential synergy between the enzymes can be important for the digestibility of different starch types (Wang et al., 2022). Considering the final ethanol yields achieved during SSF and CBP, the recombinant strains produced higher ethanol concentrations than the SSF control on rice by-products (Fig. 2**A, B**). In contrast, the final ethanol concentrations were almost identical on the potato substrates (Fig. 2**C, D**). Potato waste was more amenable to hydrolysis by commercial enzymes, indicating that enzyme synergy is an important consideration when optimizing the conversion of different starchy materials. This phenomenon was also reported by Myburgh et al. (2019), with recombinant strains that outperformed the SSF control in broken rice fermentations.

3.5.0.2. Fermentation configurations: SSF vs CBP

Starch characteristics played an important role in digestibility and resulted in noticeable differences in the fermentation kinetics between corn starch, rice and potato substrates during SSF and CBP. The lag phase was very prominent on raw corn starch (Fig. 1) as the recombinant strains had to produce amylases before hydrolysis, resulting in low ethanol production at the start of the fermentation. However, there was a shorter lag phase during the rice and potato fermentations (Fig. 2), with free sugars positively affecting ethanol production. The extra *temG_Opt* gene cassettes provided the ER T12.7 strain with significantly higher activity on starch and improved performance under raw starch fermentation conditions using a CBP approach.

ER T12.7′s superior amylolytic activity and ability to efficiently convert a variety of untreated starchy feedstocks to ethanol represent a significant achievement for CBP. The ethanol productivity was also enhanced, reducing the lag phase observed during the first 24–48 h of CBP fermentation. These results demonstrated that the ER T12.7 strain could bring CBP closer to directly converting starchy biomass without requiring exogenous amylase addition. Alternatively, ER R12.7 could represent a possible "drop-in" yeast for more conventional SSF starch-to-ethanol processes - thus decreasing the costs associated with enzyme addition. In addition to cost-effectiveness, using yeast to produce recombinant amylases offers a promising approach for enhancing the conversion efficiency and sustainability of biofuel production from starch. It capitalizes on the strengths of yeast fermentation and recombinant protein secretion to contribute to developing a more economical and environmentally friendly biofuel industry. Techno-economical assessment of the overall proof-of-concept will be crucial for selecting the most efficient process and feedstock for the large-scale conversion of starchy waste steams into bioethanol.

3.6. Genome mining of s. Cerevisiae ER T12.7 for relevant features

Numerous yeast engineering studies have employed δ-elements for gene insertions because of their abundance in the *S. cerevisiae* genome (Cripwell et al., 2020; Da Silva and Srikrishnan, 2012; Den Haan et al., 2021). Strain ER T12.7 exhibited the highest amylolytic activity during liquid assays on soluble corn starch and displayed the best ethanol productivity on raw corn starch under fermentative conditions (Tables 2, 3 and Fig. 1).

Table 6
Gene copy number estimated for integrated *temA* and *temG_Opt* genes (bold) in strains ER T12 and ER T12.7, considering the ratio between the average coverage of the recombinant genes and the average coverage of four housekeeping genes.

Gene	ER T12[a]		ER T12.7	
	Coverage	Copy number	Coverage	Copy number
temA	152	***4.46***	440	***4.37***
temG_Opt	250	***7.20***	1106	***11.21***
ALG9	34	1.00	97	0.98
TFC1	34	1.00	98	0.99
PGK1	34	1.00	101	1.02
ACT1	35	1.03	99	1.00
Average housekeeping genes	34	–	99	–

[a] Data from Illumina sequencing by Cripwell et al. (2019a).

Based on sequencing results using the Illumina NovaSeq platform, the newly engineered ER T12.7 strain had an estimated 11 copies of *temG_Opt* and retained the four copies of the *temA* α-amylase gene in ER T12. Furthermore, the 1:275 amylase/glucoamylase ratio for ER T12.7 was closer to the baseline ratio of 1:2.5 reported by Gronchi et al. (2022); therefore supporting the previous findings that higher glucoamylase activity is required for improved CBP of raw starch. The newly constructed ER T12.7 contained four more glucoamylase genes than strain ER T12 (Cripwell et al., 2019a), a 1.5-fold increase in gene copies (Table 6). The high-quality hybrid *de novo* assembly of 11.7 Mb, comprising 61 scaffolds with an N_{50} of 53659, confirmed this result. The BLAST alignment for both recombinant genes in the assembly indicated that ER T12.7 has 4 and 11 copies of *temA* and *temG_Opt*, respectively. Since gene location plays an important role in how the recombinant strain performs, it may be unreliable to correlate the specific activity to the number of gene copies. However, the additional four glucoamylase gene copies facilitated a significant increase in total amylase activity, suggesting some positive correlation that enhanced the ER T12.7 strain's CBP hydrolytic capabilities for raw starch conversion.

4. Conclusion

The amylolytic ER T12 strain was engineered to have increased glucoamylase activity by using a delta integration technique to introduce an additional four copies of the *temG_Opt* gene. The selected recombinant demonstrated significant improvements in ethanol productivity on raw starch substrates. Both rice- and potato-based substrates were efficiently converted into ethanol, thus proving their potential in establishing a starch bioeconomy. Compared to the STARGEN™ 002 cocktail saccharification, the recombinant amylases were more effective on rice-based substrates than potato waste and peels. Overall, unripe rice represented an attractive substrate for ethanol production with the CBP approach.

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CRediT authorship contribution statement

Rosemary A. Cripwell: Conceptualization, Investigation, Formal analysis, Writing – original draft, Resources. **Rebecca My:** Formal analysis. **Laura Treu:** Software, Formal analysis. **Stefano Campanaro:** Formal analysis. **Lorenzo Favaro:** Conceptualization, Writing – review & editing, Supervision, Resources. **Willem H. van Zyl:** Supervision, Resources. **Marinda Viljoen-Bloom:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rosemary Cripwell reports financial support, equipment, drugs, or supplies, and travel were provided by National Research Foundation. Favaro Lorenzo reports travel was provided by Government of Italy Ministry of Foreign Affairs and International Cooperation. Willem H. van Zy reports financial support, equipment, drugs, or supplies, and travel were provided by National Research Foundation. Rosemary Cripwell and Willem H. van Zy has patent RECOMBINANT YEAST AND USE THEREOF issued to Assignee.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Adams, J.M.M., Teunissen, P.J.M., Robson, D.-C., 2012. Scanning electron microscopy and fermentation studies on selected known maize starch mutants using STARGEN™ enzyme blends. Bioenerg. Res. 5, 330–340.

Arapoglou, D., Varzakas, T., Vlyssides, A., Israilides, C., 2010. Ethanol production from potato peel waste (PPW). Waste Manag. 30, 1898–1902.

Basile, A., De Pascale, F., Bianca, F., Rossi, A., Frizzarin, M., De Bernardini, N., Bosaro, M., Baldisseri, A., Antoniali, P., Lopreiato, R., Treu, L., Campanaro, S., 2021. Large-scale sequencing and comparative analysis of oenological *Saccharomyces cerevisiae* strains supported by nanopore refinement of key genomes. Food Microbiol. 97, 103753.

Brojanigo, S., Parro, E., Cazzorla, T., Favaro, L., Basaglia, M., Casella, S., 2020. Conversion of starchy waste streams into polyhydroxyalkanoates using *Cupriavidus necator* DSM 545. Polymers 12, 1496.

Brojanigo, S., Gronchi, N., Cazzorla, T., Wong, T.S., Basaglia, M., Favaro, L., Casella, S., 2022. Engineering *Cupriavidus necator* DSM 545 for the one-step conversion of starchy waste into polyhydroxyalkanoates. Bioresour. Technol. 347, 126383.

Chohan, N.A., Aruwajoye, G.S., Sewsynker-Sukai, Y., Gueguim Kana, E.B., 2020. Valorisation of potato peel wastes for bioethanol production using simultaneous saccharification and fermentation: process optimization and kinetic assessment. Renew. Energy 146, 1031–1040.

Cripwell, R., Favaro, L., Rose, S.H., Basaglia, M., Cagnin, L., Casella, S., van Zyl, W.H., 2015. Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amylolytic yeast. Appl. Energy 160, 610–617.

Cripwell, R.A., Rose, S.H., Favaro, L., van Zyl, W.H., 2019a. Construction of industrial *Saccharomyces cerevisiae* strains for the efficient consolidated bioprocessing of raw starch. Biotechnol. Biofuels 12, 201.

Cripwell, R.A., Rose, S.H., Viljoen-Bloom, M., van Zyl, W.H., 2019b. Improved raw starch amylase production by *Saccharomyces cerevisiae* using codon optimization strategies. FEMS Yeast Res. 19, 1–14.

Cripwell, R.A., Favaro, L., Viljoen-Bloom, M., van Zyl, W.H., 2020. Consolidated bioprocessing of raw starch to ethanol by *Saccharomyces cerevisiae*: achievements and challenges. Biotechnol. Adv. 42, 107579.

Da Silva, N.A., Srikrishnan, S., 2012. Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. FEMS Yeast Res. 12, 197–214.

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M., Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics 34, 2666–2669.

Den Haan, R., Rose, S.H., Cripwell, R.A., Trollope, K.M., Myburgh, M.W., Viljoen-Bloom, M., van Zyl, W.H., 2021. Heterologous production of cellulose-and starch-degrading hydrolases to expand *Saccharomyces cerevisiae* substrate utilization: lessons learnt. Biotechnol. Adv. 53, 107859.

Dongyu, Q., 2022. Role and potential of potato in global food security challenges of global food security contribution of potato to the world potential of global potato production strategies for promoting potato development. Food and Agriculture Organization of the United Nations. Available online: https://www.fao.org/3/cc0330en/cc0330en.pdf (accessed on 23 August 2023).

Ebrahimian, F., Denayer, J.F.M., Karimi, K., 2022. Potato peel waste biorefinery for the sustainable production of biofuels, bioplastics, and biosorbents. Bioresour. Technol. 360, 127609.

Favaro, L., Basaglia, M., Saayman, M., Rose, S., van Zyl, W.H., Casella, S., 2010. Engineering amylolytic yeasts for industrial bioethanol production. Chem. Eng. Trans. 20, 97–102.

Favaro, L., Cagnin, L., Basaglia, M., Pizzocchero, V., van Zyl, W.H., Casella, S., 2017. Production of bioethanol from multiple waste streams of rice milling. Bioresour. Technol. 244, 151–159.

Gomes, D., Cruz, M., de Resende, M., Ribeiro, E., Teixeira, J., Domingues, L., 2021. Very high gravity bioethanol revisited: main challenges and advances. Fermentation 7 (1), 38.

Görgens, J.F., Bressler, D.C., van Rensburg, E., 2015. Engineering *Saccharomyces cerevisiae* for direct conversion of raw, uncooked or granular starch to ethanol. Crit. Rev. Biotechnol. 35, 369–391.

Gronchi, N., Favaro, L., Cagnin, L., Brojanigo, S., Pizzocchero, V., Basaglia, M., Casella, S., 2019. Novel yeast strains for the efficient saccharification and fermentation of starchy by-products to bioethanol. Energies 12, 714.

Gronchi, N., De Bernardini, N., Cripwell, R.A., Treu, L., Campanaro, S., Basaglia, M., Foulquié-Moreno, M.R., Thevelein, J.M., van Zyl, W.H., Favaro, L., Casella, S., 2022. Natural *Saccharomyces cerevisiae* strain reveals peculiar genomic traits for starch-to-bioethanol production: the design of an amylolytic consolidated bioprocessing yeast. Front. Microbiol. 12, 768562.

Gupte, A.P., Basaglia, M., Casella, S., Favaro, L., 2022. Rice waste streams as a promising source of biofuels: feedstocks, biotechnologies and future perspectives. Renew. Sust. Energ. Rev. 167, 112673.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29, 1072–1075.

Hashem, M., Asseri, T.Y.A., Alamri, S.A., Alrumman, S.A., 2019. Feasibility and sustainability of bioethanol production from starchy restaurants' bio-wastes by new yeast strains. Waste Biomass Valoriz. 10, 1617–1626.

Izmirlioglu, G.G., Demirci, A., 2012. Ethanol production from waste potato mash by using *Saccharomyces cerevisiae*. Appl. Sci. 2, 738–753.

Izmirlioglu, G., Demirci, A., 2015. Enhanced bio-ethanol production from industrial potato waste by statistical medium optimization. Int. J. Mol. Sci. 16, 24490–24505.

Kim, H.R., Im, Y.K., Ko, H.M.C., 2011. Raw starch fermentation to ethanol by an industrial distiller's yeast strain of *Saccharomyces cerevisiae* expressing glucoamylase and α-amylase genes. Biotechnol. Lett 33, 1643–1648.

Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H., Phillippy, A.M., 2017. Canu: scalable and accurate long-read assembly via adaptive *k*-mer weighting and repeat separation. Genome Res. 27, 722–736.

Kowsik, P.V., Mazumder, N., 2018. Structural and chemical characterization of rice and potato starch granules using microscopy and spectroscopy. Microsc. Res. Tech. 81, 1533–1540.

Kumar, A., Duhan, J.S., 2011. Production and characterization of amylase enzyme isolated from *Aspergillus niger* MTCC-104 employing solid state fermentation. Int. J. Pharma. Bio Sci. 2, 250–258.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of becteriophage T4. Nature 227, 680–685.

Li, J., Zhao, R., Xu, Y., Wu, X., Bean, S.R., Wang, D., 2022. Fuel ethanol production from starchy grain and other crops: An overview on feedstocks, affecting factors, and technical advances. Renew. Energy 188, 223–239.

Liang, S., McDonald, A.G., 2014. Chemical and thermal characterization of potato peel waste and its fermentation residue as potential resources for biofuel and bioproducts production. Agric. Food Chem. 62 (33), 8421–8429.

Malherbe, S.J.M., Cripwell, R.A., Favaro, L., van Zyl, W.H., Viljoen-Bloom, M., 2023. Triticale and sorghum as feedstock for bioethanol production via consolidated bioprocessing. Renew. Energy 206, 498–505.

McBride, J.E.E., Deleault, K.M., Lynd, L.R., Pronk, J.T., 2008. Recombinant yeast strains expressing tethered cellulase enzymes. Patent. PCT/US2007/085390.

Milessi, T.S., Antunes, F.A., Chandel, A.K., Silva, S.S., 2013. Rice bran extract: an inexpensive nitrogen source for the production of 2G ethanol from sugarcane bagasse hydrolysate. 3. Biotech 3, 373–379.

Moreira, V.R., Rocha Lebron, Y.A., Freire, S.J., Palladino, F., de Souza Santos, L.V., Jacob, R.S., 2019. Evaluation of rice bran as a supplement for production of bioethanol by *Saccharomyces cerevisiae*. Floresta e Ambiente 26, 1–8.

Myburgh, M.W., Cripwell, R.A., Favaro, L., van Zyl, W.H., 2019. Application of industrial amylolytic yeast strains for the production of bioethanol from broken rice. Bioresour. Technol. 294, 122222.

Myburgh, M.W., Rose, S.H., Viljoen-Bloom, M., 2020. Evaluating and engineering *Saccharomyces cerevisiae* promoters for increased amylase expression and bioethanol production from raw starch. FEMS Yeast Res. 20 (6), 1–14.

O'Connell, K., Stults, J.T., 1997. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. Electrophoresis 18, 349–359.

Oliveira, H.M., Pinheiro, A.Q., Fonseca, A.J., Cabrita, A.R., Maia, M.R., 2019. Flexible and expeditious assay for quantitative monitoring of alpha-amylase and amyloglucosidase activities. MethodsX 6, 246–258.

Sakwa, L., Cripwell, R.A., Rose, S.H., Viljoen-Bloom, M., 2018. Consolidated bioprocessing of raw starch with *Saccharomyces cerevisiae* strains expressing fungal alpha-amylase and glucoamylase combinations. FEMS Yeast Res. 18 (7), 1–10.

Schneider, R.C.S., Seidel, C., Fornasier, F., De Souza, D., Corbellini, V.A., 2018. Bioethanol production from broken rice grains. Interciencia 43, 846–851.

Singh, A., Singhania, R.R., Soam, S., Chen, C.W., Haldar, D., Varjani, S., Chang, J.S., Dong, C.D., Patel, A.K., 2022. Production of bioethanol from food waste: Status and perspectives. Bioresour. Technol. 360, 127651.

Tiwari, S., Jadhav, S.K., Tiwari, K.L., 2015. Bioethanol production from rice bran with optimization of parameters by *Bacillus cereus* strain McR-3 Int. J. Environ. Sci. Technol. 12, 3819–3826.

Van Zyl, W.H., Bloom, M., Viktor, M.J., 2012. Engineering yeasts for raw starch conversion. Appl. Microbiol. Biotechnol. 95, 1377–1388.

Wang, F., Jiang, Y., Guo, W., Niu, K., Zhang, R., Hou, S., Wang, M., Yi, Y., Zhu, C., Jia, C., Fang, X., 2016. An environmentally friendly and productive process for bioethanol production from potato waste. Biotechnol. Biofuels 9, 1–10.

Wang, X., Liao, B., Li, Z., Liu, G., Diao, L., Qian, F., Yang, J., Jiang, Y., 2021. Reducing glucoamylase usage for commercial-scale ethanol production from starch using glucoamylase expressing *Saccharomyces cerevisiae*. Bioresour. Bioprocess. 8, 20.

Wang, Y., Ral, J.P., Saulnier, L., Kansou, K., 2022. How does starch structure impact amylolysis? Review of current strategies for starch digestibility study. Foods 11, 1–19.