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Consolidated bioprocessing of starchy substrates into ethanol by industrial *Saccharomyces cerevisiae* strains secreting fungal amylases

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Abstract

The development of a yeast strain that converts raw starch to ethanol in one step (called Consolidated Bioprocessing, CBP) could significantly reduce the commercial costs of starch-based bioethanol. An efficient amylolytic *Saccharomyces cerevisiae* strain suitable for industrial bioethanol production was developed in this study. Codon-optimized variants of the *Thermomyces lanuginosus* glucoamylase (*TLG1*) and *Saccharomycopsis fibuligera* α-amylase (*SFA1*) genes were δ-integrated into two *S. cerevisiae* yeast with promising industrial traits, i.e. strains M2n and MEL2. The recombinant M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] yeast displayed high enzyme activities on soluble and raw starch (up to 8118 and 4461 nkat/g dry cell weight, respectively) and produced about 64 g/L ethanol from 200 g/L raw corn starch in a bioreactor, corresponding to 55% of the theoretical maximum ethanol yield (g of ethanol/g of available glucose equivalent). Their starch-to-ethanol conversion efficiencies were even higher on natural sorghum and triticale substrates (62 and 73% of the theoretical yield, respectively). This is the first report of direct ethanol production from natural starchy substrates (without any pre-treatment or commercial enzyme addition) using industrial yeast strains co-secreting both a glucoamylase and α-amylase.

Keywords: Consolidated Bioprocessing (CBP); industrial yeast; codon optimization; raw starch; sorghum; triticale;

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Introduction

Plant biomass is an abundant and renewable feedstock for the sustainable production of biofuels and plant-derived chemicals. Biofuels, which includes bioethanol, can be obtained from dedicated crops (e.g. sugarcane and corn), by-products of agricultural processing activities (e.g. sugarcane bagasse) or even organic municipal waste. Lignocellulosic biomass is the preferred substrate as it is more abundant and less expensive than sucrose and starch substrates (Demirbas, 2009; Jang et al. 2012). However, the limitations associated with lignocellulosic ethanol production include the slow rate of enzymatic degradation, high enzyme cost and the requirement of inhibitor-tolerant industrial yeast strains (den Haan et al. 2013; Favaro et al. 2013a). Consequently, starch is still the most commonly used feedstock for ethanol production, with a relatively mature technology developed for corn in the USA (Brehmer et al. 2008) that produced about 52.5 billion litres of bioethanol in 2012, an increase from 49.2 billion litres in 2010 (Renewable Fuels Association, Falling walls & rising tides - 2012 Ethanol industry outlook, Washington).

Besides wheat and corn grains, starchy by-products such as wasted crop, cereal bran, cassava pulp and brewery-spent grains, have been proposed as alternative low-cost feedstocks for the production of bioethanol (Apiwatanapiwat et al. 2011; Favaro et al. 2012a; Favaro et al. 2013b; Kim and Dale 2004). However, current starch-to-ethanol processes require an energy-intensive liquefaction step as well as substantial amounts of exogenous amylases for enzymatic hydrolysis of raw starch; both these significantly impact the economic viability of starch as feedstock (van Zyl et al. 2012).

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Starch hydrolysing enzymes are abundant in the animal, microbial and plant kingdoms, but only a selected few are able to hydrolyse raw starch (van Zyl et al. 2012). Species of *Aspergillus*, *Fusarium*, *Lipomycetes*, *Mucor*, *Penicillium*, *Rhizopus* and *Rhizomucor* express α- and/or glucoamylases (Sun et al. 2010) and some *Aspergillus* and *Rhizopus* spp. have already been exploited for the commercial production of glucoamylases in the food industry (Jin et al. 1999; Koutinas et al. 2003). Raw starch degrading enzymes (RSDE), that both liquefy and saccharify raw starch, can significantly reduce the energy requirements and simplify the production of starch-based biofuels (Robertson et al. 2006). However, a limited number of RSDE have been cloned and characterized, e.g. α-amylases from *Lipomyces kononenkoae* (Eksteen et al. 2004; Knox et al. 2004; Ramachandran et al. 2008), *Streptomyces bovis* (Yamada et al. 2010a), *Cryptococcus* and *Bacillus* (Gupta et al. 2003; Sun et al. 2010), as well as glucoamylases from *Rhizopus oryzae* (Yamada et al. 2010a), *Corticium rolfsii, Saccharomycopsis fibuligera* (Eksteen et al. 2004; Sun et al. 2010), *Aspergillus awamori* (Favaro et al. 2012b) and *Aspergillus tubingensis* (Viktor et al. 2013).

Cost-effective conversion of raw starch to biofuels requires the production of starch-hydrolysing enzymes by a fermenting yeast to achieve liquefaction, hydrolysis and fermentation (Consolidated Bioprocessing, CBP) in a single organism. The yeast *Saccharomyces cerevisiae* remains the preferred host for ethanol production due to its high ethanol, osmo- and inhibitor tolerance in industrial processes, but it lacks the enzymes for the hydrolysis of starch (Favaro et al. 2013c; van Zyl et al. 2012). This could potentially be overcome by engineering *S. cerevisiae* strains for heterologous expression of the enzymes required for starch utilization.

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Co-expression of α-amylases and glucoamylases through extracellular secretion or tethering of enzymes on the cell surface of mainly *S. cerevisiae* laboratory yeast strains has previously been reported (reviewed in van Zyl et al. 2012). Although several amylolytic *S. cerevisiae* strains displayed a high conversion rate for raw starch, it was mostly demonstrated at low starch loadings (1 to 2% w/v) that will not be economically viable on an industrial scale (Den Haan et al. 2013). A polyploid *S. cerevisiae* strain, secreting both the *Aspergillus awamori* GA1 and *Debaryomyces occidentalis* AMY, converted 80% of 200 g/L raw starch with 80 g/L ethanol produced after 6 days, equating to 0.56 g/L/h (Kim et al. 2011). Similarly, Viktor et al. (2013) reported that the semi-industrial *S. cerevisiae* Mnuα1 strain, expressing the *A. tubingensis* α-amylase and glucoamylase genes, was able to completely hydrolyse 200 g/L raw corn starch within 5 days, producing 70 g/L ethanol (0.58 g/L/h). Both recombinant strains were only evaluated on small-scale, whereas bioreactor experiments are essential to proof the concept of raw starch CBP.

The challenge remains to engineer a robust industrial yeast strain that can effectively liquefy and saccharify high concentrations of raw starch, while simultaneously fermenting the sugars to ethanol (van Zyl et al. 2012). Industrial yeast strains are more robust than laboratory strains and display more valuable traits, including higher ethanol productivity and yield, thermostability and higher tolerance to acids, ethanol and sugar (Favaro et al. 2013a,c). They are also reasonably stable in a variety of manufacturing conditions, including drying and long-term storage. Their genetic engineering, however, is challenging and the use of episomal plasmids is undesirable as their maintenance depends on selectable markers (Romanos et al. 1992). Reiterated DNA sequences such as δ-sequences of the Ty retrotransposon and ribosomal DNA have been efficiently used as target sites to ensure the

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integration of multiple gene copies and therefore high expression levels (Favaro et al. 2010; Yamada et al. 2010b).

In this study, two novel robust *S. cerevisiae* strains were engineered to simultaneously produce and secrete the *Thermomyces lanuginosus* glucoamylase, TLG1, and the *S. fibuligera* α-amylase, SFA1, for raw starch hydrolysis and fermentation. The sequences were codon-optimized and the recombinant enzymes partially characterized by extracellular amylolytic activity and SDS-PAGE. The hydrolysis and fermentation of raw corn starch were evaluated in a bioreactor configuration at high substrate loading (200 g/L) and compared to the natural starchy substrates, sorghum and triticale.

Materials and methods

Media and growth conditions

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Recombinant plasmids were constructed and amplified in *E. coli* DH5α. The bacterial strains were cultured at 37°C on a rotating wheel in Terrific Broth or on LB agar (Sambrook and Russel, 2001). Ampicillin was added to a final concentration of 100 µg/mL for the selection of plasmid-bearing bacteria. The *S. cerevisiae* strains were cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose). Recombinants were selected on YPD agar plates containing 200 – 300 µg/mL geneticin (G418, Sigma-Aldrich, UK), and screened for starch hydrolysis on synthetic complete (SC) starch plates containing 6.7 g/L yeast nitrogen base (Sigma-Aldrich, UK), 20 g/L corn starch (Sigma-Aldrich, UK) and 20 g/L agar.

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For bioreactor studies, a modified YPD containing 5 g/L glucose, 100 mg/L ampicillin and 15 mg/L streptomycin (to inhibit bacterial contamination), 3 mL/L ethanol, 3 mL/L Tween 20 and 18 mg/L ergosterol was used. Raw corn starch (Sigma-Aldrich, UK), triticale (*X Triticosecale* Wittmack, cultivar US2007) or sweet sorghum (*Sorghum bicolor* L., cultivar PAN8816) was added at a concentration of 200 g/L. The triticale and sorghum seeds (provided by Dr. Willem Botes, Department of Genetics, Stellenbosch University) were milled and sieved, with the fractions smaller than 500 µm pooled and used as substrate.

Strains and plasmids

The genotype and origin of plasmids, yeast and bacterial strains used in this work are summarized in Table I.

DNA manipulations and plasmid construction

Restriction enzyme digestion, electrophoresis, DNA ligation, transformation and DNA preparation from *E. coli* were performed using standard methods (Sambrook and Russel, 2001). Enzymes for restriction digests and ligations were sourced from Roche Applied Science (Germany) and used as recommended by the supplier. DNA fragments were purified from agarose gels using the Gene Clean kit (Qbiogene Inc., USA).

The synthetically designed *T. lanuginosus TLG1* and *S. fibuligera SFA1* genes (GenBank accession number EF545003.1 and E03536.1, respectively) were codon-optimized (GenArt Corporation, USA) for expression in *S. cerevisiae* (Sharp and Cowe, 1991) with the native

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secretion signals intact. The *Pac*I and *Asc*I restriction sites were added to the 5' and 3'-ends of the sequences, respectively.

The synthetic *SFA1* gene was subcloned into the *Pac*I and *Asc*I sites of pBKD1 to create plasmid pSFA1, whereas the synthetic *TLG1* gene was subcloned in the same restriction sites on pBKD2 to obtain plasmid pTLG1 (Figure 1). The $ENO1_P$-*TLG1*-$ENO1_T$ cassette was excized from pTLG1 with *Spe*I and *Not*I digestion and subcloned into the corresponding sites of pSFA1 to generate pSFA1-TLG1 (Figure 1).

Bacterial and fungal transformations

Recombinant plasmids were transformed into chemically competent *E. coli* cells, followed by selection on LB-ampicillin agar plates. The industrial *S. cerevisiae* strains were engineered by means of electroporation (Favaro et al. 2012b). The plasmids were digested with *Xho*I prior to transformation and recombinant yeast cells were selected on YPD-geneticin agar plates supplemented with 1 M sorbitol.

The *S. cerevisiae* strains were transferred onto SC-starch plates and cultured for 4 days at 30°C. Plates were transferred to 4°C to allow precipitation of the residual starch, with a clear zone around the colony indicative of starch hydrolysis.

For quantitative assays, yeast recombinants were aerobically cultivated in 50 mL YPD medium at 30°C with agitation at 200 rpm with sampling at 24 h intervals. The supernatant was obtained by centrifugation (5 min, 2,235 x *g*) and extracellular enzymatic activities were determined.

The total amylase activity of strains expressing both α-amylase and glucoamylase was determined in liquid assays using the reducing sugar assay with glucose as standard (Miller,

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1959). The optimal enzyme pH was assessed at 50°C with 50 µL of the supernatant and 450 µL of the substrate (0.1% soluble potato starch or 2% raw corn starch) suspended in 0.05 M citrate-phosphate buffer at pH values from 3.5 to 6.5. The optimal assay temperature was determined at pH 4.5 using temperatures ranging from 30 to 70°C. The enzymatic reactions were conducted for 10 min and terminated by boiling in a waterbath for 15 min. The colorimetric changes were measured spectrophotometrically at 540 nm with a microtitre plate reader (Tecan Spectrafluor, Milan, Italy). Similar procedures were used to quantify the glucose released from soluble and raw corn starch, with the peroxidase-glucose oxidase method using the D-Glucose assay kit (Boehringer Mannheim-R-Biopharm, Germany). Enzymatic activities were expressed as nanokatals per gram dry cell weight (nkat/g DCW), which is defined as the enzyme activity required to produce 1 nmol of glucose per second per gram dry cell weight. All experiments were carried out in triplicate.

Electrophoresis and zymogram analysis

Recombinant *S. cerevisiae* strains were cultivated in 20 mL SC medium and the supernatant was harvested after 3 days. Two micrograms of lyophilized supernatant were separated by SDS-PAGE using two duplicate 8% separation gels (Laemmli, 1970). Electrophoresis was carried out at 100 V for 90 min at room temperature and protein species on the one gel was visualized with the silver staining method (O'Connell and Stults, 1997). The unstained gel was washed with citrate-phosphate buffer (pH 4.5) for 30 min at room temperature with gentle agitation to remove the SDS before transfer onto a plate containing 2% soluble starch (pH 6). The gel was removed after 24 hours at 30°C and the starch plate stained with a 10% iodine solution.

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Small-scale and bioreactor fermentation studies with high substrate loading

Small-scale fermentations were conducted in 120 mL serum bottles containing 100 mL YPD with 200 g/L glucose inoculated with 50 g/L wet cell weight of yeast cultures grown for 72 h at 30°C. The fermentations were carried out under oxygen-limited conditions and the bottles, equipped with a bubbling CO_2 outlet, were incubated at 30°C on a magnetic stirrer. Samples were taken through a capped syringe needle pierced through the bottle stopper.

For bioreactor experiments, pre-cultures were cultivated in 200 mL YPD medium (in 2 L Erlenmeyer flasks) for 48 h at 30°C on a shaker platform (100 rpm). Bioreactor fermentations were performed in a 2L MultiGen Bioreactor (New Brunswick Scientific Corporation, Edison, New Jersey, USA) with a wet cell loading of 50 g/L in 1 L modified YPD supplemented with 200 g/L raw corn starch, triticale or sweet sorghum as carbon source. The wet cell weight was determined by weighing a cell pellet obtained from centrifugation of the pre-culture at 3000 x g for 5 min. The triticale and sorghum substrates contained 63% and 73.5% starch per dry weight (DW), respectively. Fermentations were carried out at 30°C with stirring at 100 rpm and regular sampling of fermentation broth through a designated sampling port.

Analytical methods and calculations

Ethanol, glycerol, maltose and glucose concentrations were quantified with HPLC (Shimadzu, Japan) equipped with a refractive index detector. A cation-H refill cartridge

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(Bio-Rad, Hercules, USA) preceding the Aminex HPX-87H column (Bio-Rad, Hercules, USA), which was run at 65°C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.5 mL/min.

The ethanol yield (g of ethanol/g of available sugar) was calculated considering the amount of glucose equivalent available at the beginning of the fermentation. The theoretical CO_2 yields were calculated based on the ethanol concentrations, assuming that equimolar ethanol and CO_2 are produced. The percentage starch converted to glucose, maltose, glycerol, ethanol and CO_2 was calculated on a mole carbon basis. The volumetric productivity (Q) was based on grams of ethanol produced per litre of culture medium per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity displayed.

Results

Cloning and genomic integration of amylase genes into industrial strains

The *T. lanuginosus TLG1* and *S. fibuligera SFA1* genes were codon-optimized for expression in *S. cerevisiae* and cloned individually or combined in pBKD1 and pBKD2-derived plasmids (Figure 1, Table I). The genes were first integrated individually into the genome of the semi-industrial *S. cerevisiae* M2n strain to evaluate their respective starch hydrolysing activities. Co-expression of *TLG1* and *SFA1* was subsequently evaluated in *S. cerevisiae* M2n and in the industrial *S. cerevisiae* MEL2 strain, previously described for its promising industrial fitness (Favaro et al. 2013b). All the recombinant SFA-strains

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produced hydrolysis zones (Figure 2a); zones were neither expected nor observed for M2n[TLG1] expressing the exo-type glucoamylase *TLG1* (Figure 2a).

Characterization of recombinant amylases

Characterization of protein species by SDS-PAGE indicated that the TLG1 protein (predicted molecular size of 67 kDa) was glycosylated to yield a product of 90 kDa, whereas the recombinant SFA1 size was similar to the expected 56 kDa (Figure 2b). Zymogram analysis confirmed that the recombinant SFA1 was active (clear hydrolysis zones appeared after iodine staining of the starch plate). The TLG1 protein did not produce starch hydrolysis zones, in line with the absence of hydrolysis halos on the soluble starch plate (Figure 2a).

Both the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains displayed maximum total soluble starch hydrolysis at pH 4.5 (Figure 2c), with a continuous decrease in activity as the pH values increased above 5.5. At the optimal pH of 4.5, the enzymatic activity peaked at 60°C, with lower temperatures resulted in reduced activities (Figure 2d). Raw and soluble starch hydrolysis by the recombinant strains was therefore evaluated at pH 4.5 and either 60°C (optimal temperature for enzyme activity) or 30°C (yeast cultivation temperature). Both total amylase and glucoamylolytic assays indicated that starch hydrolysis at 30°C corresponded to 26% of the activity at 60°C (Table II). Furthermore, the activity on raw corn starch was approximately 53% of that obtained on soluble starch. The *S. cerevisiae* M2n[TLG1-SFA1] strain displayed higher enzymatic values than the MEL2[TLG1-SFA1] strain under all the assay conditions (Table II).

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Fermentation studies

The parental and recombinant yeast strains were first evaluated for their ability to ferment glucose at a high substrate loading under oxygen-limited conditions in 120 mL fermentation bottles (Figure 3). Parental strains performed slightly better than the recombinant yeasts, with a noticeable difference for the MEL2. After 96 h, the wild types MEL2 and M2n strains produced 96.45 and 94.60 g/L ethanol, respectively, while the recombinant counterparts yielded 91.00 and 92.31 g/L (Figure 3).

The *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains were subsequently evaluated for the direct conversion of raw corn starch to ethanol in 1 L bioreactor batch fermentations through a simulated consolidated bioprocessing (CBP) of 200 g/L raw starch and 5 g/L glucose (Figure 4). The *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] yeast produced 64.00 and 52.43 g/L of ethanol, respectively (corresponding to 55 and 45% of the theoretical yield) after 240 h of fermentation (Figure 4, Table III). As expected, the parental yeast strains did not utilise the raw starch for ethanol production (data not shown). Raw starch conversion by *S. cerevisiae* MEL2[TLG1-SFA1] strain was slower than *S. cerevisiae* M2n[TLG1-SFA1], probably due to the 36 hour lag phase observed for the former (Figure 4). The low residual levels of glucose and maltose in the fermentation broth indicate a rapid sugar uptake by the engineered strains (Table III).

As reported in Table III, although the final volumetric productivity (Q) was comparable between the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains (0.27 and 0.22 g/L/h, respectively), the Q_{max} of M2n[TLG1-SFA1] (0.59 g/L/h after 48 h), was approximately 1.8-fold higher than that of MEL2[TLG1-SFA1] (0.30 g/L/h after 132 h).

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Starch conversion by *S. cerevisiae* M2n[TLG1-SFA1] was also superior, with almost 75% of the polysaccharide converted compared to 62% by MEL2[TLG1-SFA1] (Table III).

Sorghum and triticale were subsequently evaluated as potential CBP substrates for the recombinant yeast (Figure 4, Table III). The *S. cerevisiae* M2n[TLG1-SFA1] strain converted 80% of the raw starch (147.5 g/L) present in 200 g/L sorghum within 5 days (Figure 4a, Table III) with the production of 50.67 g/L ethanol, whereas *S. cerevisiae* MEL2[TLG1-SFA1] only reached similar ethanol levels after 10 days (Figure 4b, Table III). The volumetric productivity of *S. cerevisiae* M2n[TLG1-SFA1] was therefore higher, peaking at 0.78 g/L/h after 24 h, compared to *S. cerevisiae* MEL2[TLG1-SFA1] that only achieved 0.46 g/L/h after 36 h (Table III). At the end of the fermentation, starch conversion by *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] was 85 and 79%, respectively, with ethanol yields of 62 and 57% of the theoretical, respectively (Table III).

Triticale was effectively converted into ethanol with both the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains producing similar levels of ethanol, i.e. 51.48 and 49.24 g/L, respectively from 200 g/L triticale (126.0 g/L raw starch) after 10 days (Figure 4). However, the volumetric productivity for M2n[TLG1-SFA1] was higher after 5 days (Table III), with a maximum of 1.04 g/L/h observed after 24 h, about 1.8-fold greater than the highest volumetric productivity (0.58 g/L/h) for MEL2[TLG1-SFA1] (Table III). It was therefore clear that the *S. cerevisiae* M2n strain was superior in terms of starch utilization and ethanol yields, being able to convert 99% of the available starch and produce 73% of the theoretical ethanol yield. The higher conversion of triticale starch relative to sorghum and corn starch can partly be ascribed to high levels of native plant amylolytic enzymes present in triticale (Pejin et al. 2009).

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Discussion

Sorghum and triticale are important cereal grains due to their drought resistance and the relatively low input costs required for cultivation thereof. However, both cereals have a relatively low cash value if sold directly as feed grain (Hoseney et al. 1981; Rooney and Awika 2005) and new industrial applications should be developed to improve their market significance. Given the relatively high starch content of the two grains, they can be considered as a potential feedstock for bioethanol production. This would, however, require consolidated bioprocessing (i.e. enzyme production and fermentation by the same organism) to reduce the input costs typically associated with starch liquefaction and hydrolysis.

The development of a CBP yeast for the effective conversion of starchy substrates into ethanol requires robust strains to be engineered for the production of raw starch hydrolysing enzymes in adequate quantities. The *S. cerevisiae* MEL2 and M2n strains that displayed promising industrial fitness (Favaro et al. 2013b, Viktor et al. 2013) were therefore chosen as hosts for the production of the recombinant enzymes.

Since codon optimization can significantly improve gene expression levels and the subsequent functionality of the enzymes, the *TLG1* (*T. lanuginosus* glucoamylase) and *SFA1* (*S. fibuligera* α-amylase) genes were codon optimized for expression in *S. cerevisiae*. The synthetic sequences were cloned individually (Figure 1) and expressed in *S. cerevisiae* M2n (creating strains M2n[TLG1] and M2n[SFA1]) with their respective activity confirmed on soluble starch (Figure 2a). This was followed by the construction of the raw starch fermenting *S. cerevisiae* M2n[TLG1-SFA1] and *S. cerevisiae* MEL2[TLG1-SFA1] strains that displayed clearing zones on starch plates (Figure 2a), as opposed to a smaller halo for *S. cerevisiae* M2n[SFA1] and none for M2n[TLG1].

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Based on the deduced amino acid sequences, molecular weights of 67 kDa and 56 kDa were predicted for the unglycosylated recombinant TLG1 and SFA1, respectively. SDS-PAGE analysis of the supernatant indicated that only TLG1 was glycosylated in both strains (Figure 2b).

The combined amylase activity of the recombinant yeast strains performed well between pH 3.5 and 5.5 with only 53% residual activity detected at pH 6.5 (Figure 2c). The amylases acted effectively between 50 and 70°C, with less than 30% relative activity at the optimal fermentation temperature (30°C). These conditions are in agreement with those reported for other raw starch degrading α-amylases and glucoamylases (Robertson et al. 2006; Sun et al. 2010). The enzymatic activity was influenced by the incubation temperature and nature of the substrate (Table II). As expected, the hydrolytic activities were significantly lower on the more recalcitrant raw starch compared to soluble starch, whereas the higher temperature of 60°C increased the enzyme activity approximately 4-fold irrespective of the strain and substrate. The *S. cerevisiae* M2n[TLG1-SFA1] strain performed slightly better than *S. cerevisiae* MEL2[TLG1-SFA1] at both 30 and 60°C on either soluble or raw starch. This could be ascribed to different copy numbers or site(s) of integration for the synthetic genes, but further genetic studies are required to confirm these hypotheses.

Delta-integration of the synthetic *TLG1* and *SFA1* genes slightly affected the fermentation ability of the recombinant strains (Figure 3). This is in agreement with previous reports by Favaro et al. (2012b) and Kang et al. (2003) indicating that the high number of integrations targeted to the δ-elements did not significantly impair the growth rate of the recombinant strains on glucose.

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This study is one of only a few that demonstrated the concept of consolidated bioprocessing of raw starch to ethanol in fermenters using a high gravity feed of 200 g/L raw starch, but it represents the first report on CBP of unprocessed starchy substrates with recombinant industrial yeast strains at a bioreactor scale. Other researches were based mainly on laboratory strains, which make direct comparison with the current work difficult. The *S. cerevisiae* YF237 laboratory strain, displaying the *R. oryzae* glucoamylase on its surface and secreting the *Streptococcus bovis* α-amylase, produced 51 g/L of ethanol from 100 g/L of raw corn starch after 60 h of fermentation (Khaw et al. 2006). The laboratory *S. cerevisiae* YF207, co-expressing the *R. oryzae* glucoamylase and *S. bovis* α-amylase on the cell surface, yielded about 55 g/L of ethanol from 200 g/L of raw corn starch after 10 days of fermentation (Chen et al. 2008). The latter compared well with the 64 and 52 g/L ethanol obtained by the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains (Figure 4, Table III) from 200 g/L raw corn starch after 10 days. In contrast to the reports mentioned above, the enzymes in this study were not tethered to the cell wall, but secreted during cultivation on raw corn starch. The volumetric productivity of both the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains could be further improved upon by means of repeated fermentations as described for other recombinant strains (van Zyl et al. 2012).

Sorghum and triticale were selected as natural starchy substrates to evaluate the fermentative capabilities of the recombinant *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains. The starch component of both materials has similar properties to corn starch and should therefore be suitable as feedstock for an integrated bioethanol process. Both grains were efficiently converted to ethanol (Figure 4), in particular by the M2n[TLG1-SFA1] strain, with starch conversion rates and ethanol production (relative to

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theoretical yield) exceeding those from raw corn starch (Table III). This could be attributed to the presence of relatively high concentrations of metal ions in triticale and sorghum, which stabilise α-amylase in the presence of high ethanol concentrations (Abdel-Aal and Wood, 2005; Stoner et al. 2005; Yamada et al. 2011). Such stabilization would ensure the continued functioning of SFA1 and may account for greater and more rapid saccharification of the starch, thus resulting in higher ethanol yields. Furthermore, native amylolytic enzymes (mainly α-amylase) in both grains will supplement the recombinant enzymes. Results from the MEL2[TLG1-SFA1] strain seem to confirm this hypothesis as ethanol was readily detected after 12 h of incubation from both triticale and sorghum, whereas ethanol production from corn starch, which does not contain native amylases, was delayed (Figure 4b).

The *S. cerevisiae* M2n[TLG1-SFA1] strain displayed comparable and high volumetric productivities on all the three substrates towards the end of the fermentation (Figure 4a), confirming that the high enzymatic activities (Table II) supported the effective saccharification of all three starchy substrates. The *S. cerevisiae* MEL2[TLG1-SFA1] strain was inferior to the M2n[TLG1-SFA1] strain (Figure 4b) due to lower levels of enzymatic activity (Table II) and produced approximately 20% less glucose on raw corn starch at 30°C, which hampered the fermentation process.

To our knowledge, only Yamada et al. (2011) have thus far reported CBP of real starchy biomass applying the tetraploid amylolytic MNIV/δGS strain (combining δ-integration and polyploidization of laboratory strains) on brown rice. The reported ethanol yield and volumetric productivity were about 100% and 0.65 g/L/h, respectively, and compared well with those achieved by the diploid semi-industrial *S. cerevisiae* M2n[TLG1-SFA1] strain for a similar time frame. Considering the higher ploidy of the MNIV/δGS laboratory strain,

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the recombinants constructed in this study might be further improved upon by polyploidization (Yamada et al. 2010b).

In conclusion, this is the first report of the simultaneous expression of synthetic (codon-optimized) copies of TLG1 and SFA1 in a foreign host. The resulting recombinants demonstrated ethanol production in excess of 60 g/L using a high gravity feed of 200 g/L corn starch, triticale and sorghum substrates without any pre-treatment or exogenous enzyme addition. For the first time, industrial strains, co-producing glucoamylase and alpha-amylase enzymes were described for efficient CBP of natural starchy biomass. The starch conversion in triticale (approached 100%) exceeded those of sorghum (85%) and corn starch (75%), suggesting a particular efficient hydrolysis of triticale starch.

The engineered strains' ethanol performance will be evaluated on other starch-containing substrates, such as wheat bran or potato peels, and repeated fermentations are likely to further enhance the efficiency of the recombinant strains. Since these feedstocks also contain other polysaccharides such as cellulose and hemicellulose, the addition of cellulases and hemicellulases would further improve the release of fermentable sugars and therefore the ethanol yield from cereal grains. Bioethanol production from such substrates by means of an amylolytic yeast strain will thus benefit from the addition of these enzymes via heterologous expression or exogenous addition.

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Table I. Summary of plasmids and strains constructed for the development of an efficient amylolytic *S. cerevisiae* strain.

Plasmid/Strains	Relevant genotype or phenotype	Source
pDRIVE	*bla*	Qiagen (USA)
pBKD1	*bla* δ-sites-$PGK1_P$-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites $_T$	McBride et al. (2008)[b]
pBKD2	*bla* δ-sites-$ENO1_P$-$ENO1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	McBride et al. (2008)[b]
pSFA1	*bla* δ-sites-$PGK1_P$-*SFA1*-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	This work
pTLG1	*bla* δ-sites-$ENO1_P$-*TLG1*-$ENO1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	This work
pTLG1-SFA1	*bla* δ-sites-$PGK1_P$-*SFA1*-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a] $ENO1_P$-*TLG1*-$ENO1_T$ -δ-sites	This work
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [*F'proAB lacq ZΔM15 Tn10(tet)*]	Stratagene (USA)
S. cerevisiae M2n	Semi-industrial strain	Viktor et al. 2013
S. cerevisiae MEL2	Industrial strain with high fermentative vigour	Favaro et al. 2013b
S. cerevisiae M2n[TLG1]	*TLG1 multiple copy integration*	This study
S. cerevisiae M2n[SFA1]	*SFA1 multiple copy integration*	This study
S. cerevisiae M2n[TLG1-SFA1]	*TLG1 and SFA1 multiple copy integration*	This study
S. cerevisiae MEL2[TLG1-SFA1]	*TLG1 and SFA1 multiple copy integration*	This study

[a]*TEF1* promoter and terminator from *Ashbya gossypii*. [b] McBride JEE, Deleault KM, Lynd LR, Pronk JT .2008. Recombinant yeast strains expressing tethered cellulase enzymes. Patent PCT/US2007/085390.

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Table II. Soluble and raw starch hydrolysing activities (nkat/DCW) of the engineered *S. cerevisiae* strains when grown in YPD broth for 72 h. The assays were performed at 30° and 60°C in citrate-phosphate buffer at pH 4.5 with either 0.1% soluble starch or 2% raw starch. The values are the means of the results obtained from two experiments conducted in triplicate (± SD). Parental strains did not give any starch-degrading activities.

	Soluble starch		Raw starch	
	60°C	30°C	60°C	30°C
Total Amylase activity (Reducing sugar assay[1])				
S. cerevisiae M2n[TLG1-SFA1]	8110 ± 474	2076 ± 168	4461 ± 381	1124 ± 97
S. cerevisiae MEL2[TLG1-SFA1]	7125 ± 335	1817 ± 127	3883 ± 338	971 ± 90
Released Glucose (Glucose kit assay[2])				
S. cerevisiae M2n[TLG1-SFA1]	5061 ± 385	1284 ± 98	2634 ± 239	674 ± 62
S. cerevisiae MEL2[TLG1-SFA1]	4165 ± 300	1037 ± 68	2161 ± 214	541 ± 55

[1]Reducing sugar assay detects all reducing sugars (mono saccharides and oligosaccharides)
[2]Glucose kit assay only detects glucose

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Table III. Conversion of starch to ethanol and by-products by recombinant *S. cerevisiae* strains

Component	***S. cerevisiae* M2n[TLG1-SFA1]**		***S. cerevisiae* MEL2[TLG1-SFA1]**	
Substrate: 200 g/L raw starch + 5 g/L glucose = a glucose equivalent of 227 g/L				
Product (g/L)	**120 h**	**240 h**	**120 h**	**240 h**
Glucose	-	-	-	-
Maltose	-	0.69 ± 0.02	1.40 ± 0.04	-
Glycerol	2.50 ±0.20	2.90 ± 0.60	2.47 ± 0.17	3.29 ± 0.03
Ethanol	55.81 ± 0.10	64.00 ± 0.10	33.46 ± 1.52	52.43 ± 1.03
CO_2	52.97	61.30	32.05	50.22
Total carbon	**111.28**	**128.89**	**69.38**	**105.95**
Carbon conversion (mol C)	65%	75%	40%	62%
Ethanol (% theoretical)	48%	55%	29%	45%
Q (g/L/h)	0.47	0.27	0.28	0.22
Q_{max} (g/L/h)	0.59 after 48 h		0.30 after 132 h	
Substrate: 147.5 g/L sorghum starch + 5 g/L glucose = a glucose equivalent of 169.0 g/L				
Product (g/L)	**120 h**	**240 h**	**120 h**	**240 h**
Glucose	-	-	-	-
Maltose	-	-	0.45 ± 0.09	-
Glycerol	2.84 ± 0.25	3.07 ± 0.05	3.42 ± 0.12	4.30 ± 0.03
Ethanol	50.67 ± 1.75	53.87 ± 1.55	43.46 ± 0.80	49.58± 1.42
CO_2	48.54	51.60	41.63	47.49
Total carbon	**102.05**	**108.54**	**88.97**	**101.37**
Carbon conversion (mol C)	80%	85%	69%	79%
Ethanol (% theoretical)	59%	62%	50%	57%
Q (g/L/h)	0.42	0.22	0.36	0.21
Q_{max} (g/L/h)	0.78 after 24 h		0.46 after 36 h	
Substrate: 126.0 g/L triticale starch + 5 g/L glucose = a glucose equivalent of 145.0 g/L				
Product (g/L)	**120 h**	**240 h**	**120 h**	**240 h**
Glucose	-	1.32 ± 0.09	-	-
Maltose	0.81 ± 0.45	1.93 ± 0.05	1.31 ± 0.14	0.27 ± 0.03
Glycerol	2.76 ± 0.04	2.86 ± 0.07	4.07 ± 0.08	4.17 ± 0.18
Ethanol	49.73 ± 1.75	51.48 ± 1.99	43.02 ± 1.78	49.24± 2.62
CO_2	47.64	49.31	41.21	47.17
Total carbon	**100.94**	**106.91**	**89.62**	**100.85**
Carbon conversion (mol C)	92%	99%	81%	91%
Ethanol (% theoretical)	67%	73%	59%	67%
Q (g/L/h)	0.41	0.21	0.36	0.21
Q_{max} (g/L/h)	1.04 after 24 h		0.58 after 36 h	

Q: ethanol productivity; Q_{max}: maximum ethanol productivity

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Figure 1. A schematic representation of the final vector constructs used in this study for codon-optimized amylase expression. The *S. fibuligera SFA1* was cloned under the regulation of the *PGK1* promoter and terminator sequences, whereas the *T. lanuginosus TLG1* was cloned between the *ENO1* promoter and terminator sequences. The $ENO1_P$-*TLG1*-$ENO1_T$ cassette was obtained from pTLG1 and subcloned onto pSFA to generate plasmid pTLG1-SFA1.

Figure 2. (a) Soluble starch plate assay indicates hydrolysis zones surrounding the *S. cerevisiae* M2n[SFA1], M2n[SFA1-TLG1] and MEL2[SFA1-TLG1] strains, whereas the reference strains (*S. cerevisiae* M2n and MEL2) and *S. cerevisiae* M2n[TLG1] indicated no α-amylase activity. (b) SDS-PAGE of the supernatant of *S. cerevisiae* M2n (lane 1), *S. cerevisiae* MEL2 (lane 2), *S. cerevisiae* M2n[SFA1-TLG1] (lane 3), *S. cerevisiae* MEL2[SFA1-TLG1] (lane 4) after silver staining. On the right the iodine stained starch plate indicating hydrolysis after exposure to the proteins in the SDS-PAGE gel. The protein size marker is depicted on the left hand side. The effect of (c) pH and (d) incubation temperature on the relative amylase activity of (□) *S. cerevisiae* M2n[SFA1-TLG1] and (◆) *S. cerevisiae* MEL2[SFA1-TLG1] grown in YPD medium containing 20 g/L glucose.

Figure 3. Ethanol production (closed symbols) and glucose consumption (open symbols) by (▲) *S. cerevisiae* M2n, (■) *S. cerevisiae* MEL2, (◆) *S. cerevisiae* M2n[SFA1-TLG1] and (●) *S. cerevisiae* MEL2[SFA1-TLG1] were monitored over time under oxygen-limited conditions.

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Figure 4. Ethanol production in 1 L bioreactor from YPD broth supplemented with 5 g/L glucose and 200 g/L raw corn starch (■), sorghum (●) or triticale (▲) by *S. cerevisiae* M2n[SFA1-TLG1] (a) and *S. cerevisiae* MEL2[SFA1-TLG1] (b). Values represent the mean of three repeats and error bars represent the standard deviation.

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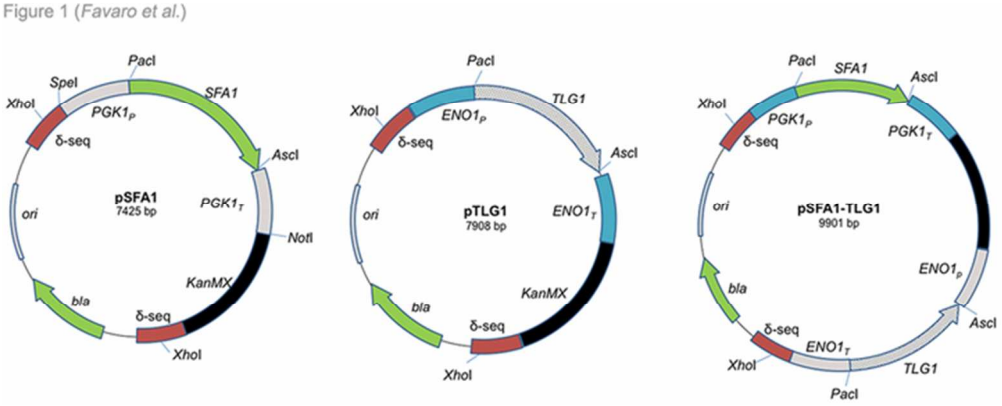

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Figure 2 (*Favaro et al*)

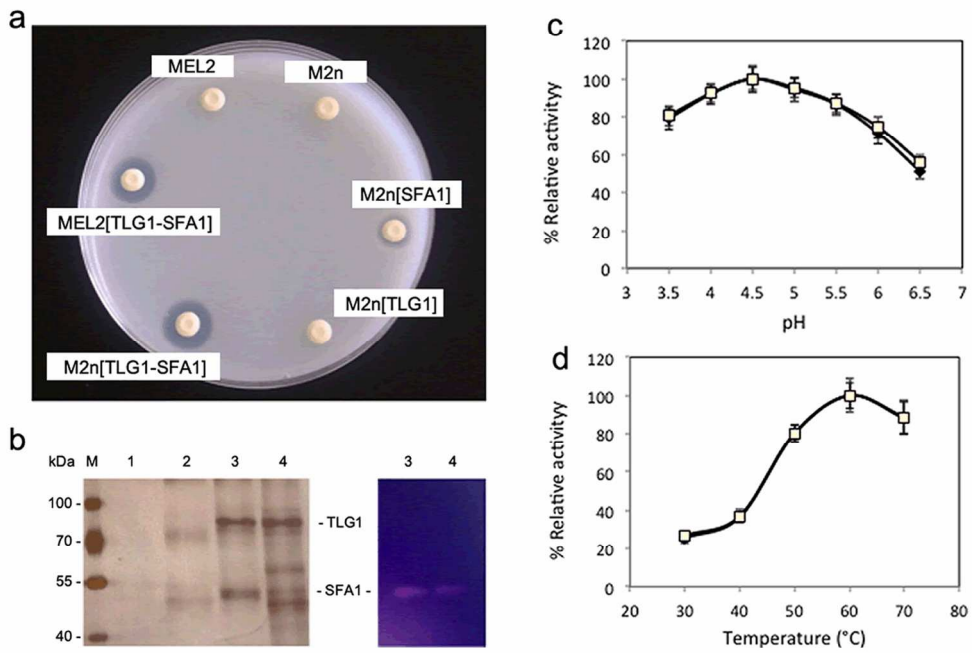

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Figure 3 (*Favaro et al*)

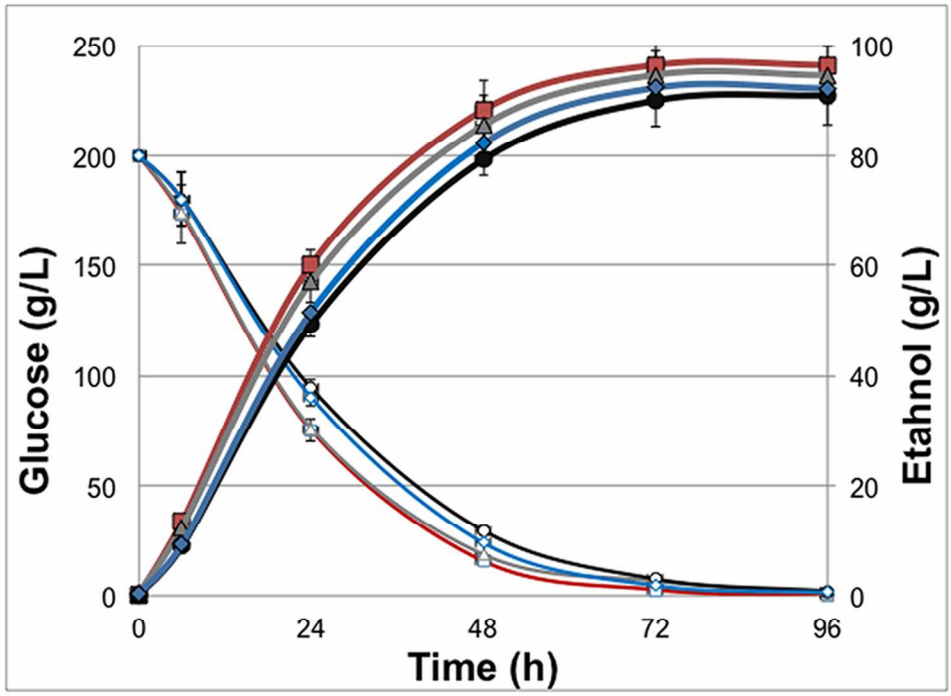

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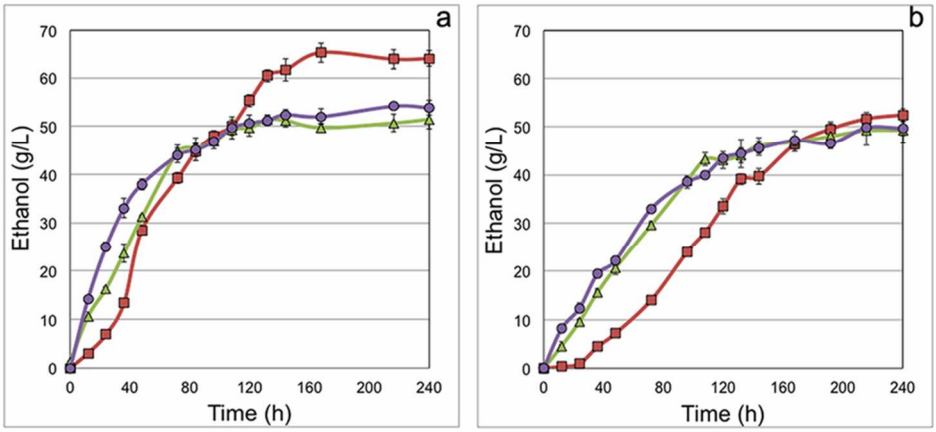
Figure 4 (*Favaro et al*)



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