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(Article begins on next page)

**Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases
and amylolytic yeast**

Rosemary Cripwell^a, Lorenzo Favaro^{b*}, Shaunita H. Rose^a, Marina Basaglia^b, Lorenzo Cagnin^b,
Sergio Casella^b, Willem van Zyl^a

^a *Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland,
Stellenbosch, South Africa.*

^b *Department of Agronomy Food Natural resources Animals and Environment, DAFNAE,
Università di Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro (PD), Italy.*

*Corresponding author: **Lorenzo Favaro, Dr**

Department of Agronomy Food Natural resources Animals and Environment (DAFNAE)

Agripolis - University of Padova

Viale dell'Università, 16

35020 Legnaro, PADOVA, ITALY

Tel. 049-8272926

Fax 049-8272929

e-mail: lorenzo.favaro@unipd.it

Abstract

Wheat bran, generated from the milling of wheat, represents a promising feedstock for the production of bioethanol. This substrate consists of three main components: starch, hemicellulose and cellulose. The optimal conditions for wheat bran hydrolysis have been determined using a recombinant cellulase cocktail (RCC), which contains two cellobiohydrolases, an endoglucanase and a β -glucosidase. The 10% (w/v, expressed in terms of dry matter) substrate loading yielded the most glucose, while the 2% loading gave the best hydrolysis efficiency (degree of saccharification) using unmilled wheat bran. The ethanol production of two industrial amyolytic *Saccharomyces cerevisiae* strains, MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], were compared in a Simultaneous Saccharification and Fermentation (SSF) for 10% wheat bran loading with or without the supplementation of optimised RCC. The recombinant yeast *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] completely hydrolysed wheat bran's starch producing similar amounts of ethanol (5.3 ± 0.14 g/L and 5.0 ± 0.09 g/L, respectively). Supplementing SSF with RCC resulted in additional ethanol production of about 2.0 g/L. Scanning electron microscopy confirmed the effectiveness of both RCC and engineered amyolytic strains in terms of cellulose and starch depolymerisation.

This study demonstrated that untreated wheat bran could be a promising ready-to-use substrate for ethanol production. The addition of crude recombinant cellulases improved ethanol yields in the SSF process and *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] strains can efficiently convert wheat bran's starch to ethanol.

Keywords: bioethanol; wheat bran; recombinant cellulase cocktail; industrial engineered amyolytic yeast; simultaneous saccharification and fermentation.

51 **1. Introduction**

52

53 Lignocellulosic biomass is the preferred substrate for bioethanol as it is more abundant and
54 less expensive than sucrose and starch substrates [1]. However, the limitations associated
55 with lignocellulosic ethanol production include the slow rate of enzymatic degradation, high
56 enzyme cost and the requirement of inhibitor-tolerant industrial yeast strains [2, 3].
57 Consequently, starch is still the most commonly used feedstock for ethanol production, with a
58 relatively mature technology developed for corn in the USA [4] that produced about 52.5
59 billion litres of bioethanol in 2012, an increase from 49.2 billion litres in 2010 [5].

60 Current starch-to-ethanol processes require an energy-intensive liquefaction step, as well as
61 substantial amounts of exogenous amylases for enzymatic hydrolysis of raw starch; both
62 these significantly impact the economic viability of starch as feedstock [6]. In order to
63 implement the large scale ethanol production from raw starch, the development of an
64 industrial yeast that converts starch to ethanol in one step (called consolidated bioprocessing -
65 CBP) is needed [7-10].

66 Recently, few studies reported the use of yeast strains for the fermentation of natural starchy
67 substrates at a bioreactor scale. Favaro and colleagues described the direct ethanol production
68 at bioreactor scale from natural starchy substrates (raw starch, sorghum and triticale), using
69 industrial yeast strains co-secreting glucoamylase and α -amylase enzymes [11]. Yamada et al.
70 [12] achieved the CBP of brown rice by the amylolytic laboratory strain MNIV/ δ GS strain
71 producing almost 80 g/L of alcohol from 200 g/l of brown rice after 120h. Although the
72 above reports pave the way for the industrial CBP of raw starch to ethanol, their focus was on
73 substrates composed only of starch, meanwhile many industrial starch-rich by-products are
74 available in great quantities with different compositions in terms of cellulose and
75 hemicellulose. These polysaccharides first have to be converted into sugars, in order to
76 achieve high ethanol efficiencies and make the overall process economically viable. This is

the case with wasted crop, cereal bran, cassava pulp, sago pith residues and brewery-spent grains, which have been proposed as low-cost materials for bioethanol, mainly by means of chemical pre-treatment, commercial cellulases, xylanase and amylases addition and subsequent fermentation [13-18]. The previously mentioned studies, though achieving promising results, demonstrate that the total exploitation of such substrates still needs to be addressed and that there is an opportunity to further increase the hydrolysis and fermentation yields from agricultural by-products containing different polysaccharides. Cheap and plentiful residual biomass has been investigated as promising renewable material to be converted into fuels, polymers, enzymes and bulk chemicals [19-22].

This study focused on wheat bran as an abundant and inexpensive starchy substrate, with a high potential for bioethanol due to its low pre-treatment cost [13, 14]. In addition to the starch content (15-30% dry matter), the hemicellulose and cellulose fractions can also be used for bioethanol production [23]. Although wheat bran does not require costly pre-treatments for hydrolysis [14, 24], not many studies have used this substrate for ethanol production [25]. Therefore, there is scope to optimise current technologies.

The hydrolysis of cellulose, starch and hemicellulose requires commercial enzymes that are very costly and not feedstock specific. Banerjee and colleagues [26] have developed a core set of recombinant enzymes for the hydrolysis of ammonia fibre expansion (AFEX) treated corn stover, using *Trichoderma reesei* enzymes produced in *Pichia pastoris*. However, there is still limited information available on the use of feedstock specific recombinant enzyme cocktails. An advantage of recombinant cocktails over commercial cocktails is that they are defined mixtures and do not contain unnecessary proteins.

In this present study, we examine the use of recombinant cellulolytic enzymes and engineered amylase-secreting strains for the hydrolysis and saccharification of wheat bran's cellulose and starch. The first objective was to investigate the simultaneous hydrolysis of cellulose

using a recombinant cellulase cocktail (RCC) produced by engineered yeast and fungal strains. For the first time, the crude enzymes secreted in the supernatant were directly used to optimize the hydrolysis of wheat bran in terms of glucose yield. Once the optimization of hydrolysis was achieved, the industrial *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] strains (both secreting the *Thermomyces lanuginosus* glucoamylase, TLG1, and the *Saccharomycopsis fibuligera* α -amylase, SFA1) were utilised for the simultaneous saccharification and fermentation (SSF) process in the presence of RCC resulting in high ethanol yields. This is the first report describing the conversion of starchy and cellulosic substrate into ethanol using crude recombinant enzymes and engineered amylolytic strains.

2. Material and methods

2.1 Strains, media and cultivations

The genotype and origin of strains used in this work are summarised in Table 1. The wild type *S. cerevisiae* MEL2 and M2n, with their respective recombinant strains MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], were utilised for wheat bran fermentation. The engineered strains contained the *TLG1* gene (glucoamylase from *Thermomyces lanuginosus*) expressed under the control of the *ENO1* promoter and the *SFA1* gene (α -amylase from *Saccharomycopsis fibuligera*) expressed under the control of the *PGK1* promoter sequences [11]. Both genes were codon optimised for expression in *S. cerevisiae* and integrated into the delta sequences on the genomes of the industrial *S. cerevisiae* MEL2 and M2n strains [11]. . Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

124 **Table 1.** Strains and recombinant enzymes used in this study

125

Strains	Relevant enzyme [*]	Source organism	Reference
RCC^{**}			
<i>S. cerevisiae</i> Y294[CbhI]	cellobiohydrolase I (CbhI)	<i>Talaromyces emersonii</i>	[27]
<i>S. cerevisiae</i> Y294[CbhII]	cellobiohydrolase II (CbhII)	<i>Chrysosporium lucknowense</i>	[27]
<i>Aspergillus niger</i> D15[EgA]	endoglucanase I (EgA) ^{***}	<i>Aspergillus niger</i>	[28]
<i>S. cerevisiae</i> Y294[Pcbgl1B]	β-glucosidase (Bgl)	<i>Phanerochaete chrysosporium</i>	[29]
SSF			
<i>S. cerevisiae</i> MEL2	-	Industrial strain for bioethanol	[14]
<i>S. cerevisiae</i> M2n	-	Semi-industrial strain	[30]
<i>S. cerevisiae</i> MEL2[TLG1-SFA1]	Glucoamylase (TLG1)	<i>T. lanuginosus</i>	[11]
	α-Amylase (SFA1)	<i>S. fibuligera</i>	
<i>S. cerevisiae</i> M2n[TLG1-SFA1]	Glucoamylase (TLG1)	<i>T. lanuginosus</i>	[11]
	α-Amylase (SFA1)	<i>S. fibuligera</i>	

126

127

128

129

^{*}All enzymes were secreted using their native secretion signal, with the exception of Pcbgl1B (using the *T. reesei* Xyn2 secretion signal)

^{**}RCC (Recombinant cellulase cocktail) [31]

^{***}*EgA* was expressed using the native DNA sequence, whereas all other genes were codon optimised for expression in *S. cerevisiae*

The *S. cerevisiae* strains (used for the recombinant enzymes) were maintained on either solid SC^{URA} agar plates (containing 6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories], 20 g/L glucose and yeast synthetic drop-out medium supplements (Sigma-Aldrich (Germany) or solid YPD (Yeast Peptone Dextrose) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar). Culture medium (6.7 g/L yeast nitrogen base, 20 g/L peptone and 20 g/L glucose, 0.05 mM citric acid buffer, pH5) was used to prepare the yeast inocula for the fermentation studies. Fermentation medium is similar to the cultivation medium, but contained 0.5 g/L glucose and 10% unmilled wheat bran. The *A. niger* D15[EgA] strain was maintained on spore plates and cultivated in double strength minimal media (2x MM, with 100 g/L glucose, lacking uridine) [32].

2.2 Chemical analysis of wheat bran

Wheat (*Triticum aestivum* L.) was grown in the area of Rovigo (Italy, 45°4'51''N, 11°47'38''E), harvested at 6 months, processed by Grandi Molini Italiani (Rovigo, Italy) and stored in plastic bags at 4°C. The wheat bran had a geometric mean diameter of 0.79 mm [13]. The dry matter content (903.4 g/kg) was obtained by drying triplicate samples for 48 h in an oven at 100°C. Wheat bran was analysed in terms of ash, starch, hemicellulose, cellulose, lignin and protein content according to international standard methods [33]. The same procedures were adopted to determine the content in terms of starch, hemicellulose, cellulose, lignin in the spent SSF wheat bran samples.

2.3 Pre-treatment of wheat bran

Raw wheat bran was homogenised to a geometric mean diameter of 0.45 mm, using a laboratory knife mill to obtain milled wheat bran. Unmilled and milled wheat bran were pre-treated with 1% sulphuric acid (w/w dry wheat bran) at 121°C. Dry matter concentration was adjusted to 51 g/kg with deionised water. Pre-treatment vessels were filled with 100 mL of the resulting slurry and autoclaved at 121°C for 30 min [14].

157 2.4 Enzymes

158 A recombinant cellulase cocktail (RCC) (Table 1), with a protein ratio of 114:102:1:637
159 (CbhI:CbhII:EgA:Bgl) [31] was used for wheat bran hydrolysis. The total activity (on
160 carboxymethyl cellulose (CMC)) and protein concentration for RCC was 7.45 nkat/mL and 16.11
161 mg/mL, respectively.

162 2.5 Determination of protein content

164 The protein content was determined with the Bio-Rad protein reagent (BioRad, USA), as directed
165 by the manufacturer with bovine serum albumin (BSA) as standard. Protein concentration was
166 expressed as milligram of protein per mL.

167 2.6 Enzymatic hydrolysis

169 Hydrolysis trials were carried out to investigate the effect of pre-treatment, substrate loading, and
170 enzyme loading on the enzymatic hydrolysis of wheat bran. The extent of bran starch hydrolysis
171 with the amylolytic enzymes secreted by *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1]
172 was also investigated. Hydrolysis trials were performed in a 5 mL working volume in McCartney
173 bottles, with 0.05 M citric acid buffer (pH 5), 0.02% NaN₃ (to prevent contamination), 2, 5, 10%
174 (w/v) substrate loading and the RCC cocktail. Reactions were incubated at 30°C in a laboratory
175 rotary-shaker-incubator (10 rpm), with sampling (0.1 mL) at time zero and at regular intervals. All
176 substrate loadings are expressed as w/v, based on dry weight.

177 In the case of bran starch hydrolysis, yeast cultures of *S. cerevisiae* MEL2[TLG1-SFA1] and
178 M2n[TLG1-SFA1] were sampled after 72 h cultivation in YPD broth and their supernatant
179 collected after centrifugation at 16000 g for 3 min. The glucose content of the samples was
180 determined (in duplicate) using the Roche D-Glucose Kit (Boehringer Mannheim, Germany)
181 according to the manufacturer's instructions. Absorbance was measured by a spectrophotometer at
182 340 nm (Boehringer Mannheim/R-Biopharm). All the experiments were performed in triplicate.

183 Data was analysed by three ways factorial ANOVA (Analysis Of Variance) using Duncan test *post*
184 *hoc* means differentiation.

185 186 **2.7 Fermentation studies on wheat bran**

187 Inocula for *S. cerevisiae* strains were prepared in 200 mL culture medium in 500 mL Erlenmeyer
188 flasks and incubated on a rotary shaker (30°C) at 150 rpm for 60 h. An SSF was performed using
189 fermentation medium containing 10% (w/v) unmilled wheat bran and an initial inoculum of 0.3 g
190 dry weight/L. Control fermentations (without enzyme addition) were run in parallel to the SSF
191 reactions using the fermentation medium, supplemented with 30 g/L glucose, since wheat bran
192 typically contains 10% cellulose and 20% starch. In addition, hydrolysis controls with RCC and
193 wheat bran were run in parallel to the SSF reactions under the same conditions except for the
194 inoculum.

195 Unmilled wheat bran was used as the substrate and different filter-sterilised enzyme combinations
196 were compared: (1) no enzymes and (2) RCC. Fermentations and control reactions were conducted
197 at a working volume of 50 mL (pH 5) in a 55 mL serum bottle for 10 days at 30°C on a magnetic
198 stirrer. Serum bottles were equipped with a bubbling CO₂ outlet and fermentations were carried out
199 under oxygen-limited conditions. Ampicillin (100 mg/L) and streptomycin (75 mg/L) were added to
200 prevent contamination.

201 Samples were taken daily during the course of the fermentation and analysed for glucose, cellobiose
202 and ethanol content, using ultra High Performance Liquid Chromatography (Nexera – Shimadzu
203 Italia SRL, Milan, Italy) with a hydrogen column (Rezex R0A) at 60°C and 5 mM H₂SO₄ as the
204 mobile phase at a flow rate of 0.6 mL/min. The compounds were detected with a refractive-index
205 detector (RID 6A; Shimadzu, Kyoto, Japan). Experiments were performed in triplicate.

206 207 **2.8 Scanning electron microscopy analysis**

208 Scanning electron microscopy (SEM) images were obtained from hydrolysis and SSF samples of
209 wheat bran. Wheat bran was dehydrated in ethanol solutions at increasing concentrations (10, 20,

210 30, 50, 70, 80, 90, 95% and absolute) and applied to a specimen stub. Samples were then coated
211 with gold and observed using a Jeol JSM-6490 Scanning Electron Microscope at 15 kV.

212 **2.9 Calculations**

214 Glucose concentrations were used to calculate the degree of saccharification (DS). DS_{glucan}
215 represents the soluble glucose released after hydrolysis (soluble sugars determined at time zero
216 were deducted). DS_{starch} was based on the total sugar concentration in the hydrolysate (corrected for
217 glucose concentration measured at time zero) with respect to the initial starch concentrations. A
218 conversion factor of 0.9 (162/180) was applied due to the difference in the mass between the
219 anhydroglucose ring and glucose, as a water molecule is added during the hydrolysis.

$$220 \quad DS_{glucan} = \frac{[glucose \text{ g/L}] \times 0.9}{[cellulose \text{ g/L}]} \times 100\%$$

$$221 \quad DS_{starch} = \frac{[glucose \text{ g/L}] \times 0.9}{[starch \text{ g/L}]} \times 100\%$$

222 The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilised glucose/polysaccharide) was calculated
223 considering the amount of glucose/cellulose/starch consumed during the fermentation and compared
224 to the maximum theoretical yield of 0.51 g of ethanol/g of consumed glucose and 0.56 g of
225 ethanol/g of consumed starch and/or cellulose. The volumetric productivity (Q) was based on grams
226 of ethanol produced per liter of culture medium per hour (g/L/h) and the maximum volumetric
227 productivity (Q_{max}) was defined as the highest volumetric productivity displayed by the *S.*
228 *cerevisiae* strains.

229 **3. Results and discussion**

231 **3.1 Wheat bran composition**

232 The composition of the bran used in this work is reported in Table 2. Other than starch and cellulose
233 (both nearly 11% of dry matter), the substrate was particularly rich in hemicellulose, with a value
234 (39%) quite similar to those previously reported [34]. Interestingly, starch content was low if

235 compared to that of other reports [13,14,25] indicating different and variable efficiency of starch
236 extraction during milling processes.

237
238 | **Table 2.** Composition (% of the dry matter) of unmilled and milled wheat bran used in this study
239

Component	Unmilled (%)	Milled (%)
Hemicellulose	39.06	38.99
Starch	11.01	11.01
Cellulose	10.68	10.91
Protein	17.94	17.88
Lignin	4.98	5.08
Ash	0.05	0.04

240

241 Bran is also composed of a large protein fraction (17.9%). The values agree well with recently
242 published results [13,14] and lignin content (about 5%) was similar to that reported by Palmarola-
243 Adrados *et al.* [25]. This study focused on the conversion of wheat bran's hexose-containing
244 polysaccharides into ethanol meanwhile the hydrolysis and fermentation of bran's hemicellulose is
245 currently being addressed towards the complete exploitation of wheat bran for bioethanol
246 production.

247 3.2 Cellulose wheat bran hydrolysis by crude recombinant cellulase cocktail (RCC)

248 In order to achieve high yields in the hydrolysis of wheat bran cellulose, several recombinant
249 enzymes were screened for their saccharifying activities (data not shown). The following four
250 cellulases were selected for their high hydrolytic potential, confirming their promise in terms of
251 cellulose depolymerisation, as previously reported in our research outcomes [27-29]: namely, the
252 cellobiohydrolase I (CbhI) of *Talaromyces emersonii*, the cellobiohydrolase II (CbhII) of
253 *Chrysosporium lucknowense* and the β -glucosidase (Pcbgl1B) of *Phanerochaete chrysosporium*
254 secreted by *S. cerevisiae* Y294 together with the endoglucanase I (EgA) of *Aspergillus niger*
255 heterologously produced by *A. niger* D15[EgA]. The enzymes were found to be effective once
256 formulated in a cocktail, hereafter referred as RCC, with the protein concentration ratio of

Formattato: Colore carattere: Rosso

114:102:1:637 (CbhI:CbhII:EgA:Bgl). The influence of chemical pre-treatment, substrate and enzymatic loading on hydrolysis yield was then tested.

- 3.2.1 Effect of pre-treatment

Milled and unmilled wheat bran was pre-treated at 121°C for 30 min with or without low sulphuric acid addition (1% w/w dry wheat bran) and RCC applied to the resulting pre-treated materials in order to select the most promising substrates.

As expected, the structural analysis conducted on the four different substrates revealed that, after the mild pre-treatment, most of the cellulose was still intact and limited solubilisation of hemicellulose also took place mainly in the sulphuric acid pre-treated materials with the highest degree of depolymerisation detected in the milled wheat bran (data not shown). However, no significant differences in terms of glucose levels and degree of saccharification (DS_{glucan}) were measured after the hydrolysis with RCC of the four materials (data not shown). As a result, since physico-chemical pre-treatment adds extra cost to the process, unmilled wheat bran, not-sulphuric acid pre-treated, was used for the remainder of the study.

- 3.2.2 Effect of substrate loading

Hydrolysis trials on unmilled wheat bran were subsequently performed with different substrate loadings (Fig. 1a). As expected, higher substrate loadings resulted in greater levels of glucose released ($p < 0.001$). However, the lower wheat bran concentrations, the higher saccharification yields were achieved: the DS_{glucan} obtained after 144 h was 34, 24 and 18% for the 2, 5 and 10% substrate loadings, respectively (Fig. 1b).

Overall, as reported in Fig. 1, the increase in glucose release and DS_{glucan} is not linear indicating a plateauing effect. The lower DS_{glucan} obtained for the higher substrate loadings corresponds to previous observations on several substrates [35-37] and can be ascribed to possible inhibition of the enzymes as a result of the accumulating glucose, and/or reduced accessibility of the cellulose. However, the amount of glucose released using a 10% substrate loading (Fig. 1a) is the highest ($p < 0.001$) and enough to support the growth of *S. cerevisiae*. Therefore, such a loading would be

283 better suited for SSF process. Increasing the substrate concentration above 10% was not possible, as
 284 the reaction mixture would become too viscous, compromising proper mixing.

285

286 **Fig. 1.** Effect of three substrate loadings (2, 5 and 10%) on the hydrolysis of wheat bran cellulose using the RCC.
 287 Released glucose (a) and degree of saccharification (DS_{glucan}) (b) were calculated for wheat bran hydrolysis at 2, 5 and
 288 10% substrate loadings. Statistical evaluation (c) by ANOVA of the effect of different substrate loadings, time (h) and
 289 their interaction on hydrolysis after 144 hours (**p<0.01).

290 - 3.3.3 Effect of enzyme loading

291 The effect of enzyme dosages was investigated on 5 and 10% substrate loading (Fig. 2a). When the
 292 enzyme loading was doubled (2 x RCC), the glucose yield after 24 h increased by 86 and 49% for
 293 the 5 and 10% substrate loadings, respectively. At 144 h, the increase was 51 and 9%, respectively
 294 (Fig. 2a). The highest DS_{glucan} (37%) was achieved with a 2 x RCC and 5% substrate loading (Fig.
 295 2b), which was nearly 13% higher than for the reaction with 5% substrate loading and RCC. A
 296 slight increase (<2%) in DS_{glucan} was observed when the enzyme concentration was doubled using a
 297 10% substrate loading, however, this was not statistically relevant and possibly ascribed to the
 298 accumulation of glucose in 2x RCC condition, thus inhibiting the enzymes activity. The ANOVA
 299 test revealed a significant improvement of the glucose yield when the substrate loading, the enzyme
 300 loading, or treatment time increased (Fig. 2c).

Fig. 2. Effect of substrate and enzyme loadings on enzymatic hydrolysis of wheat bran cellulose. Experiments were carried out with 5 and 10% substrate loading (w/v) of unmilled wheat bran and two different enzyme loadings: 1x RCC and a 2 x RCC. Released glucose (a) and degree of saccharification (DS_{glucan}) (b) were calculated. Statistical evaluation (c) by ANOVA of the effect of substrate loading, enzymatic loading and incubation time (h), as well as their interactions on hydrolysis (ns: not significant; ** $p < 0.01$).

3.3 Wheat bran's starch hydrolysis using crude recombinant amylases secreted by the engineered amyolytic strains

The amyolytic enzymes secreted by *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1], to be used in the SSF of wheat bran, were assessed in terms of hydrolysis on wheat bran's starch in trials with three different substrate loadings: 2, 5 and 10% (Fig. 3). The recombinant amylases secreted by both industrial strains were effective in hydrolysing the starch content of wheat bran and, at the tested substrate dosages, displayed similar glucose release which appears to be linear (Fig. 3). After 90 h of incubation, the DS_{starch} was approximately 49 and 42% in all the substrate loadings for MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], respectively, suggesting a slightly higher saccharification ability for the former yeast.

Fig. 3. Wheat bran's starch hydrolysis using the supernatant of recombinant *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1]. Three different substrate loadings were used (2, 5 and 10% w/v). Data shown are the mean values of three replicates and standard deviations are included.

3.4 Fermentation studies on wheat bran

A substrate loading of 10% was used for the wheat bran SSF, as it gave the highest glucose levels in the hydrolysis trials (Fig. 1a, 3). As described in the 2.7 Material and Methods section, reference fermentations were performed with both recombinant (*S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1]) and wild type (*S. cerevisiae* M2n and MEL2) strains in broth containing 30 g/L glucose to simulate wheat bran composition (Fig. 4a, Table 3).

Fig. 4. Fermentation products during SSF of 10% (w/v) unmilled wheat bran. Ethanol levels by wild type *S. cerevisiae* MEL2 (●) and M2n (○) and their respective recombinant *S. cerevisiae* MEL2[TLG1-SFA1] (■) and *S. cerevisiae* M2n[TLG1-SFA1] (□) in control fermentation with 30 g/L glucose (a). Ethanol levels (b) and cellobiose accumulation (c) from wheat bran by *S. cerevisiae* MEL2 (●) and MEL2[TLG1-SFA1] (■) with (dash lines) or without (continuous lines) RCC addition. The results obtained for *S. cerevisiae* M2n and M2n[TLG1-SFA1] were not reported in (b) and (c) as the data were similar to those of the MEL2 and MEL2[TLG1-SFA1]. Data shown are the mean values of three replicates and standard deviations are included.

334 **Table 3.** Conversion of glucose and wheat bran's starch and/or cellulose to ethanol by wild type *S. cerevisiae* yeast (MEL2 and M2n) and their respective
335 engineered strains: MEL2[TLG1-SFA1] and M2n[TLG1-SFA1]. SSF of wheat bran (10% w/v) was conducted with or without RCC (Recombinant Cellulase
336 Cocktail).

Strain	Highest ethanol concentration (g/L)	Glucose utilisation (%)	Starch utilisation (%)	Cellulose utilisation (%)	$Y_{E/S}$ (g/g)	Q (g/L/h)	Q_{max} (g/L/h)
Glucose (30 g/L) medium							
MEL2	14.29	100	-	-	0.48 (94%)	0.22	0.74
MEL2[TLG1-SFA1]	14.12	100	-	-	0.47 (93%)	0.21	0.73
M2n	14.18	100	-	-	0.47 (93%)	0.21	0.73
M2n[TLG1-SFA1]	13.92	100	-	-	0.47 (91%)	0.21	0.72
Wheat bran without RCC							
MEL2	0.18	-	0	0	-	-	-
MEL2[TLG1-SFA1]	5.26	-	100	0	0.48 (85%)	0.07	0.18
M2n	0.23	-	0	0	-	-	-
M2n[TLG1-SFA1]	5.01	-	100	0	0.45 (81%)	0.07	0.17
Wheat bran with RCC							
MEL2	2.30	-	0	41	0.50 (89%)	0.03	0.09
MEL2[TLG1-SFA1]	7.30	-	100	37	0.50 (89%)	0.10	0.22
M2n	2.29	-	0	40	0.50 (89%)	0.03	0.09
M2n[TLG1-SFA1]	7.00	-	100	37	0.49 (88%)	0.10	0.20

337 $Y_{E/S}$, ethanol yield per gram of consumed substrate calculated on the highest ethanol production and % of theoretical maximum indicated in brackets

338 The yeast showed similar fermentative performances: all the glucose was metabolised within 18 h
339 and the maximum ethanol concentrations ranged from 13.92 to 14.29 g/L, with an average ethanol
340 yield of about 93% of the theoretical (Table 3). Moreover, as reported in Table 3, both maximum
341 and final volumetric productivities were comparable for the two parental and recombinant yeast.
342 During SSF of wheat bran without RCC addition, only the engineered strains were able to produce
343 ethanol (Fig. 4b, Table 3). The recombinant yeast MEL2[TLG1-SFA1] yielded, after 72 h, 5.26 g/L
344 ethanol (Fig. 4b) while *S. cerevisiae* M2n[TLG1-SFA1], displaying similar volumetric productivity,
345 produced up to 5.01 g/L ethanol in the same timeframe (Table 3). Starch was not detected by the
346 chemical analysis performed on spent wheat bran at the end of the SSF, indicating that both strains
347 completely hydrolysed the polysaccharide (Table 3). The resulting ethanol yield per gram of
348 consumed starch was higher than 85 and 81% for MEL2[TLG1-SFA1] and M2n[TLG1-SFA1],
349 respectively, with productivity values comparable for the engineered strains (Table 3). Their starch-
350 to-ethanol conversion efficiencies were similar to those recently described for the same engineered
351 strains from raw corn starch, sorghum and triticale [11].
352 SEM of wheat bran samples during the SSF confirmed the ability of the recombinant yeast to break
353 down the starch granules, which were abundantly present at the beginning of the fermentation (Fig.
354 5a), limited in number but still visible after 44 h of incubation (Fig. 5b) and completely disappeared
355 after 72 h of fermentation by MEL2[TLG1-SFA1] (Fig. 5c).
356 Supplementing the SSF with the optimised RCC was effective for cellulose hydrolysis, since high
357 glucose levels were released by the enzymes (data not shown). As a result, both wild type and
358 engineered strains were supported for ethanol production and, after 72 h, the ethanol level by
359 MEL2[TLG1-SFA1] exceeded 7.30 g/L, which was 1.4-fold of the amount produced in the absence
360 of the RCC (Table 3). On the other hand, the parental MEL2, unable to produce ethanol from wheat
361 bran in the absence of external enzymes addition, obtained up to 2.30 g/L thanks to RCC. As
362 reported in Table 3, similar ethanol levels were achieved by the wild type M2n and the engineered
363 M2n[TLG1-SFA1].

364

365

366 **Fig. 5.** SEM micrographs of wheat bran at the beginning (a,d), after 44 h (b,e) and 72 h (c,f) of SSF with RCC and *S.*
367 *cerevisiae* MEL2[TLG1-SFA1].

368

369 Overall, the use of RCC and engineered amyolytic strains proved to be strategic, since additional
370 ethanol production was achieved by the recombinant strains and, in the case of MEL2[TLG1-SFA1]
371 and M2n[TLG1-SFA1], alcohol levels were above 3-fold those of the parental yeast strains (Table
372 3). The ethanol yield were higher than 88% of the theoretical for all the strains and compared well
373 with those reported for SSF of other cellulosic materials, such as wheat straw, willow and paper
374 sludge [34]. Furthermore, the volumetric productivity values were significantly greater for the
375 recombinant yeast, exhibiting a Q_{max} of about 0.21 g/L/h instead of 0.09 g/L/h as detected for the
376 parental strains (Table 3).

377 Efficient biomass hydrolysis is dependent on β -glucosidase, as this enzyme is needed for the final
378 step of hydrolysis by converting the cellobiose to glucose [39]. However, an increase of about 1.17
379 g/L cellobiose was observed after RCC addition to the fermentation with both *S. cerevisiae*
380 MEL2[TLG1-SFA1] and MEL2 (Fig. 4b) indicating insufficient β -glucosidase activity of Bgl from
381 *P. chrysosporium*. In order to avoid commercial β -glucosidase supplementation (which is costly),

382 recombinant β -glucosidase needs to have improved abilities such as increased specific activity [40]
383 and further investigations are in progress to enhance the β -glucosidase activity in RCC.
384 Despite the suboptimal cellobiose-splitting activity, RCC was able to hydrolyse about 37% of the
385 cellulose content as pointed out by the chemical analysis of wheat bran fermented by the engineered
386 amyolytic strains. The efficiency of cellulose hydrolysis was similar also in the SSF of wheat bran
387 using the parental yeast (Table 3). Considering that RCC was composed by crude supernatant and
388 not purified enzymes, this efficiency has to be considered high and further improvable.
389 Cellulose depolymerisation was verified by SEM conducted during the wheat bran SSF of all the
390 strains in the presence of RCC. At the beginning of the experiment, the structure of wheat bran was
391 still intact with a rough surface (Fig. 5d), while cellulose damages increased with the incubation
392 time (Fig. 5e after 44 h) and were clearly evident at the end of the SSF (Fig. 5f); thus the RCC was
393 successful in hydrolysing the cellulose and simultaneously exposing the starch to the recombinant
394 amylases secreted by *S. cerevisiae* MEL2[TLG1-SFA1] and by *S. cerevisiae* M2n[TLG1-SFA1].
395 Overall, SEM analysis showed that significant changes occurred in the structure of wheat bran after
396 SSF with the RCC and amyolytic yeast, proving their effectiveness in terms of starch and cellulose
397 depolymerisation (Fig. 5).

398 4. Conclusions

399 In this study, we demonstrated an SSF whereby the cellulose component of wheat is hydrolysed by
400 recombinant cellulases, while at the same time the starch fraction is depolymerised by amyolytic
401 yeast. These results pointed out that recombinant enzyme cocktails and recombinant strains, both
402 tailored for a given substrate, play a key role for the efficient ethanol production from agricultural
403 by-products. Crude enzyme and substrate loading were optimised to define a proficient SSF of
404 wheat bran. *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] completely converted wheat
405 bran starch to ethanol with high yields and RCC supplementation resulted in additional alcohol
406 production. This research showed that untreated wheat bran can be a ready-to-use substrate for

ethanol production by SSF and further techno-economical evaluations will be undertaken to determine the actual feasibility of the whole process for the conversion of such by-product into bioethanol.

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Conflict of Interest

The Authors declare no conflict of interest.

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