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

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 amylolytic *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 amylolytic 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 amylolytic yeast; simultaneous saccharification and fermentation.

1. Introduction

Lignocellulosic biomass is the preferred substrate for bioethanol as it is more abundant and less expensive than sucrose and starch substrates [1]. 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 [2, 3]. Consequently, starch is still the most commonly used feedstock for ethanol production, with a relatively mature technology developed for corn in the USA [4] that produced about 52.5 billion litres of bioethanol in 2012, an increase from 49.2 billion litres in 2010 [5].

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 [6]. In order to implement the large scale ethanol production from raw starch, the development of an industrial yeast that converts starch to ethanol in one step (called consolidated bioprocessing - CBP) is needed [7-10].

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

77 the case with wasted crop, cereal bran, cassava pulp, sago pith residues and brewery-spent
78 grains, which have been proposed as low-cost materials for bioethanol, mainly by means of
79 chemical pre-treatment, commercial cellulases, xylanase and amylases addition and
80 subsequent fermentation [13-18]. The previously mentioned studies, though achieving
81 promising results, demonstrate that the total exploitation of such substrates still needs to be
82 addressed and that there is an opportunity to further increase the hydrolysis and fermentation
83 yields from agricultural by-products containing different polysaccharides. Cheap and
84 plentiful residual biomass has been investigated as promising renewable material to be
85 converted into fuels, polymers, enzymes and bulk chemicals [19-22].
86 This study focused on wheat bran as an abundant and inexpensive starchy substrate, with a
87 high potential for bioethanol due to its low pre-treatment cost [13, 14]. In addition to the
88 starch content (15-30% dry matter), the hemicellulose and cellulose fractions can also be used
89 for bioethanol production [23]. Although wheat bran does not require costly pre-treatments
90 for hydrolysis [14, 24], not many studies have used this substrate for ethanol production [25].
91 Therefore, there is scope to optimise current technologies.
92 The hydrolysis of cellulose, starch and hemicellulose requires commercial enzymes that are
93 very costly and not feedstock specific. Banerjee and colleagues [26] have developed a core
94 set of recombinant enzymes for the hydrolysis of ammonia fibre expansion (AFEX) treated
95 corn stover, using *Trichoderma reesei* enzymes produced in *Pichia pastoris.* However, there
96 is still limited information available on the use of feedstock specific recombinant enzyme
97 cocktails. An advantage of recombinant cocktails over commercial cocktails is that they are
98 defined mixtures and do not contain unnecessary proteins.
99 In this present study, we examine the use of recombinant cellulolytic enzymes and engineered
100 amylase-secreting strains for the hydrolysis and saccharification of wheat bran's cellulose
101 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).

Table 1. Strains and recombinant enzymes used in this study

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

*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

163 **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

168 **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% NaN_3 (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.

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

2.7 Fermentation studies on wheat bran

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

Unmilled wheat bran was used as the substrate and different filter-sterilised enzyme combinations were compared: (1) no enzymes and (2) RCC. Fermentations and control reactions were conducted at a working volume of 50 mL (pH 5) in a 55 mL serum bottle for 10 days at 30°C on a magnetic stirrer. Serum bottles were equipped with a bubbling CO_2 outlet and fermentations were carried out under oxygen-limited conditions. Ampicillin (100 mg/L) and streptomycin (75 mg/L) were added to prevent contamination.

Samples were taken daily during the course of the fermentation and analysed for glucose, cellobiose and ethanol content, using ultra High Performance Liquid Chromatography (Nexera – Shimadzu Italia SRL, Milan, Italy) with a hydrogen column (Rezex R0A) at 60°C and 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The compounds were detected with a refractive-index detector (RID 6A; Shimadzu, Kyoto, Japan). Experiments were performed in triplicate.

2.8 Scanning electron microscopy analysis

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

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

2.9 Calculations

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

$$DS_{glucan} = \frac{[glucose\ g/L]\ x\ 0.9}{[cellulose\ g/L]}\ x\ 100\%$$

$$DS_{starch} = \frac{[glucose\ g/L]\ x\ 0.9}{[starch\ g/L]}\ x\ 100\%$$

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

3. Results and discussion

3.1 Wheat bran composition

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

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

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

Formattato: Colore carattere: Rosso

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

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

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

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

257 114:102:1:637 (CbhI:CbhII:EgA:Bgl). The influence of chemical pre-treatment, substrate and
258 enzymatic loading on hydrolysis yield was then tested.

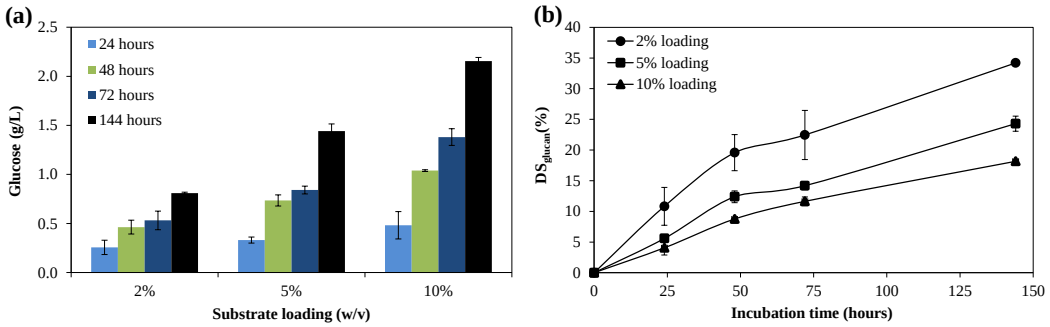
259 - **3.2.1 Effect of pre-treatment**

260 Milled and unmilled wheat bran was pre-treated at 121°C for 30 min with or without low sulphuric
261 acid addition (1% w/w dry wheat bran) and RCC applied to the resulting pre-treated materials in
262 order to select the most promising substrates.
263 As expected, the structural analysis conducted on the four different substrates revealed that, after
264 the mild pre-treatment, most of the cellulose was still intact and limited solubilisation of
265 hemicellulose also took place mainly in the sulphuric acid pre-treated materials with the highest
266 degree of depolymerisation detected in the milled wheat bran (data not shown). However, no
267 significant differences in terms of glucose levels and degree of saccharification (DS_{glucan}) were
268 measured after the hydrolysis with RCC of the four materials (data not shown). As a result, since
269 physico-chemical pre-treatment adds extra cost to the process, unmilled wheat bran, not-sulphuric
270 acid pre-treated, was used for the remainder of the study.

271 - **3.2.2 Effect of substrate loading**

272 Hydrolysis trials on unmilled wheat bran were subsequently performed with different substrate
273 loadings (Fig. 1a). As expected, higher substrate loadings resulted in greater levels of glucose
274 released ($p < 0.001$). However, the lower wheat bran concentrations, the higher saccharification
275 yields were achieved: the DS_{glucan} obtained after 144 h was 34, 24 and 18% for the 2, 5 and 10%
276 substrate loadings, respectively (Fig. 1b).
277 Overall, as reported in Fig. 1, the increase in glucose release and DS_{glucan} is not linear indicating a
278 plateauing effect. The lower DS_{glucan} obtained for the higher substrate loadings corresponds to
279 previous observations on several substrates [35-37] and can be ascribed to possible inhibition of the
280 enzymes as a result of the accumulating glucose, and/or reduced accessibility of the cellulose.
281 However, the amount of glucose released using a 10% substrate loading (Fig. 1a) is the highest
282 ($p<0.001$) 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 became too viscous, compromising proper mixing.



285

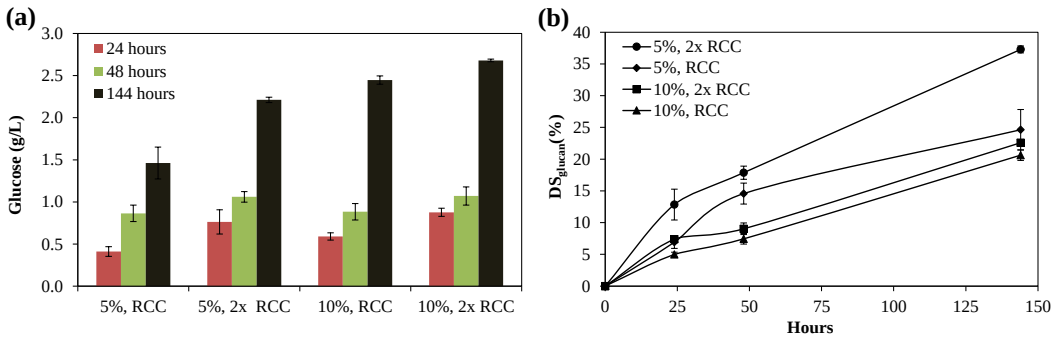
(c)

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	235.74	<0.001	**
Incubation time (h)	256.24	<0.001	**
Substrate loading x h	26.12	<0.001	**

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).



(c)

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	28.60	<0.001	**
Enzyme loading	216.26	<0.001	**
Incubation time (h)	470.42	<0.001	**
Substrate loading x Enzyme loading	<0.001	0.957	Ns
Substrate loading x h	15.25	<0.001	**
Enzyme loading x h	19.56	<0.001	**

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 amylolytic strains

The amylolytic 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.

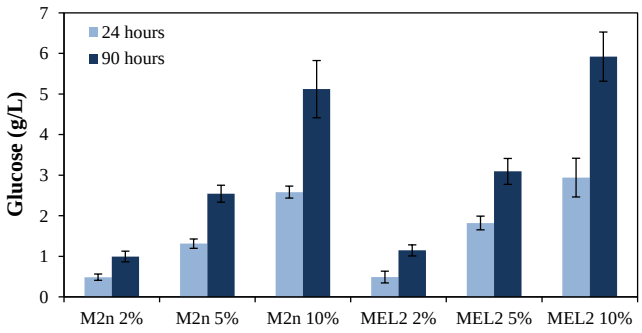


Fig. 3. Wheat bran's starch hydrolysis using the supernatant of recombinant *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1]. Three different substrate loadings were used (2, 5 and 10% w/v). Data shown are the mean values of three replicates and standard deviations are included.

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).

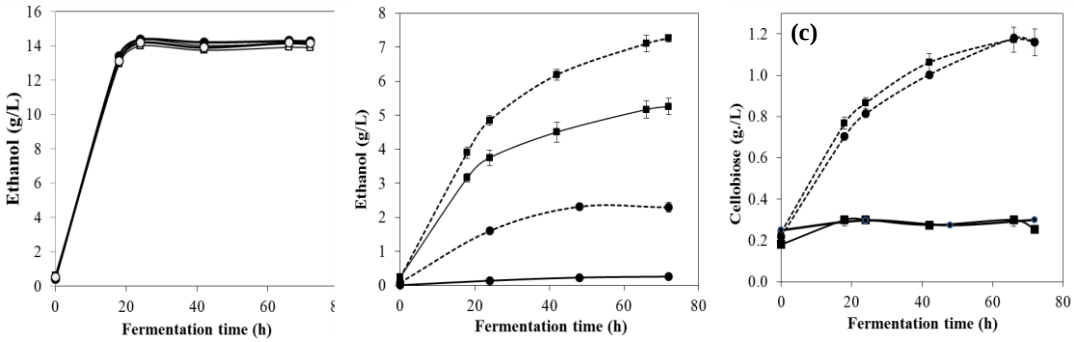


Fig. 4. Fermentation products during SSF of 10% (w/v) unmilled wheat bran. Ethanol levels by wild type *S. cerevisiae* MEL2 (●) and M2n (○) and their respective recombinant *S. cerevisiae* MEL2[TLG1-SFA1] (■) and *S. cerevisiae* M2n[TLG1-SFA1] (□) in control fermentation with 30 g/L glucose **(a)**. 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.

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 engineered strains: MEL2[TLG1-SFA1] and M2n[TLG1-SFA1]. SSF of wheat bran (10% w/v) was conducted with or without RCC (Recombinant Cellulase 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

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

The yeast showed similar fermentative performances: all the glucose was metabolised within 18 h and the maximum ethanol concentrations ranged from 13.92 to 14.29 g/L, with an average ethanol yield of about 93% of the theoretical (Table 3). Moreover, as reported in Table 3, both maximum and final volumetric productivities were comparable for the two parental and recombinant yeast.

During SSF of wheat bran without RCC addition, only the engineered strains were able to produce ethanol (Fig. 4b, Table 3). The recombinant yeast MEL2[TLG1-SFA1] yielded, after 72 h, 5.26 g/L ethanol (Fig. 4b) while *S. cerevisiae* M2n[TLG1-SFA1], displaying similar volumetric productivity, produced up to 5.01 g/L ethanol in the same timeframe (Table 3). Starch was not detected by the chemical analysis performed on spent wheat bran at the end of the SSF, indicating that both strains completely hydrolysed the polysaccharide (Table 3). The resulting ethanol yield per gram of consumed starch was higher than 85 and 81% for MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], respectively, with productivity values comparable for the engineered strains (Table 3). Their starch-to-ethanol conversion efficiencies were similar to those recently described for the same engineered strains from raw corn starch, sorghum and triticale [11].

SEM of wheat bran samples during the SSF confirmed the ability of the recombinant yeast to break down the starch granules, which were abundantly present at the beginning of the fermentation (Fig. 5a), limited in number but still visible after 44 h of incubation (Fig. 5b) and completely disappeared after 72 h of fermentation by MEL2[TLG1-SFA1] (Fig. 5c).

Supplementing the SSF with the optimised RCC was effective for cellulose hydrolysis, since high glucose levels were released by the enzymes (data not shown). As a result, both wild type and engineered strains were supported for ethanol production and, after 72 h, the ethanol level by MEL2[TLG1-SFA1] exceeded 7.30 g/L, which was 1.4-fold of the amount produced in the absence of the RCC (Table 3). On the other hand, the parental MEL2, unable to produce ethanol from wheat bran in the absence of external enzymes addition, obtained up to 2.30 g/L thanks to RCC. As reported in Table 3, similar ethanol levels were achieved by the wild type M2n and the engineered M2n[TLG1-SFA1].

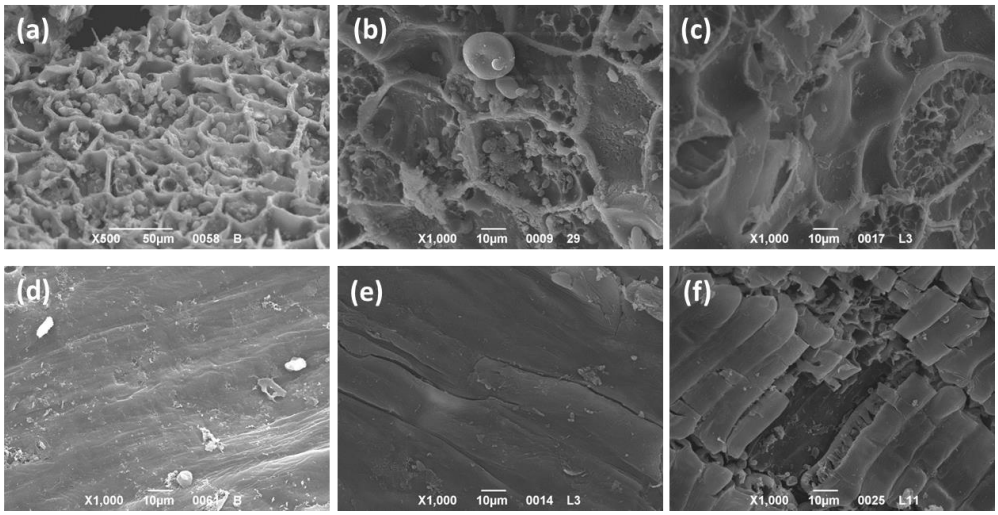


Fig. 5. SEM micrographs of wheat bran at the beginning (a,d), after 44 h (b,e) and 72 h (c,f) of SSF with RCC and *S. cerevisiae* MEL2[TLG1-SFA1].

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

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

recombinant β-glucosidase needs to have improved abilities such as increased specific activity [40] and further investigations are in progress to enhance the β-glucosidase activity in RCC.

Despite the suboptimal cellobiose-splitting activity, RCC was able to hydrolyse about 37% of the cellulose content as pointed out by the chemical analysis of wheat bran fermented by the engineered amylolytic strains. The efficiency of cellulose hydrolysis was similar also in the SSF of wheat bran using the parental yeast (Table 3). Considering that RCC was composed by crude supernatant and not purified enzymes, this efficiency has to be considered high and further improvable.

Cellulose depolymerisation was verified by SEM conducted during the wheat bran SSF of all the strains in the presence of RCC. At the beginning of the experiment, the structure of wheat bran was still intact with a rough surface (Fig. 5d), while cellulose damages increased with the incubation time (Fig. 5e after 44 h) and were clearly evident at the end of the SSF (Fig. 5f); thus the RCC was successful in hydrolysing the cellulose and simultaneously exposing the starch to the recombinant amylases secreted by *S. cerevisiae* MEL2[TLG1-SFA1] and by *S. cerevisiae* M2n[TLG1-SFA1]. Overall, SEM analysis showed that significant changes occurred in the structure of wheat bran after SSF with the RCC and amylolytic yeast, proving their effectiveness in terms of starch and cellulose depolymerisation (Fig. 5).

4. Conclusions

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

Acknowledgements

This work was partially supported by: the research project funded by the University of Padova entitled 'Engineering Consolidated Bioprocessing yeasts for the one-step conversion of cellulosic substrates into bioethanol'; the research project "BioRivaluta" funded by Regione Veneto (PSR 2007-2013, Misura 124, n. 2307660); the bilateral joint research project N. ZA11MO2 entitled 'Development of robust yeast strains for bioethanol production from starchy and cellulosic plant biomass'; and the National Research Foundation (South Africa). Mr Federico Fontana (Padova University, Italy) is acknowledged for HPLC analysis.

Conflict of Interest

The Authors declare no conflict of interest.

References

[1] Balat M, Balat H. Recent trends in global production and utilization of bioethanol fuel. Appl Energy 2009;86:2273-82. doi.org/10.1016/j.apen- ergy.2009.03.015.

[2] Favaro L, Basaglia M, Trento A, van Rensburg E, García-Aparicio M, van Zyl WH, Casella S. Exploring grape marc as trove for new thermotolerant and inhibitor-tolerant *Saccharomyces cerevisiae* strains for second-generation bioethanol production. Biotechnol Biofuels 2013;6:168.

[3] den Haan R, Kroukamp H, Mert M, Bloom M, Görgens JF, van Zyl WH. Engineering *Saccharomyces cerevisiae* for next generation ethanol production. J Chem Technol Biotechnol 2013;88:983-991.

[4] Brehmer B, Bals B, Sanders J, Dale B. Improving the corn-ethanol industry: Studying protein separation techniques to obtain higher value-added product options for distillers grains. Biotechnol Bioeng 2008;101:49–61. doi:10.1002/bit.21881.

[5] Renewable Fuels Association, Falling walls & rising tides - 2012 Ethanol industry outlook, Washington.

[6] Van Zyl WH, Bloom M, Viktor MJ. Engineering yeasts for raw starch conversion. Appl Microbiol Biotechnol 2012;95:1377–88. doi:10.1007/s00253-012-4248-0.

[7] Favaro L, Basaglia M, Saayman M, Rose SH, van Zyl WH, Casella S. Engineering amylolytic yeasts for industrial bioethanol production. Chemical Engineering Transactions 2010;20:97-102. doi:10.3303/CET1020017.

[8] Kim H-R, Im Y-K, Ko H, Chin J-E, Kim I-C, Lee HB, Bai S. Raw starch fermentation to ethanol by an industrial distiller's yeast strain of *Saccharomyces cerevisiae* expressing glucoamylase and α-amylase genes. Biotechnol Lett 2011;33:1643–1648. doi:10.1007/s10529-011-0613-9.

[9] Favaro L, Jooste T, Basaglia M, Rose SH, Saayman M, Görgens JF, Casella S, van Zyl WH. Codon-optimized glucoamylase sGAI of *Aspergillus awamori* improves starch utilization in an industrial yeast. Appl Microbiol Biotechnol 2012;95:957-968.

[10] Favaro L, Jooste T, Basaglia M, Rose SH, Saayman M, Görgens JF, Casella S, van Zyl WH. Designing industrial yeasts for the consolidated bioprocessing of starchy biomass to ethanol. Bioeng 2013c;4:1-6.

[11] Favaro L, Viktor MJ, Rose SH, Viljoen-Bloom M, van Zyl WH, Basaglia M, Cagnin L, Casella S. Consolidated bioprocessing of starchy substrates into ethanol by industrial *Saccharomyces cerevisiae* strains secreting fungal amylases (2015). Biotechnol Bioeng doi:10.1002/bit.25591.

[12] Yamada R, Yamakawa S-I, Tanaka T, Ogino C, Fukuda H, Kondo A. Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that

express amylases. Enzyme Microb Technol 2011;48:393–6. doi:10.1016/j.enzmictec.2011.01.002.

[13] Favaro L, Basaglia M, Casella S. Processing wheat bran into ethanol using mild treatments and highly fermentative yeasts. Biomass Bioenerg 2012;46:605-617. doi.org/10.1016/j.biombioe.2012.07.001.

[14] Favaro L, Basaglia M, van Zyl WH, & Casella S. Using an efficient fermenting yeast enhances ethanol production from unfiltered wheat bran hydrolysates. Appl Energy 2013;102:170–8. dx.doi.org/10.1016/j.apenergy.2012.05.059.

[15] Kim S, Dale BE. Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenerg 2004;26:361-75.

[16] White JS, Yohannan BK, Walker GM. Bioconversion of brewer's spent grains to bioethanol. FEMS Yeast Research 2008;8:1175–84. doi:10.1111/j.1567-1364.2008.00390.x.

[17] Thangavelu SK, Ahmed AS, Ani FN. Bioethanol production from sago pith waste using microwave hydrothermal hydrolysis accelerated by carbon dioxide. Appl Energy 2014;128:277–83. doi:10.1016/j.apenergy.2014.04.076.

[18] Virunanon C, Ouephanit C, Burapatana V, Chulalaksananukul W. Cassava pulp enzymatic hydrolysis process as a preliminary step in bio-alcohols production from waste starchy resources. J Clean Prod 2013;39:273–9. doi:10.1016/j.jclepro.2012.07.055.

[19] Rødsrud C, Lersch M, Sjöde A. History and future of world's most advanced biorefinery in operation, Biomass Bioenerg 2012;46: 46-59.

[20] Favaro L, Alibardi L, Lavagnolo MC, Casella S, Basaglia M. Effects of inoculum and indigenous microflora on hydrogen production from the organic fraction of municipal solid waste. Int J Hydrogen Energy 2013;38:11774-9.

[21] Romanelli MG, Povolo S, Favaro L, Fontana F, Basaglia M, Casella S. Engineering Delftia acidovorans DSM39 to produce polyhydroxyalkanoates from slaughterhouse waste. International Journal of Biological Macromolecules 2014;71:21-7.

[22] Schirru S, Favaro L, Mangia MN, Basaglia M, Casella S, Comunian R, Fancello F, Gombossy de Melo Franco BD, de Souza Oliveira RP, Todorov SD. Comparison of bacteriocins production from *Enterococcus faecium* strains in cheese whey and optimised commercial MRS medium. Ann Microbiol 2014:64:321-31.

[23] Chotěborská P, Palmarola-Adrados B, Galbe M, Zacchi G, Melzoch K, Rychtera M. Processing of wheat bran to sugar solution. J. Food Eng. 2004;61:561–5.

[24] Liu Z, Ying Y, Li F, Ma C, Xu P. Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. J Ind Microbiol Biotechnol 2010;37:495–501.

[25] Palmarola-Adrados B, Choteborská P, Galbe M, Zacchi G. Ethanol production from non-starch carbohydrates of wheat bran. Bioresour Technol 2005;96:843–50.

[26] Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Bongers M, Walton JD. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresour Technol 2010;101:9097–9105. doi:10.1016/j.biortech.2010.07.028.

[27] Ilmén M, den Haan R, Brevnova E, McBride J, Wiswall E, Froehlich A, Voutilainen SP, Siika-Aho M, la Grange DC, *et al*. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. Biotechnol Biofuels 2011;4:30.

[28] Rose SH, van Zyl WH. Exploitation of *Aspergillus niger* for the heterologous production of cellulases and hemicellulases. The Open Biotechnology Journal 2008;2:167-75.

[29] Njokweni AP, Rose SH, van Zyl, WH. Fungal β-glucosidase expression in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 2012;39:1445-52.

[30] Viktor MJ, Rose SH, van Zyl WH, Viljoen-Bloom M. Raw starch conversion by *Saccharomyces cerevisiae* expressing *Aspergillus tubingensis* amylases. Biotechnol for Biofuels 2013;6:167. doi:10.1186/1754-6834-6-167.

508 [31] Dobson RA. (2014). The development of a recombinant fungal enzyme cocktail for the
509 hydrolysis of paper sludge and evaluation on other cellulosic substrates. M.Sc. thesis.
510 Microbiology Department, Stellenbosch University. South Africa.
511 [32] Rose SH, van Zyl W. Constitutive expression of the gene *Trichoderma reesei* β-1,4-
512 xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egl*) in *Aspergillus niger* in
513 molasses and defined glucose media. Appl Microbiol Biotechnol 2002;58:461–8.
514 [33] AOAC (Association of Official Analytical Chemists). Official methods of analysis. 18th
515 ed. AOAC, Arlington, VA; 2010.
516 [34] Maes C, Delcour JA. Alkaline hydrogen peroxide extraction of wheat bran non-starch
517 polysaccharides. J Cereal Sci 2001;34:29-35.
518 [35] Zhu M, Zhu Z., Li X. Bioconversion of paper sludge with low cellulosic content to ethanol
519 by separate hydrolysis and fermentation. African Journal of Biotechnology
520 2011;10:15072–83. doi:10.5897/AJB11.1644.
521 [36] Modenbach A, Nokes S. Enzymatic hydrolysis of biomass at high-solids loadings: a
522 review, Biomass Bioenergy 2013;56:526–44. doi:10.1016/j.biombioe.2013.05.031.
523 [37] Caspeta L, Caro-Bermudez MA, Ponce-Noyola T, Martinez A. Enzymatic hydrolysis at
524 high-solids loadings for the conversion of agave bagasse to fuel ethanol. Appl Energy
525 2014;113:277–86. doi:10.1016/j.apenergy.2013.07.036.
526 [38] Olofsson K, Bertilsson M, Liden G. A short review on SSF - an interesting process option
527 for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 2008, 1:7.
528 doi:10.1186/1754-6834-1-7
529 [39] Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. Role and significance of
530 beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour
531 Technol 2013;127:500-7. doi: 10.1016/j.biortech.2012.09.012.
532 [40] Sørensen A, Lübeck M, Lübeck P, Ahring B. Fungal beta-glucosidases: a bottleneck in
533 industrial use of lignocellulosic materials. Biomolecules 2013;3:612–31.