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Original Citation:

Availability:
This version is available at: 11577/3251351 since: 2017-12-07T13:41:22Z

Publisher:
Elsevier Ltd

Published version:
DOI: 10.1016/j.biortech.2017.07.108

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Production of bioethanol from multiple waste streams of rice milling

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Abstract

This work describes the feasibility of using rice milling by-products as feedstock for bioethanol. Starch-rich residues (rice bran, broken, unripe and discolored rice) were individually fermented (20% w/v) through Consolidated Bioprocessing by two industrial engineered yeast secreting fungal amylases. Rice husk (20% w/v), mainly composed by lignocellulose, was pre-treated at 55°C with alkaline peroxide, saccharified through optimized dosages of commercial enzymes (Cellic® CTec2) and fermented by the recombinant strains.

Finally, a blend of all the rice by-products, formulated as a mixture (20% w/v) according to their proportions at milling plants, were co-processed to ethanol by optimized pre-treatment, saccharification and fermentation by amylolytic strains.

Fermenting efficiency for each by-product was high (above 88% of the theoretical) and further confirmed on the blend of residues (nearly 52 g/L ethanol). These results demonstrated for the first time that the co-conversion of multiple waste streams is a promising option for second generation ethanol production.

Keywords: rice milling by-products; alkaline peroxide pre-treatment; enzymatic saccharification; consolidated bioprocessing; multiple residues co-fermentation

1. Introduction

Bioethanol produced from biomass is regarded as an attractive fuel to reduce dependence on oil and decrease carbon dioxide emissions (Gnansounou and Dauriat, 2010; Hamelinck et al., 2005). One of the main costs in bioethanol and other bio-commodities production is the substrate and the use of cheap materials such as energy-crops, food processing residues, agricultural and forest waste is crucial (Alibardi et al., 2012; Ishola et al., 2013; Kougias et al., 2017; Rai et al., 2014; Romanelli et al., 2014; Schirru et al., 2014; Shah et al., 2016; Tsapekos et al., 2017).

Lignocellulosic biomass is the most promising raw material for bioethanol considering its great availability and limited price (Salehi Jouzani and Taherzadeh, 2015). Despite these advantages, lignocellulose is very expensive to process because of the need for costly pre-treatments and large dosages of commercial enzymes. Therefore, more efficient and cost-effective methods for the conversion of lignocellulosic biomass to ethanol are needed (Parachin et al., 2011; Sindhu et al., 2016). Routes fermenting lignocellulosic and starchy biomass to ethanol in one step without supplementation with externally produced enzymes are of evident appeal. Indeed, such 'consolidated bioprocessing' (CBP) is widely acknowledged as the ultimate configuration for low cost hydrolysis and fermentation of biomass (Favaro et al., 2013b; Olson et al., 2012).

To meet the increasing ethanol demand it is fundamental both to select a suitable and abundant residual biomass and to exploit it more efficiently by converting all the components, including lignocellulosic residues (Zabed et al., 2016). Furthermore, in the case of using waste from industrial processes as material, ethanol plants should be in close proximity of the by-products, thus reducing cost and greenhouse gas emission related to their transport.

This study targeted the wide residual streams of rice processing plant, namely

broken, unripe and discolored rice as well as rice bran and rice husk with the final aim of producing bioethanol. Every year millions of tons of rice by-products are wasted, with relevant environmental concerns (Abbas and Ansumali, 2010). According to FAO (Food and Agriculture Organization), the global paddy market amounted to nearly 745 million tons in 2014, resulting in the worldwide availability of about 150, 50, 45, 30 and 7 million tons of rice husk, rice bran, broken, unripe and discolored rice, respectively. Such enormous amount of cheap biomass would be directly accessible and/or collectable at rice processing sites.

Overall, rice by-products could be divided into two main categories: starch-rich (rice bran, broken, unripe and discolored rice) and lignocellulosic substrate (rice husk). Although the first cluster is currently valorized in feed formulations, more valuable applications should be developed to further improve the economics of rice milling industry. Moreover, rice husk, the main residue of rice milling, is currently considered a combustible waste for energy recovery because of its limited value as animal feed, mainly due to its low digestibility and bulk density. Thus, rice husk could serve as a promising lignocellulosic feedstock for ethanol production as it contains about 35% cellulose, 15% hemicellulose and high quantities of lignin (16%) and ash (20%) together with a little amount of starch (up to 7%).

The objective of this work is to develop the first method for the efficient simultaneous conversion of all the by-products of rice milling plants into ethanol. To this purpose, novel CBP yeast secreting fungal enzymes have been used as fermenting strains and efficiently applied for the CBP of each single starchy substrate. Moreover, rice husk has been pre-treated with alkaline peroxide, saccharified by optimized dosages of commercial Cellic® CTec2 and fermented by CBP strains. Finally, all the by-products have been fermented in a mixture formulated according to their proportions of production in a typical milling plant. This is the first report describing the one step co-

conversion of starchy and lignocellulosic substrates into bioethanol.

2. Material and methods

2.1 Strains, media and cultivations

Two industrial strains, *S. cerevisiae* MEL2 and M2n, with their respective recombinant strains MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], were utilized for fermentation studies. The parental yeast, previously described for their promise in terms of bioethanol applications (Favaro et al., 2013a; Viktor et al., 2013), have been lately engineered for the co-expression of the *TLG1* (encodes glucoamylase from *Thermomyces lanuginosus*) and the *SFA1* (encodes α-amylase from *Saccharomycopsis fibuligera*) genes. Both genes were integrated into the chromosomes of *S. cerevisiae* MEL2 and M2n, resulting in high amylolytic activities and fermenting abilities on pure starchy substrates, such as corn, wheat and triticale grains (Favaro et al., 2015).

The strains were maintained on YPD (Yeast Peptone Dextrose) plates. Culture broth (20 g/L peptone, 20 g/L glucose, 6.7 g/L yeast nitrogen base, 0.05 mM citric acid buffer, pH 5) was used to grow yeast inocula for the fermentation kinetics. Fermentation medium composition was similar to the cultivation broth, with the exception of glucose concentration (0.5 g/L).

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

2.2 Chemical analysis of feedstocks

Rice by-products (bran, husk, broken, discolored and unripe rice) were obtained from La Pila (Isola della Scala, Italy), dried in a forced-air oven at 55 °C for 24 h and milled in a hammer mill to seep throughout a 1.25 mm screen. The milled feedstocks were stored at room temperature and their composition in terms of ash, starch, hemicellulose,

cellulose, lignin and protein was determined according to international standard methods (Horwitz et al., 1975). The same procedures were used to assess the content of starch, hemicellulose, cellulose and lignin in the spent fermentation samples.

2.3 Alkaline peroxide pre-treatment of rice husk

Milled rice hulls were slurried in water (20%, w/v) containing H_2O_2 (0, 2.5, 5, 7.5 or 10.0%, v/v), adjusted to pH 11 using NaOH and shaken in an incubator at 150 rpm at 55 °C for 24 h. The pH of the pre-treated rice husk was adjusted to 5.5 using concentrated HC1 before enzymatic saccharification. Samples (4 mL), withdrawn after 0, 1, 2, 4 and 24 h, were stored at -20 °C before analysis.

2.4 Enzymatic saccharification

The enzymatic saccharification of the alkaline peroxide pre-treated rice husk was performed by shaking gently (100 rpm) at 50 °C for 96 h after adjusting the pH to 5.5 with HC1 and adding three dosages of Cellic® CTec2, 1.5, 3 and 6 % w/w (g/g cellulose) according to the instructions of the supplier. Samples (4 mL) were withdrawn after 0, 12, 24, 36, 48, 60, 72 and 96 h, heat denatured (boiled for 10 min) to inactivate the enzymes, then kept at -20 °C for before HPLC (High Performance Liquid Chromatography) analysis.

2.5 Fermentation studies

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 24 h. Small-scale fermentations, conducted in 120-mL serum bottles containing 100 mL of fermentation medium with 20% (w/v) dry substrate, were inoculated with 50 g/L wet cell weight (corresponding to about 5×10^8 CFU/mL) of cells grown at 30°C for 60 h.

Fermentation bottles were provided with a needle for the CO_2 removal and fermentations were performed under oxygen-limited conditions. Ampicillin (100 mg/L) and streptomycin (75 mg/L) were added to prevent bacterial contamination. Samples (2 mL), taken daily during the course of the fermentation, were kept at -20 °C. Two types of fermentation studies were conducted: Simultaneous Saccharification and Fermentation (SSF) on pre-treated rice husk and CBP on rice bran, broken, discolored and unripe rice. SSF fermentation was performed also on 20% (w/v) of mixed rice residues according to the ratio in which they are usually produced at milling plants: 20:7:6:4:1.5 for husk, bran, broken rice, unripe rice, discolored rice, respectively. The loading of Cellic® CTec2 in all SSF studies was 3% w/w (g/g cellulose). Experiments were performed in triplicate.

2.6 Analytical methods, calculations and statistical analysis

Samples taken from liquid fraction during pre-treatment, enzymatic hydrolysis and fermentation were analysed for arabinose, galactose, glucose, xylose, mannose, sucrose, maltose, cellobiose, acetic acid, formic acid, furfural and HMF (5-hydroxymethyl-2-furaldehyde). Samples, filtered through 0.22-µm, were diluted prior to HPLC analysis. Liquid chromatography analysis was performed using a Shimadzu Nexera HPLC system, equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The chromatographic separations were performed using a Phenomenex Rezex ROA-Organic Acid H^+ (8%) column (300mm×7.8mm). The column temperature was set at 65 °C and the analysis was performed at a flow rate of 0.6 mL/min using isocratic elution, with 0.01 M H_2SO_4 as a mobile phase. Analytes were identified by comparing their retention times and the concentrations were calculated using calibration curves of the corresponding external standard.

Sugars concentrations were used to calculate the degree of saccharification (DS) of cellulose, hemicellulose and both cellulose and hemicellulose in pre-treated rice husk. DS represents the soluble sugars (glucose or arabinose, galactose and xylose) released after hydrolysis of rice husk. Total sugars yield was calculated considering the total sugars released over the sum of cellulose and hemicellulose available. A conversion factor of 0.9 was applied, as a water molecule is added during the hydrolysis (Cripwell et al., 2015).

$$DS = \frac{[sugar(s)\ g/L]\ x\ 0.9}{[polysaccharide(s)\ g/L]}\ x\ 100\%$$

The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilized glucose equivalent) was determined 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 equivalent. The volumetric productivity (Q) was calculated as grams of produced ethanol 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.

The theoretical CO_2 yields were determined based on the ethanol produced by each yeast strain, assuming that equimolar ethanol and CO_2 are produced. The percentage of carbon converted to glucose, maltose, glycerol, ethanol, and CO_2 was obtained on a mole carbon basis.

Statistical analyses were assessed using the Graphpad Prism 5 package (Graphpad Software, Inc., San Diego, California). Descriptive statistics, mean values and standard

deviations were calculated. Data were analysed also by two ways factorial ANOVA (Analysis Of Variance) with Duncan test.

3. Results and discussion.

3.1 Rice by-products composition

The composition of rice by-products used in this work is reported in Table 1. Cellulose was abundant (38.6%) only in rice husk, which had hemicellulose (16.1) and lignin (16.7) as other main components. Hemicellulose content was much lower in bran, unripe, broken and discoloured rice. On the other hand, starch levels were greatly higher in bran, unripe, broken and discolored rice. Protein and ash were one of the significant fractions in rice bran (13.9%) and husk (15.9%), respectively.

Overall, the values reported in Table 1 agreed well with published results (Abbas and Ansumali, 2010; Nakano et al., 2012; Saha and Cotta, 2007; Saunders, 1985; Singh et al., 2011) and clearly confirmed that rice milling by-products could be sorted into two groups: starch- and lignocellulosic rich- materials. In the case of the former category the efficient Consolidated Bioprocessing (CBP) by means of engineered amylolytic strains would be a sustainable strategy (Favaro et al., 2010; Favaro et al., 2013b; Salehi Jouzani and Taherzadeh, 2015; van Zyl et al., 2012). On the other hand, the need for optimized pre-treatment and saccharification of rice husk is required, given the high cellulose and lignin content (Gnansounou and Dauriat, 2010; Parachin et al., 2011; Sindhu et al., 2016; Singh et al., 2011).

With the final aim of processing all the rice milling by-products simultaneously into ethanol, specific fermentation experiments on each starchy and lignocellulosic substrate were performed using two industrial strains secreting fungal amylases, recently described for their efficient starch-to-ethanol route (Favaro et al., 2015).

3.2 Consolidated bioprocessing of starchy rice milling by-products

The parental and recombinant yeast strains were first evaluated for their ability to ferment starchy materials at a high substrate loading (20%, w/v) under oxygen-limited conditions in 120-mL fermentation bottles (Figure 1, Table 2). As reported in Table 2, each fermentation differed in the amount of glucose equivalent according to the concentration of starch and simple hexose sugars (mainly glucose) available in the feedstock.

As expected, the parental yeast strains did not utilize the raw starch for ethanol production and only produced limited amounts of ethanol from the simple sugars occurring in the feedstocks (Figure 1 a,b and Table 3).

On the contrary, both recombinant strains, *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1], produced high levels of ethanol during the CBP of all tested rice starchy by-products. The low residual amounts of glucose and maltose in the fermentation broth indicate a rapid sugar uptake by the recombinant strains. Moreover, limited glycerol concentrations were detected suggesting that the carbon metabolism was mainly directed to ethanol production (Table 2).

Rice bran was the most efficiently used feedstock: *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] fermented about 90 g/L of available starch to 42.06 and 41.05 g/L alcohol, respectively (corresponding to 92 and 90% of the theoretical yield) (Figure 1a,b and Table 3). Raw starch conversion kinetics of engineered strains was similar, with *S. cerevisiae* MEL2[TLG1-SFA1] being slightly slower (Figure 1a,b). As reported in Table 2, although the final volumetric productivity (Q) was comparable between the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains, the Q_{max} of M2n[TLG1-SFA1] (1.11 g/L/h after 24 h), was approximately 1.20-fold higher than that of MEL2[TLG1-SFA1] (0.98 g/L/h after 24 h). Moreover, M2n[TLG1-SFA1], both

after 72 and 144 h of fermentation, exhibited higher carbon conversion efficiency (about 96%) than that of MEL2[TLG1-SFA1] (nearly 90%).

S. cerevisiae M2n[TLG1-SFA1] efficiently converted the raw starch present in 20% (w/v) of discolored rice (Figure 1a) with the production of 78.82 g/L ethanol, whereas lower ethanol levels (up to 68.28 g/L) were obtained by *S. cerevisiae* MEL2[TLG1-SFA1] (Figure 1b). The volumetric productivity of *S. cerevisiae* M2n[TLG1-SFA1] was therefore higher, peaking at 0.82 g/L/h after 72 h, compared to *S. cerevisiae* MEL2[TLG1-SFA1] that achieved 0.67 g/L/h only after 84 h (Table 2). Moreover, carbon conversion rate was much higher in the case of M2n[TLG1-SFA1] during the CBP kinetic (Table 2). At the end of the fermentation, starch consumption by *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] was 84 and 74%, respectively, with ethanol yields of 91 and 88% of the theoretical, respectively (Table 3).

Broken rice was processed to ethanol by both *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains, with the former confirming higher performances: M2n[TLG1-SFA1] produced 49.09 and 74.54 g/L ethanol after 3 and 6 days of fermentation, respectively from 200 g/L broken rice (Figure 1a, Table 2). On the contrary, ethanol levels achieved by MEL2[TLG1-SFA1] were 42.04 and 67.97 g/L after 3 and 6 days of incubation (Figure 1b, Table 2). As a result, the volumetric productivity for M2n[TLG1-SFA1] was higher, with a maximum of 0.74 g/L/h detected after 48 h, about 1.4-fold greater than the highest volumetric productivity (0.61 g/L/h after 84 h) for MEL2[TLG1-SFA1] (Table 2). Carbon conversion efficiency was higher in the case of M2n[TLG1-SFA1] both after 72 and 144 h (Table 2). Moreover, *S. cerevisiae* M2n[TLG1-SFA1] was superior in terms of starch utilization and ethanol yields, being able to convert 88% of the available starch and exhibit 89% of the theoretical ethanol yield. MEL2[TLG1-SFA1] displayed lower starch-utilizing and fermenting abilities, consuming 80% of raw starch and producing about 0.44 g of

ethanol per g of utilized glucose equivalent, corresponding to 85% of the theoretical (Table 3).

The engineered strains confirmed their distinct fermentation patterns also in the case of unripe rice (Figure 1a,b). The starch-to-ethanol conversion of M2n[TLG1-SFA1] was again more efficient than that of MEL2[TLG1-SFA1], with 65.99 and 60.49 g/L ethanol produced by the former and latter strain, respectively (Table 3). Ethanol productivity was greater for *S. cerevisiae* M2n[TLG1-SFA1] too (Table 2), exhibiting values 16% higher than those detected for MEL2[TLG1-SFA1]. As described in Table 2 for the other starchy by-products, most of the carbon conversion took place within the first 72 h of incubation and M2n[TLG1-SFA1] proved to be the most efficient also on unripe rice, capable of 63% of carbon conversion instead of 55% by MEL2[TLG1-SFA1]. Therefore, starch utilization and final ethanol yield from unripe rice were higher for M2n[TLG1-SFA1], with almost 91% of consumed starch and 0.45 g of ethanol per g of used glucose equivalent whereas the other engineered strain was able to hydrolyze about 84% of the polysaccharide available and produce ethanol with a slightly lower efficiency (Table 3).

Taken together, the results reported in Figure 1 and Table 3 pointed out that the four starchy substrates were effectively converted into ethanol, with the following order of increasing performance: rice bran > discolored > broken > unripe rice. The highest carbon conversion yield and ethanol efficiency detected in both strains from rice bran could be ascribed to the amount of protein (13.9% dry matter) available in bran (Table 1), significantly higher ($p \leq 0.01$) than those found in the other three substrates, having, on average, protein levels of nearly 8.7% dry matter. This observation is in agreement with several papers in literature describing that both nature and concentration of nitrogen significantly influenced recombinant proteins production by engineered yeast strains (Favaro et al., 2012; Hahn-Hägerdal et al., 2005; Silva et al., 2010).

The extent of starch utilization in the two recombinant strains (Table 3) was found to be inversely proportional to the overall amount of polysaccharide in the system: starch in rice bran, which accounts for 28.5% of the dry matter (Table 1) has been completely utilized whereas in discoloured rice, containing the highest amount of starch (84.6%), the average starch utilization level was significantly lower (79%, $p \leq 0.01$). This finding could also be related to different degrees of starch digestibility within the tested materials (Singh et al., 2010). For instance, it was already reported that whole grain rice with intact bran, such as unripe rice, could withstand hydrolysis longer than well-polished rice grain (Englyst and Englyst, 2005).

Noteworthy, the recombinants demonstrated efficient production of ethanol from four starchy by-products with no pre-treatment nor exogenous enzyme addition, avoiding the need for expensive pre-treatments and, at least, partial enzymatic hydrolysis claimed in the majority of papers dealing with ethanol production from starchy materials (Chu-Ky et al., 2016; Gohel and Duan, 2012; Ho et al., 2013; Yuangsaard et al., 2013; Zhang et al., 2013). Considering now the performances within the two strains, *S. cerevisiae* M2n[TLG1-SFA1] was superior than MEL2[TLG1-SFA1] on all the tested starchy by-products (Figure 1 and Tables 2,3). This finding is consistent with recent fermentation studies of both strains using real starchy substrates (corn, sweet sorghum and triticale grains) and the greater starch converting performance of M2n [TLG1-SFA1] was previously credited to higher enzymatic activities (Favaro et al., 2015).

Moreover, this is the first report describing industrial yeast strains with high substrate utilization and fermentation efficiencies on a cluster of starchy by-products. Indeed, there are only few research papers on the processing of single starchy residues into ethanol using engineered amylolytic yeast (Apiwatanapiwat et al., 2011; Cripwell et al., 2015). As a result, these findings are of great interest towards the large-scale application

of the recombinant strains, mainly M2n[TLG1-SFA1], in CBP systems from both starchy substrates and by-products.

3.3 Pre-treament, saccharification and fermentation of rice husk

Rice husk used in this study contained high cellulose, hemicellulose and lignin content (Table 1) and the definition of proper pre-treatment and saccharification is crucial towards the efficient exploitation of such substrate into ethanol. In the present paper, alkaline peroxide was selected as pre-treatment option because it was already reported for the efficient decrystallization of cellulose of rice husk, without the release of significant amounts of inhibitors (Cabrera et al., 2014; Diaz et al., 2013; Saha and Cotta, 2007; Xu et al., 2016).

Initially, the effect of residence time (1, 2, 4 and 24 h at 55°C) of alkaline pre-treatment (7.5% H_2O_2, v/v, pH 11.5) on the enzymatic saccharification of 20% rice husk was investigated. The resulting yields of total sugars after enzymatic saccharification using Cellic® CTec2 (1.5% w/w cellulose) at 50°C, pH 5.0 for 96 h increased with the incubation time and was highest after 2 hours of pre-treatment (Supplementary material). Based on this finding, such residence time has been selected for the following pre-treatment experiments.

The effect of peroxide level (0-10%, v/v) on the pre-treatment of rice husk at 55°C for 2 h on the enzymatic saccharification using 1.5% Cellic® CTec2 is reported in Figure 2. The highest value of sugar release, 246.19 g/kg, and glucose yield (158.54 g/kg) were obtained in the sample treated with 7.5% H_2O_2. Small amounts of galactose and sucrose were also detected (data not shown). Both monosaccharide and total sugar yields were significantly different ($p \leq 0.01$) on pre-treated rice husk with lower amounts of peroxide (2.5 and 5%) and found to be slightly reduced in the case of 10% H_2O_2 treated sample. Therefore, the concentration of 7.5 % (v/v) was selected for subsequent

pre-treatment studies.

Interestingly, inhibitors production after alkaline pre-treatment was limited, with no furans (HMF and furfural) and acetic acid detected. This finding is consistent with other research papers where alkaline pre-treatment did not result in detectable levels of inhibitory compounds (Banerjee et al., 2011; Cabrera et al., 2014; Saha and Cotta, 2007). Nevertheless, the higher peroxide levels, the higher concentration of formic acid was created, with a level of 1.6 g/L measured in the sample treated with 10% H_2O_2 (data not shown).

In order to optimize enzyme dose, alkaline pre-treated rice husk (7.5% H_2O_2, pH 11, 55°C, 2 h) was hydrolyzed by three different loadings of Cellic® CTec2 (1.5, 3 and 6% w/w cellulose). As expected, pre-treatment alone resulted in low total sugar release but, when combined with enzymatic hydrolysis, the higher enzyme dose, the greater sugar yields were obtained (Figure 3). The most abundant sugars were glucose, 162.17, 307.22 and 343.70 g/Kg, and xylose, 57.98, 129.62 and 153.92 g/Kg, after the incubation for 96 h with 1.5, 3 and 6% Cellic® CTec2, respectively. Most of the sugars, about 90% of the total releases by the three tested enzymatic loadings, were obtained within 36 h, with sugars levels slowly increasing up to 96 h, after which no further enzymatic hydrolysis took place (data not shown).

Considering both hexoses and pentoses yields, the enzymatic hydrolysis defined in this study pointed at high saccharification efficiency of cellulose and hemicellulose. The DS values of hemicellulose (46, 88 and 95% for 1.5, 3 and 6% enzyme loading, respectively) were higher than those of cellulose (40, 72 and 82% for 1.5, 3 and 6% enzyme loading, respectively). The overall hydrolysis yields based on the total sugar release by 1.5, 3 and 6% Cellic® CTec2 were found to be 66, 89 and 94% of the theoretical, respectively. The outstanding total sugar amounts obtained with the latter two enzymatic loadings were greater than those recently described from alkaline pre-

treated rice husk by using higher enzyme dosages (Saha and Cotta, 2007) and/or much lower substrate loadings, 2-5% dry matter (Cabrera et al., 2014; Diaz et al., 2013; Singh et al., 2011).

The potential for conversion of the cellulosic components of pre-treated rice husk to ethanol was evaluated in120-mL fermentation bottles, under SSF conditions using 3 % Cellic® CTec2. Such enzymatic dosage was selected as techno-economic trade-off between sugar yield and enzyme loading (Figure 3). SSF was carried out at 30 °C on 20% w/v pre-treated rice husk inoculating *S. cerevisiae* M2n, MEL2 and the respective recombinants M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] (Figure 4, Table 2 and 3).

The two parental strains produced high ethanol levels (almost 23 g/L) from the glucose released by Cellic® CTec2. As reported in Table 3, Cellic® CTec2 addition resulted in the utilization of a great proportion of cellulose (on average 65%), with a cellulose DS slightly lower ($p \leq 0.05$) than that described for the saccharification of rice husk (72%). This finding could be ascribed to the lower temperature of incubation of SSF (30° C) compared to enzymatic hydrolysis (50 °C), as already reported in previous researches (Kelbert et al., 2016; Mutturi and Lidén, 2013). Moreover, the engineered strains obtained concentrations of ethanol significantly higher ($p \leq 0.01$) than those of their parental yeast, further confirming their great promise as starch hydrolysing microbes. The recombinants were able to completely utilize starch available in rice husk, with M2n[TLG1-SFA1] exhibiting the highest ethanol efficiency of 0.48 g/g of consumed glucose equivalent, corresponding to 94% of the theoretical (Table 3). As reported in Table 2, small amounts of glucose and maltose were detected after 48 h, indicating an efficient saccharification of both cellulose and starch by Cellic® CTec2 and recombinant secreted amylases, respectively. M2n[TLG1-SFA1] was faster than MEL2[TLG1-SFA1] in ethanol production with a volumetric productivity peaking at 1.21 g/L/h after 12 h of incubation (1.16-fold greater than the highest value for

MEL2[TLG1-SFA1]), meanwhile both strains exhibited similar carbon conversion rates (about 60%) (Table 2).

3.4 Simultaneous saccharification and fermentation of mixture of rice by-products

The fermenting yields obtained from single substrates, both starchy and cellulosic by-products (Figures 1 and 4, respectively), were found to be high and likely to be improved upon by repeated fermentations and further optimization of commercial enzyme loadings.

Moreover, this work for the first time aimed to achieve high ethanol performances from all the waste streams of a rice milling plant. To this purpose, all the by-products were formulated in a mixture and simultaneously processed to bioethanol. The resulting blend, composed mainly by starch, cellulose, hemicellulose, lignin and protein (31, 21, 11, 9 and 8% of dry matter), was fermented through SSF by using M2n[TLG1-SFA1] and MEL2[TLG1-SFA1]. For comparison SSF was performed also with the two parental strains (Figure 4).

As reported in Table 3, both wild type yeast produced similar ethanol levels (about 18 g/L), converting glucose released during SSF by Cellic® CTec2 with comparable fermenting yields (0.48 g/g). Cellulose utilization was on average 65 %, confirming the high efficiency of hydrolysis already reported for rice husk (Table 3).

Figure 4 clearly described that, within the first 12 h of fermentation, the engineered strains produced ethanol with rates and levels close to their parental strains. Later on, both recombinants secreting amylases readily converted starch to ethanol, resulting in significantly greater alcohol concentrations. M2n[TLG1-SFA1] produced almost 52 g/L of ethanol meanwhile MEL2[TLG1-SFA1] about 47 g/L. Starch utilization was completed in the former strain whereas was lower (90%) with the latter. Therefore,

ethanol yields were different with M2n[TLG1-SFA1] exhibiting the most promise (0.47 g/g, corresponding to 92% of the theoretical) (Table 3).

M2n[TLG1-SFA1] was superior also in terms of carbon conversion efficiency and volumetric productivity (Table 2). After 48 h of incubation, M2n[TLG1-SFA1] showed 78% of carbon conversion instead of 62% achieved by MEL2[TLG1-SFA1], with a volumetric productivity of 0.97 g/L/h, 1.27-fold higher than that of MEL2[TLG1-SFA1]. At the end of the fermentation, MEL2[TLG1-SFA1] partially closed the gap with M2n[TLG1-SFA1], exhibiting 79% of carbon-to-products efficiency and volumetric productivity of 0.49 g/L/h compared with that of the superior M2n[TLG1-SFA1] strain (0.54 g/L/h).

Overall, as reported in Table 3, the starch utilization extent of the by-products' was very high and cellulose saccharification could be further improved by future experiments focused on enzyme loading optimization.

4. Conclusions

This paper demonstrated that rice milling by-products could be efficiently converted into ethanol both as single feedstock and as mixture. As such, this is the first report on the biofuel production from multiple starchy and lignocellulosic-rich streams.

The final ethanol titer from the mixture was above 51 g/L and repeated fermentations together with further enzyme loading optimization are likely to foster ethanol yield.

Techno-economic and environmental evaluations are in progress to determine the viability of the whole process. Nevertheless, the results of this study indicate that the co-conversion of multiple waste is a feasible option for second generation ethanol production.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Acknowledgements

Nicolò Stefani, MSc and Elisabetta Bordignon, MSc (Padova University, Italy) are acknowledged for valuable technical support. Giuseppe Piubello, MSc provided rice by-products.

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' [grant GRIC120EG8]; the research project "BioRivaluta" funded by Regione Veneto (PSR 2007-2013, Misura 124) [grant 2307660]; and the bilateral joint research project entitled 'Development of robust yeast strains for bioethanol production from starchy and cellulosic plant biomass' [grant ZA11MO2].

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Figures captions

Fig. 1. Ethanol production from CBP of 20% (w/v) rice bran (♦), discoloured rice (■), unripe rice (●) or broken rice (▲) by *S. cerevisiae* strains: M2n and M2n[SFA1-TLG1] (a) and MEL2 and *S. cerevisiae* MEL2[SFA1-TLG1] (b). Continuous and dash lines describe ethanol production by recombinant and parental strains, respectively. Values represent the mean of three replicates and error bars represent the standard deviation.

Fig. 2. Effect of H_2O_2 concentrations applied during the pre-treatment (pH 11, 55°C, 2 h) of rice husk (20%, w/v) on enzymatic hydrolysis (50°C, pH 5, 96 h, 1.5% w/w Cellic® CTec2). Values represent the mean of three replicates and error bars represent the standard deviation.

Fig. 3. Effect of Cellic® CTec2 loading on sugar yields after 96 h hydrolysis of alkaline pre-treated (7.5%, pH 11, 55°C, 2 h) rice husk (20%, w/v). Values represent the mean of three replicates and error bars represent the standard deviation.

Fig. 4. Ethanol production during SSF from 20% (w/v) of rice husk (○) or mixture of rice by-products (◊) by *S. cerevisiae* strains: M2n and M2n[SFA1-TLG1] (a) and MEL2 and *S. cerevisiae* MEL2[SFA1-TLG1] (b). Cellic® CTec2 (3% w/w, g/g cellulose) was loaded at the beginning of SSF. Continuous and dash lines describe ethanol production by recombinant and parental strains, respectively. Values represent the mean of three replicates and error bars represent the standard deviation. The rice by-products mixture was obtained as described in subsection 2.5 Fermentation studies.

Table 1. Composition of rice by-products used in this study.

	% dry matter				
Component	**Discolored rice**	**Broken rice**	**Unripe rice**	**Rice bran**	**Rice husk**
Starch	84.6	77.7	68.6	28.5	6.9
Cellulose	0.1	0.2	1.8	4.6	38.4
Ash	0.5	0.5	1.5	8.0	15.9
Hemicellulose	0.9	0.5	3.7	8.4	16.1
Lignin	-	-	-	2.8	16.7
Protein	8.0	8.3	9.9	13.9	3.4

Table 2. Conversion of rice waste and mixtures of rice by-products to ethanol and by-products by recombinant *S. cerevisiae* strains.

Component	***S. cerevisiae* M2n[TLG1-SFA1]**		***S. cerevisiae* MEL2[TLG1-SFA1]**	
Rice bran = a glucose equivalent of 89.91 g/L and a total carbon available (mol C) of **3.00**				
Product (g/L)	**72 h**	**144 h**	**72 h**	**144 h**
Glucose	nd	nd	nd	nd
Maltose	0.04 ±0.01	nd	nd	nd
Glycerol	3.77 ±0.30	3.94 ± 0.41	3.99 ± 0.27	3.77 ± 0.23
Ethanol	**42.06 ± 1.90**	**41.55 ± 2.53**	**39.36 ± 2.76**	**38.90 ± 2.38**
CO_2	40.23	39.74	37.65	37.21
Total carbon	2.87	2.84	2.70	2.66
Carbon conversion (mol C)	96%	95%	90%	89%
Q (g/L/h)	0.58	0.29	0.55	0.27
Q_{max} (g/L/h)	1.11 after 24 h		0.98 after 24 h	
Discolored rice = a glucose equivalent of 201.52 g/L and a total carbon available (mol C) of **6.72**				
Product (g/L)	**72 h**	**144 h**	**72 h**	**144 h**
Glucose	0.55 ±0.04	nd	0.78 ±0.05	nd
Maltose	0.16 ±0.02	0.01 ±0.01	0.85 ±0.06	nd
Glycerol	3.75 ±0.29	5.50 ± 0.43	3.45 ±0.32	4.89 ± 0.48
Ethanol	**55.09 ± 1.44**	**78.82 ± 3.60**	**46.63 ± 2.97**	**68.28 ± 2.45**
CO_2	52.69	75.39	44.60	65.31
Total carbon	3.74	5.32	3.21	4.62
Carbon conversion (mol C)	56%	79%	48%	69%
Q (g/L/h)	0.77	0.55	0.65	0.47
Q_{max} (g/L/h)	0.82 after 72 h		0.67 after 84 h	
Broken rice = a glucose equivalent of 184.36 g/L and a total carbon available (mol C) of **6.15**				
Product (g/L)	**72 h**	**144 h**	**72 h**	**144 h**
Glucose	nd	nd	nd	nd
Maltose	0.91 ± 0.08	nd	1.40 ± 0.11	0.27 ± 0.03
Glycerol	2.76 ± 0.24	4.30 ± 0.37	2.47 ± 0.19	4.17 ± 0.18
Ethanol	**49.09 ± 2.95**	**74.54 ± 3.40**	**42.04 ± 3.69**	**67.97± 4.07**
CO_2	46.96	71.30	40.21	47.17
Total carbon	3.32	5.01	2.87	4.54
Carbon conversion (mol C)	54%	82%	47%	74%
Q (g/L/h)	0.68	0.52	0.58	0.47
Q_{max} (g/L/h)	0.74 after 48 h		0.61 after 84 h	
Unripe rice = a glucose equivalent of 162.94 g/L and a total carbon available (mol C) of **5.43**				
Product (g/L)	**72 h**	**144 h**	**72 h**	**144 h**
Glucose	0.16 ± 0.01	nd	0.20 ± 0.01	nd
Maltose	0.67 ± 0.04	nd	1.65 ± 0.14	0.55 ± 0.03
Glycerol	2.36 ± 0.20	3.95 ± 0.31	2.07 ± 0.18	3.12 ± 0.22
Ethanol	**51.17 ± 3.07**	**65.99 ± 3.09**	**42.04 ± 1.80**	**60.49± 2.18**
CO_2	48.95	63.12	40.21	57.86
Total carbon	3.44	4.43	3.00	4.07
Carbon conversion (mol C)	63%	82%	55%	75%
Q (g/L/h)	0.71	0.46	0.61	0.42
Q_{max} (g/L/h)	0.87 after 48 h		0.71 after 84 h	

to be continued

Rice husk = a glucose equivalent of 100.62 g/L and a total carbon available (mol C) of **3.35**				
Product (g/L)	**48 h**	**96 h**	**48 h**	**96 h**
Glucose	0.20 ± 0.01	nd	0.35 ± 0.01	nd
Maltose	0.06 ± 0.01	nd	0.09 ± 0.01	nd
Glycerol	2.99 ± 0.30	3.04 ± 0.41	2.01 ± 0.17	2.82 ± 0.21
Ethanol	**30.39 ± 1.49**	**30.10 ± 2.06**	**28.10 ± 1.67**	**29.46 ± 1.72**
CO_2	29.07	28.79	26.88	28.18
Total carbon	2.09	2.06	1.91	2.01
Carbon conversion (mol C)	62%	61%	57%	60%
Q (g/L/h)	0.63	0.31	0.55	0.27
Q_{max} (g/L/h)	1.21 after 12 h		1.04 after 12 h	
Mixture of by-products = a glucose equivalent of 122.23 g/L and a total carbon available (mol C) of **4.07**				
Product (g/L)	**48 h**	**96 h**	**48 h**	**96 h**
Glucose	0.55 ± 0.02	nd	0.25 ± 0.02	nd
Maltose	0.16 ± 0.01	nd	0.05 ± 0.01	0.09 ± 0.01
Glycerol	3.75 ± 0.25	4.00 ± 0.29	3.07 ± 0.18	3.92 ± 0.31
Ethanol	**46.71 ± 2.08**	**51.88 ± 2.62**	**36.82 ± 1.80**	**47.39 ± 2.18**
CO_2	44.68	49.62	35.22	45.33
Total carbon	3.19	3.51	2.51	3.23
Carbon conversion (mol C)	78%	86%	62%	79%
Q (g/L/h)	0.97	0.54	0.77	0.49
Q_{max} (g/L/h)	1.14 after 12 h		1.00 after 12 h	

nd: not detected; Q: ethanol productivity; Q_{max}: maximum ethanol productivity

Table 3. Conversion of rice's glucose, 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]. Substrate loading of each fermentation experiment was 20% (w/v).

Strain	***Highest ethanol concentration (g/L)***	***Glucose utilization (%)***	***Starch utilization (%)***	***Cellulose utilization (%)***	$Y_{E/S}$ **(g/g)**
Rice bran					
MEL2	15.11	100	-	-	0.49 (96%)
MEL2[TLG1-SFA1]	41.05	100	100	-	0.46 (90%)
M2n	14.96	100	-	-	0.48 (95%)
M2n[TLG1-SFA1]	42.06	100	100	-	0.47 (92%)
Discoloured rice					
MEL2	6.74	100	-	-	0.49 (96%)
MEL2[TLG1-SFA1]	68.28	100	74	-	0.45 (88%)
M2n	6.59	100	-	-	0.49 (96%)
M2n[TLG1-SFA1]	78.82	100	84	-	0.46 (91%)
Broken rice					
MEL2	5.73	100	-	-	0.49 (97%)
MEL2[TLG1-SFA1]	67.97	100	80	-	0.44 (85%)
M2n	5.58	100	-	-	0.48 (94%)
M2n[TLG1-SFA1]	74.54	100	88	-	0.46 (89%)
Unripe rice					
MEL2	5.17	100	-	-	0.49 (96%)
MEL2[TLG1-SFA1]	60.49	100	84	-	0.44 (86%)
M2n	5.05	100	-	-	0.48 (94%)
M2n[TLG1-SFA1]	65.99	100	91	-	0.45 (87%)
Rice husk					
MEL2	22.82	-	-	64	0.48 (94%)
MEL2[TLG1-SFA1]	29.50	-	100	65	0.46 (92%)
M2n	22.77	-	-	65	0.48 (94%)
M2n[TLG1-SFA1]	30.45	-	100	63	0.48 (94%)
Mixture of by-products					
MEL2	18.19	100	-	65	0.48 (94%)
MEL2[TLG1-SFA1]	47.39	100	90	66	0.46 (90%)
M2n	18.32	100	-	66	0.48 (95%)
M2n[TLG1-SFA1]	51.88	100	100	64	0.47 (92%)

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

Fig. 1

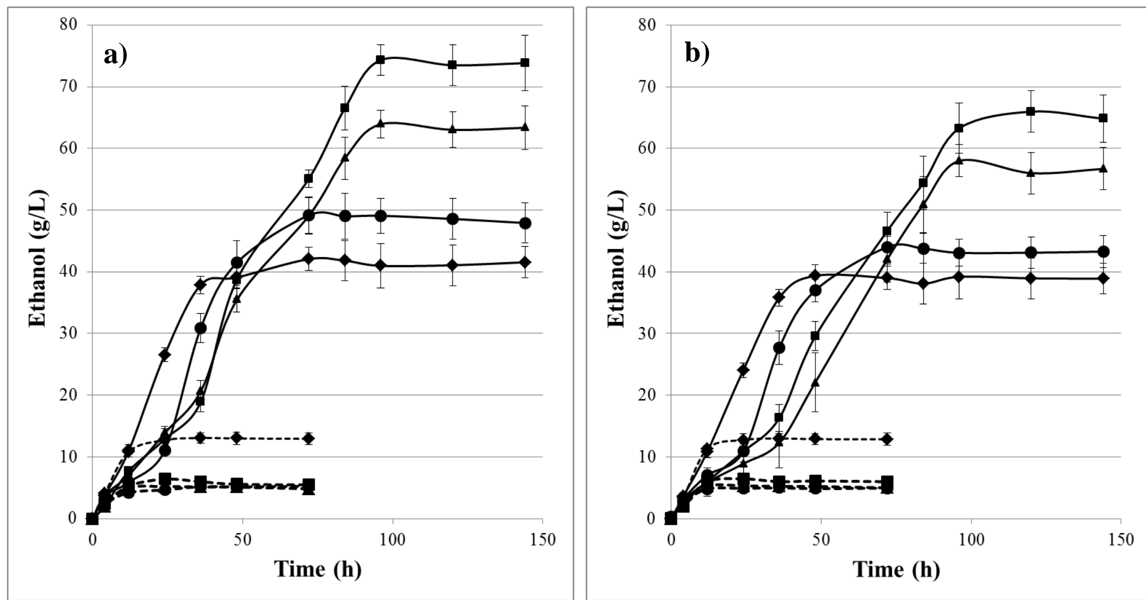

Fig. 2

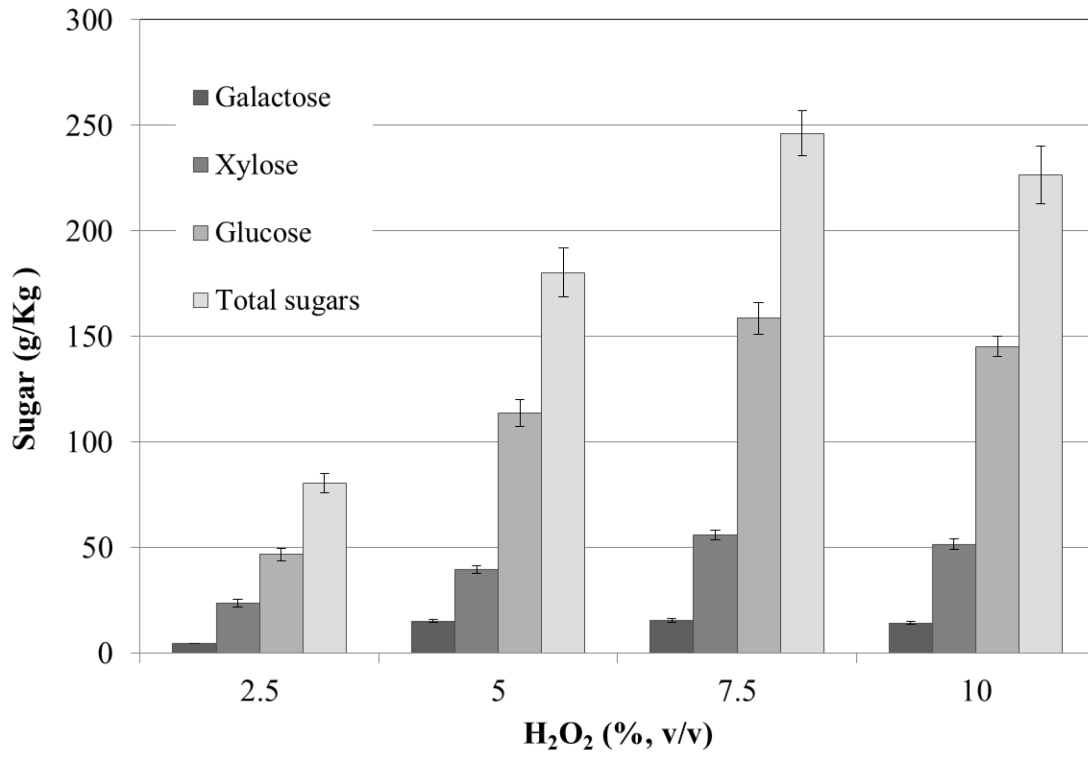

Fig. 3

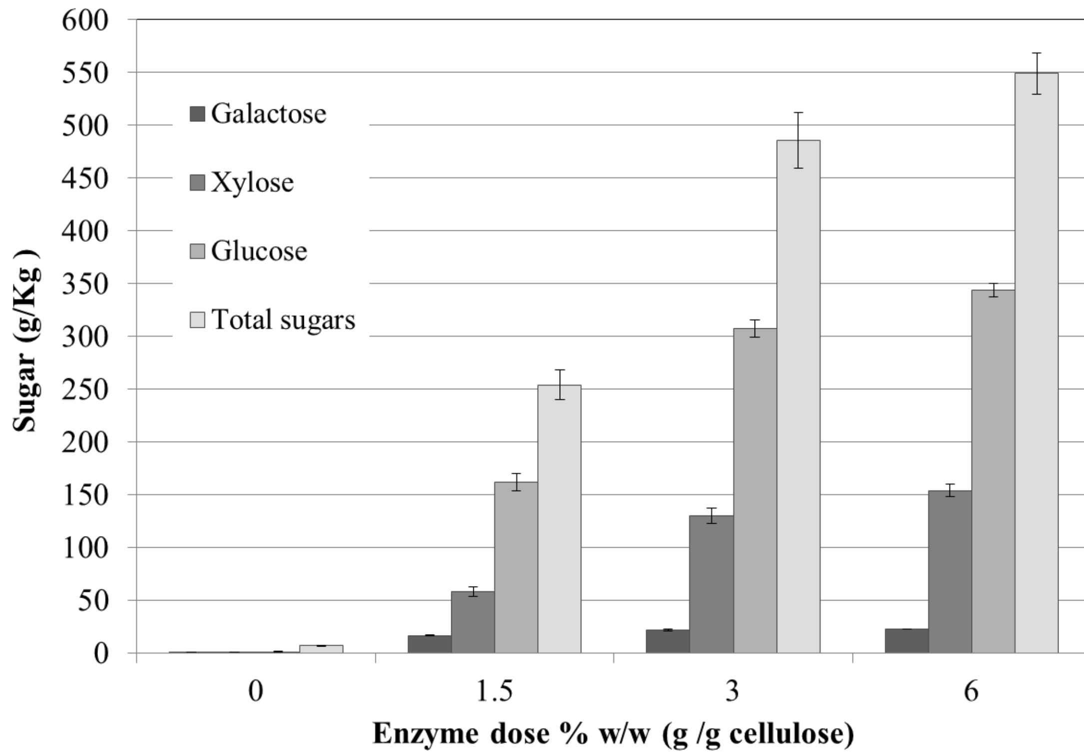

Fig. 4