Valorization of kitchen biowaste for ethanol production via simultaneous saccharification and fermentation using co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*

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Abstract

The biotransformation of the pre-dried and shredded organic fraction of kitchen waste to ethanol was investigated, via co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* (*Scheffersomyces stipitis*). Preliminary experiments with synthetic media were performed, in order to investigate the effect of different operational parameters on the ethanol production efficiency of the co-culture. The control of the pH and the supplementation with organic nitrogen were shown to be key factors for the optimization of the process. Subsequently, the ethanol production efficiency from the waste was assessed via simultaneous saccharification and fermentation experiments. Different loadings of cellulolytic enzymes and mixtures of cellulolytic with amylolytic enzymatic blends were tested in order to enhance the substrate conversion efficiency. It was further shown that for solids loading up to 40% waste on dry mass basis, corresponding to 170g.L⁻¹ initial concentration of carbohydrates, no substrate inhibition occurred, and ethanol concentration up to 45g.L⁻¹ was achieved.

**Keywords:** bioethanol, kitchen wastes, yeast co-cultures, enzymatic hydrolysis

1. **Introduction**

According to recent data, almost 90 million tonnes of food are wasted annually in the EU, with associated costs estimated at 143 billion euros (FUSIONS, 2016). Food waste (FW) is generated at various stages of the food supply chain (FSC) and considerable variations in the amounts and composition of the FW produced at each stage are reported. In developed countries, FW is mainly generated at consumer level, whereas in developing countries food is wasted mainly during the early
and middle stages of the FSC due to poor technology and infrastructure, and rapid spoilage because of difficult climatic conditions (FAO, 2011). Considering that almost one billion people become hungry every year worldwide, and another billion are undernourished (FAO, 2011), it becomes clear that wasting food is not only an economic but also an ethical issue. In addition, wasting food depletes the environment of limited natural resources, being thus an environment concern. Due to the above reasons, the European Commission has included among the Sustainable Development Goals (SDG) a target to halve the per capita generated FW at the retail and consumer level by 2030 and to reduce food losses along FSC. Such a goal could be indeed met via the exploration of different strategies to prevent FW generation or to identify ways for their exploitation i.e. by recovering nutrients, energy or high added-value products (Parthiba Karthikeyan et al., 2018).

FW is a biological matter subjectable to biodegradation with high content in protein, carbohydrates, lipids and other nutrients that could be actually recovered and/or converted to other high added-value products. Depending on the source and composition of different types of FW different strategies have so far been proposed for its valorization. Among them, domestic food waste i.e. FW occurring at the end of the food supply chain (FSC), is considered a very promising substrate for bioconversion via microbiological processes towards biofuels, such as ethanol, hydrogen and methane (Braguglia et al., 2018). This is due to the fact that domestic FW has in general a high content of readily fermentable carbohydrates, such as sugars and starch and also contains all the necessary nutrients that can support efficient growth of different types of microorganisms.

Among different biofuels, bioethanol has been most widely used during the past decades as alternative fuel for reducing CO\textsubscript{2} emissions, whereas it can also be used in the automobile sector as: a) octane enhancer in unleaded gasoline in place of the methyl–tertio-butyl ether (MTBE), b) oxygenated compound for clean combustion of gasoline and c) renewable energy carrier to partly substitute oil and to increase the security of supply (Gnansounou and Dauriat, 2005). Different types of feedstocks, mainly carbohydrate-based, have been proposed as substrates for ethanol production including energy “crops”, lignocellulosic biomass, organic residues and wastes and, more recently, algal biomass, resulting to the so called first- second- and third-generation bioethanol, respectively (Sims et al., 2010; Ho et al., 2014).

The use of co-cultures for ethanol production has been widely studied and their use was further motivated by the food-to fuel debate that has driven the research interest towards 2\textsuperscript{nd} generation bioethanol from lignocellulose. The processing of lignocellulosic materials via pretreatment and hydrolysis leads to the liberation of various sugars, with glucose and xylose being the dominant ones
(Carrere et al, 2016). As such, for sustainable production of ethanol from such complex substrates, the selection of microorganisms is crucial for their efficient valorization. Among the ethanologenic microorganisms, *Saccharomyces cerevisiae* is most commonly used for ethanol fermentation (Azhar et al, 2017). However, wild strains of *S. cerevisiae* are in general incapable of metabolizing xylose and, as such, are inefficient for ethanol production from mixed sugars. *Pichia stipitis* (*Scheffersomyces stipitis*), *Candida shehatae*, and *Pachysolen tannophilus* are found to be capable of fermenting C5 sugars, such as xylose (Antonoloulou et al, 2016, Hickert et al, 2014; Senkevich et al, 2013; Lin et al., 2012) and can be cultured with *S. cerevisiae* in a co-culture. Utilization of co-cultures for ethanol production appears to be advantageous over single cultures, since there is potential for synergistic action of the metabolic pathways of all involved strains (Bader et al, 2010). The combination of *P. stipitis* and *S. cerevisiae* is among the most commonly used, since the pH and temperature at which *S. cerevisiae* ferments glucose to ethanol are compatible with those of *P. stipitis* (Chen, 2011; Singh and Bishnoi, 2012). Microaerophilic conditions (~2 mmol.l.h⁻¹ of oxygen) are necessary for efficient ethanol formation from xylose by the xylose-fermenting yeasts, to maintain cell viability and the balance of NADH (Chen, 2011). *S. cerevisiae* on the other hand does not require oxygen to ferment glucose, but even in the presence of high oxygen levels ethanol production can be generated as soon as the external glucose concentration exceeds 0.8 mM (Verduyn et al, 1984).

In the present study the efficiency of second generation bioethanol production from the pre-dried and shredded organic fraction of food waste (food residue biomass, FORBI) was investigated, using co-cultures of the yeasts *S. cerevisiae* and *P. stipitis* as biocatalysts. Experiments with synthetic substrates were initially carried out in order to assess the effect of key parameters in alcoholic fermentation on bioconversion efficiency and the final ethanol yields. In the sequel, the effect of enzymatic loading and type of enzymes used on ethanol production was studied, in a concept of simultaneous saccharification and fermentation (SSF). To our knowledge, it is the first time that such a biomass is valorized for ethanol production using co-cultures of C6 and C5 yeasts. This is a concept that is advantageous for the maximum exploitation of its carbohydrate content, since apart from the sugars and the cellulosic and starchy substrates, hemicellulose can also be utilized.

2. Materials and Methods

2.1. Food waste
The kitchen wastes that were used for the production of FORBI were source-separated and included fermentable wastes solely. They were collected at municipality level twice a week from 240 houses of the Municipality of Halandri, Greece. Upon collection, kitchen wastes were subjected to simultaneous heat-drying (92°C) and shredding for approximately 9 hours in a GAIA GC-3000 dryer/shredder, resulting to a homogeneous biomass product (Food Residue Biomass product, FORBI) of the following characteristics: Total solids (TS), 91.28±0.75%, volatile solids (VS), 92.34±0.73%, total carbohydrates, 0.43±0.03 g.g⁻¹ TS, dissolved sugars, 0.21±0.02 g.g⁻¹ TS, starch 0.16±0.02 g.g⁻¹ TS, cellulose 0.11±0.01 g.g⁻¹ TS, hemi-cellulose 0.03±0.00 g.g⁻¹ TS, lignin 0.05±0.00 g.g⁻¹ TS, total Kjeldahl nitrogen, 1.63±0.17 g.100g⁻¹ TS, proteins, 0.11±0.01 g.g⁻¹ TS. Drying and shredding were applied in order to prevent biodeterioration of the waste and to ensure its stable and unchanging composition during storage.

2.2. Microorganisms, media and growth conditions

All fermentation tests were performed using the yeasts S. cerevisiae, CECT 1332 and P. stipitis, CECT 1922 that were both purchased from the Spanish type culture collection of microorganisms (CECT). Both strains were stored at 4°C in slant solid cultures in the following medium (g.L⁻¹): yeast extract 3; malt extract 3; myco-peptone 5; d-glucose 10; agar 20. For the startup of each experiment, slant cultures were used for the inoculation of 100 mL fresh liquid medium of the above composition under sterile conditions. Cultures were incubated at 27°C, under mechanical agitation at 150 rpm for 24 h, in order to obtain cells at the same growth stage for every experiment. The cells contained in equal volumes of S. cerevisiae and P. stipitis cultures were then harvested via centrifugation and used as inoculum for each experiment.

2.3. Fermentation tests with synthetic media

Initial fermentation tests with the co-cultures of the S. cerevisiae and P stipitis were performed in order to investigate the effect of key factors i.e. initial substrate concentration (C_{Sin}), pH and organic nitrogen addition, on the performance of the co-cultures during alcoholic fermentation. Four sets of batch experiments were performed with glucose as the sole carbon source under aseptic microaerobic conditions (Menis et al., 2017). All cultures were performed in duplicate in 100mL Erlenmeyer flasks with 50mL working volume. The first set of experiments consisted of 3 cultures in duplicate,
with the 20 g.L\textsuperscript{-1}, 40 g.L\textsuperscript{-1} and 60 g.L\textsuperscript{-1} initial glucose concentration, in which there was no adjustment of the initial pH (initial value 4.6 ± 0.2) or addition of yeast extract as organic nitrogen source, whereas KH\textsubscript{2}PO\textsubscript{4}, 1 g.L\textsuperscript{-1} was also added. In the second experiment, the initial pH was adjusted to 5, 5.4 or 5.8 using NaOH 3M and HCl 3M, the C\textsubscript{Sim} was 40 g.L\textsuperscript{-1}, there was no addition of organic nitrogen source, whereas KH\textsubscript{2}PO\textsubscript{4}, 1 g.L\textsuperscript{-1} was also added. In the third experiment, potassium phosphate buffer solutions of pH 5.5 and 6 were used in order to better control the pH during the fermentation; the C\textsubscript{Sim} was 40 g.L\textsuperscript{-1} and no organic nitrogen source was added. The fourth experiment was actually a replicate of the third one, using also organic nitrogen in the medium in the form of yeast extract, 1 g.L\textsuperscript{-1}. In all cases, the media were also supplemented with MgCl\textsubscript{2}.6H\textsubscript{2}O, 1 g.L\textsuperscript{-1} and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1 g.L\textsuperscript{-1}, and the cultures were temperature-controlled at 30\textdegree C, and were constantly agitated at 150 rpm using magnetic stirrers.

### 2.4. Fermentation tests with FORBI

FORBI is a complex waste that contains different types of carbohydrates, both soluble and insoluble. Since neither \textit{S. cerevisiae} nor \textit{P. stipitis} produce enzymes for the hydrolysis of carbohydrates, it is expected that the addition of such enzymes during fermentation of FORBI in an SSF approach, could enhance greatly its bioconversion efficiency towards ethanol. Three sets of experiments with FORBI as carbon source were performed, in order to optimize the process in terms of: a) the enzymatic loading of cellulolytic enzymes by supplementation of the 10% TS FORBI (w/v) medium with 2-30 FPU.g\textsuperscript{-1} TS of Celluclast (Novozymes\textsuperscript{®}) 1.5L and Novozyme 188 (Novozymes\textsuperscript{®}) at a ratio 3:1 (Antonopoulou et al, 2016), b) the FORBI solids loading, 10-40% TS FORBI (w/v), corresponding to different initial substrate concentration and c) the combination of cellulotytic and amylolytic enzyme mixtures. In the latter case 20% TS FORBI (w/v) were supplemented with the following enzymes/ enzymatic mixtures, the activity of which was expressed as Filter Paper Units (FPU) and Fungal Amylase Units (FAU) for cellulolytic and amylolytic enzymes, respectively i) 10 FPU.g\textsuperscript{-1} TS Celluclast 1.5L, ii) 10 FPU.g\textsuperscript{-1} TS cellulase blend (Sigma), iii) 10 FPU.g\textsuperscript{-1} TS cellulase blend (Cellic CTec2, Sigma) and 10 FAU.g\textsuperscript{-1} TS α-amylase (fungal α- amylase, ≥800 FAU/g, Sigma) iv) 10 FPU.g\textsuperscript{-1} TS cellulase blend, 10 U.g\textsuperscript{-1} TS α-amylase and 5 U.g\textsuperscript{-1} TS amyloglucosidase (Sigma). In this experiment, a 4.8 acetate buffer was used instead of deionized water for the preparation of the medium. Finally, the effect of the microorganisms in mono- and co-cultures on the bioconversion of the substrate and on the final ethanol yields was also assessed, using 20% TS FORBI (w/v) and 10 FPU.g\textsuperscript{-1} TS Celluclast 1.5L and Novozyme 188 at a ratio 3:1. In all cases, the media were also
supplemented with KH$_2$PO$_4$, MgCl$_2$.6H$_2$O, (NH$_4$)$_2$SO$_4$, 1 gL$^{-1}$ each. All experiments were performed under aseptic conditions and the cultures were incubated in 30°C, constantly agitated at 150 rpm using magnetic stirrers.

2.4. Analytical methods

TS, VS and Total Kjeldahl Nitrogen (TKN) in raw and extract-free samples were quantified according to Standard Methods (APHA, 1995). Crude protein content was determined by multiplying TKN by a factor of 6.25 (Monlau et al., 2012). Total and soluble carbohydrates were quantified according to DuBois et al. (1956). Starch content was determined via a total starch assay kit (Megazyme). Cellulose and hemicellulose content were determined according to the National Renewable Energy Laboratory (NREL)’s standard laboratory analytical procedure (LAP) for the determination of structural carbohydrates in biomass (Sluiter et al., 2008). Glucose and ethanol were quantified via HPLC-RI (Shodex) with an Aminex HPX–87H column (Biorad) at 60°C and a Cation H micro-guard cartridge (biorad Laboratories), with H$_2$SO$_4$ 0.006N mobile phase at a flow rate of 0.6mL.min$^{-1}$. 

3. Results and Discussion

3.1. Experiments with glucose as the sole carbon source

In Fig. 1 (a, b, c) substrate consumption, pH drop and ethanol generation are illustrated during the alcoholic fermentation of different $C_{Sin}$ of glucose with $S. cerevisiae$ and $P. stipitis$ co-cultures. As shown a considerable amount of glucose remains unexploitable in all cases. The amount of glucose that yeasts could uptake was similar for all $C_{Sin}$ i.e. 10.53 ±1.53 g.L$^{-1}$, 10.04 ±0.35 g.L$^{-1}$ and 9.5 ±0.05 g.L$^{-1}$ corresponding to ~53%, ~23% and only ~16 % (Table 1) consumption for $C_{Sin}$ 20 g.L$^{-1}$, 40 g.L$^{-1}$ and 60 g.L$^{-1}$, respectively. The pH drop profile is almost identical different and it can be noted that when it reaches ~2.5, growth is inhibited. Maximum ethanol concentration in the fermentation broth and also maximum ethanol yield in terms of consumed glucose ($Y_{EtOH/Gl cons}$) were observed for $C_{Sin}$ 60 g.L$^{-1}$. However, since glucose uptake was minimum for the higher $C_{Sin}$, the estimated ethanol yield in terms of initial substrate concentration ($Y_{EtOH/Gl ini}$) was actually the lowest (Table 1).
When the pH was adjusted to higher values, glucose uptake exhibited an increasing tendency, reaching 11.25 ±0.70 g.L⁻¹ and 12.79 ±0.09 g.L⁻¹ for an initial pH of 5.4 and 5.8, respectively. As shown in Table 1, the effect on $Y_{\text{EtOH/Gl cons}}$ and $Y_{\text{EtOH/Gl in}}$ increase was actually negligible. The limiting factor seems to be again the pH drop, since as illustrated in Fig1.e, the pH profiles were similar, reaching a lowest value of 2.5.

In order to further study the effect of pH on the performance of the co-cultures, the pH was adjusted via the addition of buffer solution which has the ability to prevent dramatic pH drops. This is illustrated in Fig. 2.b, where it is shown that the minimum pH values are 3.5 and 3.7 for initial pH values of 6 and 5.5, respectively, whereas the pH of control cultures (i.e with no buffer addition) dropped to 2.5. The positive effect of pH control was even more obvious when estimating the percentage of substrate uptake and $Y_{\text{EtOH/Gl in}}$, which was actually doubled and almost tripled when 5.5. and 6 pH buffers were used, respectively.

**Figure 1.** Effect of initial substrate concentration and initial pH on glucose consumption, (a,d), pH drop, (b,e) and ethanol production, (c, f) during batch co-cultures of *S. cerevisiae* and *P. stipitis* with glucose as the sole carbon source.

Subsequently, the effect of organic nitrogen on the performance of the co-culture was investigated via experiments with different pH adjustment and supplementation with yeast extract. As illustrated
in Fig.2 (d,e,f), it is apparent that the the uptake of glucose was significantly enhanced in the presence of yeast extract, even in the case that no pH control was made. The consumption of substrate for pH buffers of 5.5 and 6 was four-folded compared to the control, reaching $36.10 \pm 0.49$ g.L$^{-1}$ and $37.11 \pm 0.09$ g.L$^{-1}$, corresponding to ~84% of total glucose bioconversion. The significant increase of the substrate uptake that was observed resulted also to a four-fold increase of the $Y_{E/OH/GI}$, compared to the control, i.e. from $0.07 \pm 0.00$ to $0.31 \pm 0.00$ and $0.30 \pm 0.01$ for the cultures with pH buffer 5.5 and 6, respectively.

**Figure 2.** Effect of buffer addition and supplementation with yeast extract on glucose consumption, (a,d), pH drop, (b,e) and ethanol production, (c, f) during batch co-cultures of *S. cerevisiae* and *P. stipitis* with glucose as the sole carbon source.

The pH has a great impact on the activity of cell enzymes and can modify the chemical pathways of the biological reactions as well as their kinetics. In alcoholic fermentations, the pH tends to drop, as metabolites are accumulated in the fermentation broth, to a different extent, depending on the external pH manipulation strategy followed, or the complexity of the medium used. Ethanol does not contribute to the pH drop and it is reported that the increase of ethanol concentration from 0 to 12° alc led to an increase of pH by 0.18 in synthetic media with similar composition to natural fermentation broths. As such, the pH drop can be attributed to the acidic by-products of alcoholic fermentation and the different forms of dissolved CO$_2$ (Akin et.al, 2008). The pH drop was also
assumed to be correlated to the nitrogen consumption, during which H⁺ ions are generated by yeasts (Castillo et al., 1995). Since bioconversion leads to higher metabolites’ concentrations, inevitably in the absence of pH control, there will be a minimal pH below which the metabolism of the microorganisms will be inactivated. This lower value seems to vary for different yeast strains; pH, ranging from 2.75 to 4.25 is reported as limiting for the survival and growth of different yeasts strains (Fleet and Heard, 1993). In the case of *S. cerevisiae* and its co-culture with *P. stipitis*, a pH of 5.0 is reported to be the optimal initial pH in controlled-pH media (Zhang et al. 2015; Taniguchi et al, 1997). During the experiment with different initial glucose concentrations (20 g glucose.L⁻¹-60 g glucose.L⁻¹) it was shown that the bioconversion of ~10g.L⁻¹ of glucose in all cases resulted in pH drop to ~2.5, starting from initial pH ~5. When the initial pH in the cultures was adjusted to higher values i.e. 5.4 and 5.8, the limiting range of glucose consumed increased only slightly, whereas when potassium phosphate buffer was used for the better control of the pH drop glucose bioconversion increased considerably. As such, by comparing the bioconversion of glucose without pH control with that at optimum pH control for the same *C*<sub>Sin</sub> (40 g.L⁻¹), an almost threefold increase was observed (from 0.08 ± 0.02 to 0.21 ± 0.03 g EtOH.g⁻¹ glucose).

**Figure 3.** Total carbohydrates consumption (a), pH change (b) and ethanol production (c) during alcoholic fermentation of 10% TS FORBI (w/v) via co-cultures of *S. cerevisiae* and *P. stipitis* and different enzymatic loading of Celluclast 1.5L/Novozyme 188 mixture.

It should be also noted that for the range of *C*<sub>Sin</sub> tested (maximum *C*<sub>Sin</sub> for glucose, 60 g.L⁻¹; for FORBI, ~160g.L⁻¹), the limiting factor for the bioconversion of sugars to ethanol by the co-culture seemed to be solely the pH, since no substrate inhibition was observed in the case of FORBI (results shown in section 3.2). This assumption is also supported by previous studies according to which glucose concentrations above 150 g.L⁻¹ and xylose concentrations above 70 g.L⁻¹ are reported as
inhibitory during alcoholic fermentation of mono- and co-cultures of *S. cerevisiae* and *P. stipitis* (Thatipamala et al., 1992; Nicolic et al, 2012; Dhabhai et al., 2012; Zhang, 2015).

**Table 1.** Effect of initial substrate concentration, pH and addition of yeast extract on the consumption of glucose and the yields of produced ethanol during batch co-cultures of *S. cerevisiae* and *P. stipitis* with glucose as the sole carbon source (*no buffer, no added N*).

| Parameter tested     | range | Glucose uptake (%) | $Y_{EtOH/Gl\ cons}$ (g EtOH/g consumed glu.) | $Y_{EtOH/Gl\ in}$ (g EtOH/g initial glu.) |
|----------------------|-------|---------------------|---------------------------------------------|------------------------------------------|
|                      |       |                     |                                             |                                          |
| $C_{s.init.}$ (no pH control, no N addition) | 20g/L | 52.55 ± 0.03        | 0.31 ± 0.03                                | 0.16 ± 0.02                              |
|                      | 40g/L | 22.91 ± 0.03        | 0.35 ± 0.02                                | 0.08 ± 0.02                              |
|                      | 60g/L | 15.94 ± 0.03        | 0.37 ± 0.02                                | 0.07 ± 0.01                              |
| pH init (C$_{s.init.}:$40g/L, no N addition) | 5     | 22.35 ± 1.90        | 0.32 ± 0.07                                | 0.08 ± 0.02                              |
|                      | 5.4   | 28.98 ± 0.78        | 0.36 ± 0.02                                | 0.09 ± 0.03                              |
|                      | 5.8   | 31.80 ± 0.99        | 0.36 ± 0.01                                | 0.09 ± 0.02                              |
| pH control (C$_{s.init.}:$40g/L, no N addition) | no buffer | 21.88 ± 3.03        | 0.32 ± 0.03                                | 0.08 ± 0.00                              |
|                      | buffer, pH 5.5 | 41.20 ± 1.37 | 0.38 ± 0.01 | 0.16 ± 0.00 |
|                      | buffer, pH 6  | 56.13 ± 4.89        | 0.39 ± 0.03                                | 0.21 ± 0.03                              |
| N source (C$_{s.init.}:$40g/L) | Control* | 20.88 ± 3.03        | 0.31 ± 0.03                                | 0.07 ± 0.00                              |
|                      | no buffer, + y. e. | 54.09 ± 4.02 | 0.39 ± 0.01 | 0.20 ± 0.02 |
|                      | pH 5.5, + y. e.   | 83.81 ± 0.97        | 0.37 ± 0.01                                | 0.31 ± 0.02                              |
|                      | pH 6, + y. e.      | 84.42 ± 0.67        | 0.37 ± 0.00                                | 0.30 ± 0.00                              |

**2.1. Fermentation of FORBI**

FORBI is a complex organic substrate that contains different types of free sugars and complex carbohydrates, as well as different nitrogen sources. The FORBI that was used in the present study consisted of ~43% (w/w TS) total carbohydrates and ~21% free sugars i.e soluble monosaccharides, disaccharides and oligosaccharides. Neither *S. cerevisiae* or *P. stipitis* are able to produce enzymes for the hydrolysis of complex carbohydrates such as cellulose, hemicellulose and starch, which in total contribute up to 30% of the total carbohydrate content of the FORBI used in the present study. As such, in order to enhance the accessibility of the carbohydrate content of FORBI to yeasts for ethanol production, different hydrolytic enzymes and blends of those were assessed.
During the fermentation of complex substrates such as lignocellulosic biomass and or starchy substrates via yeasts, the supplementation of the cultures with hydrolytic enzymes is essential for their efficient bioconversion. Physicochemical pretreatment prior to hydrolysis has also proven to facilitate the liberation of sugars and thus ethanol yields and productivities, when the lignin content of the substrate is considerable (Antonopoulou et al, 2016). However, such pretreatment methods...
could lead to the degradation of sugars, already available in the feedstock (Antonopoulou et al, 2013) and to the release of inhibitory for ethanol production compounds and thus should be avoided. The FORBI that was used in the present study contained ~5% (w/w) lignin, ~11% (w/w) cellulose, ~3% (w/w) hemicelluloses and ~16% (w/w) total starch, whereas the soluble sugars corresponded to more than 20% (w/w). Based on this composition, it was considered that a physicochemical pretreatment could probably deteriorate substrate efficiency and for this reason enzymatic hydrolysis was solely applied. According to the relative content in holocellulose and starch, the use of cellulolytic, amylolytic enzymes or blends of them at various loadings have been proposed for the enhancement of ethanol production from FW (Hafid et al, 2017).

2.1.1. Effect of enzymatic loading of cellulolytic enzymes and initial substrate concentration

In order to initially assess the effect of cellulolytic enzymes on the fermentation of the contained in the FORBI cellulose, FORBI suspensions were supplemented with 2-30 FPU Celluclast.g\(^{-1}\) TS of FORBI, as well as with Novozyme at ratio 1:3 (v/v). Celluclast is a cellulase that catalyzes cellulose breakdown into glucose oligomers, cellobiose and glucose, whereas Novozyme is a β-glucosidase that breaks down oligomers and cellobiose to glucose. The effect of the enzymatic loading of Celluclast 1.5L/Novozyme 188 on the total carbohydrates uptake and subsequent ethanol generation is illustrated in Fig. 3. As shown, the overall rate of total carbohydrates consumption does not differentiate much, with the overall uptake of carbohydrates reaching 84%-85% (Table 2). It seems thus, that about 15% of the total carbohydrates contained in FORBI could not be utilized by the yeasts. Since the pH of all cultures remained within the range of 4.8 to 5.2 (Fig. 3b), which is favorable for ethanol production and no limitation of nitrogen could be expected, the observed inability of the yeasts to consume the total available carbohydrate substrates could solely be attributed to partial accessibility. Indeed, the hydrolytic enzymes were used to break down cellulose selectively, whereas the hemicellulose, the insoluble starch and probably great amount of the soluble starch contained in the waste was not accessible to the yeasts as carbon source. However, ethanol production seems to be enhanced for enzymatic loadings above 10 FPU.g\(^{-1}\) TS of cellulolytic enzymes as indicated by maximum ethanol concentrations that were achieved (Fig.3c). In order to further assess the effect of enzymatic loading on ethanol production, the ethanol yields in terms of the carbohydrates that were consumed (\(Y_{\text{EtOH/carbl con}}\)) and in terms of the initial FORBI load (\(Y_{\text{EtOH/waste in}}\)) in the medium were estimated and are presented in Table 2. As shown, the \(Y_{\text{EtOH/carbl con}}\) exhibited an increasing tendency for higher enzymatic loading (above 10 FPU.g\(^{-1}\) TS) compared to the values obtained from cultures with lower enzymatic loading, reaching 0.36 ± 0.01 g EtOH.g\(^{-1}\),
0.37 ± 0.00g EtOH.g\(^{-1}\) and 0.37 ± 0.02g EtOH.g\(^{-1}\) of consumed carbohydrates when 10, 20 and 30 FPU Celluclast.g\(^{-1}\) TS of FORBI were used, respectively. This tendency was also reflected on the \(Y_{\text{EtOH/waste in.}}\) that reached 0.13 ±0.01g EtOH.g\(^{-1}\) of initial TS FORBI for 20-30 FPU.g\(^{-1}\) TS of FORBI. It overall, it is assumed that for enzymatic loading of cellulolytic enzymes in the range of 2-10 FPU g\(^{-1}\) TS, enhancement of ethanol production is observed for higher loadings. For enzymatic loadings above 10 FPU.g\(^{-1}\) TS FORBI, however, no significant enhancement was observed and consequently 10 FPU.g\(^{-1}\) TS FORBI were selected as optimal in terms of both achieved ethanol yields and economic sustainability of the process.

In order to further investigate the effect of substrate concentration on carbohydrates uptake and their bioconversion to ethanol, an experiment with different solids loading of FORBI was performed. As such, co-cultures with 10-40% TS FORBI (w/v), supplemented with Celluclast 1.5L/Novozyme 188 enzymatic mixture (10 FPU Celluclast 1.5L. g\(^{-1}\) TS FORBI, selected by previous experiments). The initial substrate concentrations that corresponded to 10% TS FORBI, 20% TS FORBI, 30% TS FORBI and 40% TS FORBI are 43g.L\(^{-1}\), 86g.L\(^{-1}\), 129g.L\(^{-1}\) and 172 g.L\(^{-1}\) total carbohydrates, respectively, expressed as glucose equivalents. The effect of the different solids loading and consequently the different initial substrate concentrations, on the total carbohydrates uptake and subsequent ethanol generation is illustrated in Fig. 4, whereas the substrate bioconversion efficiency and ethanol yields are summarized in Table 2. It is apparent that no significant substrate inhibition is observed, even for the highest solids loading, since in all cases the bioconversion of carbohydrates is ~82%. It should be noted at this point that the delay in the consumption of carbohydrates for the cultures with 40% TS FORBI, was not due to kinetic inhibition but due to inadequate mechanical agitation of the medium. The high solids loading resulted to a medium with the form of thick slurry which was very hardly agitated. As such, the enzymes were not evenly distributed in the medium and the liquefaction of FORBI was initially spatially confined. As a result of the inability for efficient agitation during the first hours of the incubation, the yeast cells were not evenly distributed in the medium resulting in a delay in ethanol production, as shown in Fig. 4c. However, the final estimated \(Y_{\text{EtOH/carbi con.}}\) and \(Y_{\text{EtOH/waste in.}}\) were only slightly affected, whereas in the case of the highest solids loading, the ethanol concentration reached 45.4 ±1.4 g EtOH.L\(^{-1}\), which is quite important for the viability of the process since a minimal concentration of 40 g/L of ethanol is required for its efficient recovery from a fermentation broth (Wingren et al., 2004)

The above results are in agreement with previous studies during which on site produced cellulolytic enzymes were used for the saccharification of FW and subsequent fermentation towards ethanol
(Matsakas and Christakopoulos, 2015). In that study, it was shown that the use of cellulolytic enzymes led to a threefold increase in ethanol concentration, which corresponded to 38.6% of the maximum theoretically possible, based on both soluble and insoluble carbohydrates. Similarly, Matsakas et al. (2014), using household food wastes at a 45% solids loading, with the addition of 10 FPU of cellulolytic enzymes (Celluclast1.5L and Novozyme 188) achieved $C_{\text{EtOH}}$ of 42.78 ± 0.83 g.L\(^{-1}\) and 39.15 ± 0.75 g.L\(^{-1}\) via monocultures of common baker’s yeast, when performing separate and SSF, respectively. The achieved values corresponded to 57% and 52% of the maximum theoretical yields based on the maximum ethanol that could be produced from the soluble and the cellulosic sugars contained in the waste. In the present study the production of ethanol with 40% solids loading of FORBI reached 54.4% of the maximum theoretically possible, based on total available carbohydrate content of the waste, whereas for solids loadings of 10-30% ethanol production was even higher, reaching 58% of the maximum theoretical.

2.1.2. Effect of different enzymatic mixtures

As shown from the above experiments, the maximum bioconversion efficiency of the carbohydrate content was ~85%, with optimal cellulolytic enzymes loading and optimal initial substrate concentration, leaving thus a 15% of carbohydrates in FORBI unexploitable.

Since this could only be attributed to the inaccessibility of yeasts to the remaining substrates, an experiment with different enzymatic mixtures was performed in order to assess the ethanol production efficiency when hemicellulose and starch would also be converted. Four cultures were prepared in duplicates with 20% TS FORBI and the following enzymes/ enzymatic mixtures were also added prior to inoculation: i) 10 FPU.g\(^{-1}\) TS Celluclast 1.5 L, i.e cellulase that attacks cellulose producing cellobiose, oligomers of glucose and glucose; ii) 10 FPU g\(^{-1}\) TS cellulase blend (cellic) i.e. enzymatic blend of cellulases and glucosidases as well as xylanase activity, able to hydrolyze both cellulose and hemicellulose to their monomers; iii) 10 FPU g\(^{-1}\) TS cellulase blend and 10 FAU g\(^{-1}\) TS $\alpha$-amylase which can convert starch to maltose mainly and iv) 10 FPU g\(^{-1}\) TS cellulase blend, 10 FAU g\(^{-1}\) TS $\alpha$-amylase and 5 U g\(^{-1}\) TS amyloglucosidase, with the latter converting maltose to glucose. The effect of the different enzymes/mixtures on the total carbohydrates uptake and subsequent ethanol generation is illustrated in Fig.5. The effect of different enzymes on the uptake of carbohydrates is obvious, with the most complex enzymatic blend leading to faster as well as higher bioconversion (Fig. 5a).
Similarly, the $Y_{\text{EtOH/carbol,con}}$ and $Y_{\text{EtOH/waste, in}}$ were enhanced significantly by the presence of more complex enzymatic blends. In Table 2, the estimated bioconversion efficiencies and the ethanol yields from different treatments are shown. As expected, the supplementation solely with cellulases, resulted to the lowest bioconversion and the lowest ethanol yields i.e. $80.21 \pm 1.94\%$ uptake of carbohydrates and $0.12 \pm 0.01 \text{ g EtOH. g}^{-1} \text{ FORBI}$, which are actually the lowest values observed for all experiments with the FORBI of the present study. This finding indicates that the absence of glycosidase resulted to much lower liberation of glucose from the contained in the FORBI cellulase, which was degraded mostly to cellobiose. *S. sereviceae* cannot metabolize cellobiose and as such it can be assumed that the bioconversion of cellobiose to ethanol was performed solely by *P. stipitis* resulting to lower ethanol yields (Parekh and Wayman, 1986). On the other hand, when the enzyme blend (cellic) is used, it can be assumed that the holocellulosic compound of FORBI i.e. both cellulose and hemicellulose was accessible to the yeasts since both strains can consume the liberated hexoses whereas *P. stipitis* can also bioconvert the liberated from hemicellulose pentoses. The presence of amylolytic enzymes in the media enhanced further both the bioconversion efficiency and the ethanol yields which reached $95.04 \pm 1.91\%$ and $0.15 \pm 0.01 \text{ g EtOH. g}^{-1} \text{ FORBI}$.

As shown in Table 2, supplementation of FORBI with more complex enzymatic mixtures, containing both cellulytic and amyloytic enzymes had led in all cases to similar ethanol to carbohydrates yields, when estimated in terms of consumed carbohydrates i.e. $Y_{\text{EtOH/carbol,con}}$. As expected though, the estimated yields based on total available carbohydrate content of the waste i.e. were highly increased, since the bioconversion efficiency was also considerably higher. As such, the use of hydrolytic enzymes for holocellulose break down (cellic) resulted in an ethanol production 57% of the maximum theoretical, whereas their combination with $\alpha$-amylase and glucoamylase led to an ethanol production of 65.2% of the maximum theoretical. In the latter case, 95% of the total available carbohydrates were bioconverted, leading to the conclusion that the specific enzymatic combination can almost fully hydrolyze the carbohydrates of the waste. It is reported that up to 72.33% of the maximum theoretical ethanol yield could be achieved by FW using enzymatic blends (including cellulases, $\alpha$-amylase and glucoamylase) (Loizidou et al., 2017). However, those yields were obtained after separate hydrolysis and fermentation of the wastes, which is reported as advantageous by other studies as well (Matsakas et al., 2014). It is expected thus that further studies for ethanol production from FORBI with the co-cultures of *S. sereviceae*, CECT 1332 and *P. stipitis*, CECT 1922 using a separate hydrolysis and fermentation process could probably lead to complete bioconversion of the carbohydrates content of the waste. Table 2. Effect enzymatic loading, solids loading and type of enzymes on the consumption of total carbohydrates and ethanol yields during batch mono- and co-cultures of *S. cerevisiae* and *P. stipitis* with DKW as the sole carbon source.
| Parameter tested | Carbohydrates uptake (%) | $Y_{\text{EtOH}}/\text{carb. con}$ (g EtOH/g carb. consumed) | $Y_{\text{EtOH}}/\text{waste}$ (g EtOH/g initial waste) |
|------------------|--------------------------|----------------------------------------------------------|--------------------------------------------------|
| **strain**       |                          |                                                          |                                                  |
| $S. \text{cerevisiae}$ | 83.35 ± 3.91            | 0.35 ± 0.01                                              | 0.13 ± 0.01                                      |
| $P. \text{stipitis}$ | 84.15 ± 2.43            | 0.30 ± 0.01                                              | 1.01 ± 0.01                                      |
| Co-culture       | 85.02 ± 1.97            | 0.37 ± 0.01                                              | 0.13 ± 0.01                                      |
| **Enzymatic loading** |                          |                                                          |                                                  |
| 2                | 84.14 ± 2.92            | 0.34 ± 0.03                                              | 0.12 ± 0.01                                      |
| 5                | 84.26 ± 1.95            | 0.35 ± 0.00                                              | 0.12 ± 0.01                                      |
| 10               | 83.99 ± 2.67            | 0.36 ± 0.01                                              | 0.13 ± 0.01                                      |
| 20               | 84.34 ± 3.11            | 0.37 ± 0.00                                              | 0.13 ± 0.01                                      |
| 30               | 85.01 ± 1.90            | 0.37 ± 0.02                                              | 0.13 ± 0.01                                      |
| **Solids Loading** |                          |                                                          |                                                  |
| 10 %             | 82.74 ± 2.60            | 0.36 ± 0.01                                              | 0.14 ± 0.01                                      |
| 20 %             | 82.90 ± 2.12            | 0.36 ± 0.02                                              | 0.13 ± 0.01                                      |
| 30 %             | 82.54 ± 2.64            | 0.36 ± 0.01                                              | 0.12 ± 0.01                                      |
| 40 %             | 81.16 ± 3.12            | 0.34 ± 0.01                                              | 0.12 ± 0.01                                      |
| **Enzymatic mixture** |                          |                                                          |                                                  |
| Cellulase        | 80.21 ± 1.94            | 0.32 ± 0.01                                              | 0.12 ± 0.01                                      |
| Cellulase blend  | 85.68 ± 3.35            | 0.34 ± 0.01                                              | 0.14 ± 0.01                                      |
| Cellulase blend +\(\alpha\)-amyloglucosidase | 95.04 ± 1.91 | 0.35 ± 0.01 | 0.15 ± 0.01 |

### 2.1.3. Effect of type of microorganisms

In Fig. 6, the profiles of substrate consumption, pH variation and ethanol production from 10% FORBI suspensions are illustrated, corresponding to an initial concentration of carbohydrates ~40g/L, using both mono- and co- cultures of $S. \text{cerevisiae}$ and $P. \text{stipitis}$. All cultures were supplemented with 10 FPU.g\(^{-1}\) TS Celluclast 1.5L and Novozyme 188 (ratio 1:3). As shown in Fig.6c, the pH in all cultures seems to be self-controlled at ~5, as also observed for all other experiments with co-cultures in the present study, in the presence of Celluclast 1.5L and Novozyme 188. Since no pH control had taken place, it can be assumed that FORBI, due to its complex composition, can self-control the pH, maintaining it at favorable for ethanol production levels. From Fig. 6c, it is obvious that $S. \text{cerevisiae}$ leads to higher ethanol concentrations ($C_{\text{EtOH}}$) compared to $P. \text{stipitis}$, whereas the co-culture led to the highest $C_{\text{EtOH}}$. The maximum achieved $C_{\text{EtOH}}$ was 13.61 ± 1.31 g.L\(^{-1}\), which corresponds to a $Y_{\text{EtOH}}/\text{carb. con}$. of 0.37 ± 0.01 g EtOH.g\(^{-1}\) of consumed.
carbohydrates and a $Y_{\text{EtOH/waste}}$ of 0.13 ± 0.01 g EtOH. g$^{-1}$ FORBI. It seems thus, that co-culturing of $S. \text{cerevisiae}$ and $P. \text{stipitis}$ can improve ethanol productivities from FORBI, as also proved by comparative results obtained from mono-cultures of the yeasts. The latter showed that $P. \text{stipitis}$ leads to significantly lower ethanol yields compared to $S. \text{cerevisiae}$ and even lower compared to the co-culture. This is in agreement with previous studies using either synthetic media or waste. In fermentative experiments with a synthetic medium containing glucose and xylose, with free and immobilized $P. \text{stipitis}$ and $S. \text{cerevisiae}$ cells, it was observed that ethanol production was higher in the culture containing both yeast species (Dhabhai et al, 2012). This observation is also in agreement with co-culture studies with $S. \text{stipitis}$ and $P. \text{cerevisiae}$, using different lignocellulosic hydrolysates (Chandel et al, 2011). The mechanism though that leads to the better performance of co-cultures has not been fully clarified yet.

3. Conclusions

$S. \text{sereviceae}$, CECT 1332 and $P. \text{stipitis}$, CECT 1922 are compatible for the simultaneous fermentation of all types of carbohydrates contained in the FORBI, for ethanol production. The proposed concept for the valorization of FW via co-cultures of C6 and C5 yeasts seems to be quite advantageous for maximum valorization of the carbohydrates contents of the waste, since apart from the sugars and the cellulosic and starchy substrates, hemicellulose can also be exploited. Overall it was demonstrated that the FORBI is a promising substrate for ethanol production using co-cultures of the above yeast stains.

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Figure 1. Effect of initial substrate concentration and initial pH on glucose consumption, (a,d), pH drop, (b,e) and ethanol production, (c, f) during batch co-cultures of *S. cerevisiae* and *P. stipitis* with glucose as the sole carbon source.

Figure 2. Effect of buffer addition and supplementation with yeast extract on glucose consumption, (a,d), pH drop, (b,e) and ethanol production, (c, f) during batch co-cultures of *S. cerevisiae* and *P. stipitis* with glucose as the sole carbon source.

Figure 3. Total carbohydrates consumption (a), pH change (b) and ethanol production (c) during alcoholic fermentation of 10% TS FORBI (w/v) via co-cultures of *S. cerevisiae* and *P. stipitis* and different enzymatic loading of Celluclast 1.5L/Novozyme 188 mixture.

Figure 4. Total carbohydrates consumption (a), pH change (b) and ethanol production (c) during alcoholic fermentation of 10-40% TS FORBI (w/v) via co-cultures of *S. cerevisiae* and *P. stipitis* supplemented with 10FPU Celluclast/Novozyme 188 mixture.

Figure 5. Total carbohydrates consumption (a), pH change (b) and ethanol production (c) during alcoholic fermentation of 20% TS FORBI (w/v) via co-cultures of *S. cerevisiae* and *P. stipitis* supplemented with different mixtures of cellulolytic and amylolytic enzymes.

Figure 6. Total carbohydrates consumption (a), glucose concentration (b), pH change (b) and ethanol production (c) during alcoholic fermentation of 10% TS FORBI (w/v) via co-cultures of *S. cerevisiae* and *P. stipitis* supplemented with 10FPU Celluclast/Novozyme 188 mixture.