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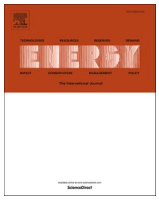

Saccharification of starchy food waste through thermochemical and enzymatic pretreatment, towards enhanced bioethanol production via newly isolated non-conventional yeast strains

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ABSTRACT

A starchy food waste containing mainly cooked wasted rice (WR) was exploited for bioethanol production using novel yeast strains was investigated. Different pretreatment schemes of the waste at solids loading 10%–30% TS WR (w/v) i.e. enzymatic, thermochemical and combined thermochemical/enzymatic pretreatment, were evaluated aiming to the maximum liberation of fermentable carbohydrates and their subsequent bioconversion to ethanol. Fermentation tests of the whole pretreated slurries were initially performed with the yeasts strains that were identified as *Kluyveromyces marxianus* isolate V3-19, *Pichia kudriavzevii* strain YF1702 and *K. marxianus* strain TTG-428, and their fermentation efficiencies (*FE*) were comparatively assessed. It was shown that the combined pretreatment led to the maximum saccharification, whereas *FEs* were higher for *K. marxianus,* V3-19, exceeding 90% of the theoretical maximum. In the case of the highest organic loading of WR, though, up to 25% of soluble carbohydrates remained unexploitable after 72 h of fermentation, indicating that kinetic restrictions occurred in the process. Further experiments with the hydrolysates that were recovered after combined pretreatment, revealed that the removal of solids enhances the consumption of sugars and leads to complete uptake for the loading 20% TS WR (w/v).

1. Introduction

The gradual depletion of fossil resources occurring during the previous decades due to the ever-increasing energy demands, has raised a significant challenge for the future energy autonomy of nations worldwide. At the same time, the rapidly increasing use of fossil fuels has also led to significant and cumulative environmental problems, such as aggravate of the greenhouse effect, global warming and subsequent climate change, with unpredictable consequences for both the planet and humanity. Taking into account the above, the European Union has drawn up a specific policy to deal with climate change, setting specific goals for the near future. Among them, the integrated climate and energy policy, which was approved by the European Council in October 2014 and revised in December 2018, attempts to achieve by 2030 reduction of greenhouse gas emissions by at least 40% compared to 1990 and increase of the amount of renewable energy sources in energy consumption up to 32%. In September 2020, the Commission not only approved the EU Climate Target Plan for 2030 but also increased the goal for reduction of greenhouse gas emissions by 2030 from 40% to 55% [1]. In order for every nation to reduce their reliance on fossil fuels, it is essential to invest in new technologies concerning alternative energy forms and renewable energy sources such as water circulation, wind, geothermal and biomass derived fuels [2]. Among them, biofuels produced via microbial processes such as bioethanol, have attracted much interest in the recent years since they are environmentally friendly compared to conventional fossil fuels and implicate mild and low cost

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technologies that do not disturb the balance of carbon cycle. Indeed, during the combustion of biofuels no toxic nitrogen and sulfur oxides (NO_x, SO_x), i.e. the mainly responsible for photochemical pollution and acid rain that have detrimental effects on the environment, are generated [3], whereas the CO_2 emission is equal to the amount of CO_2 used for photosynthesis to produce biomass, maintaining thus its balance in the atmosphere [4].

However, biofuels can contribute to EU's goals to achieve a specific amount of renewable sources in energy consumption as long as they are produced in a sustainable way i.e. without impeding the process of food production. The production of second generation biofuels by exploiting lignocellulosic biomass and wastes has, thus, been proposed to overcome the limitations of first generation biofuels coming from substrates that could be used as food or feeds [5].

Among the different biofuels, bioethanol is the first one that has been commercialized, due to its proven suitability as an oxygenate and octane enhancer when mixed with gasoline that makes it a suitable transportation fuel [6]. A great number of waste feedstock containing significant amounts of sugars, or other compounds that can be converted into sugars (i.e. hollocellulose and starch), such as food wastes and residues generated throughout the food production chain, have been proposed as possible substrates for sustainable ethanol production in line with the principles of a circular economy [7]. Nevertheless, not all of them prove eventually to be sufficient substrates to achieve the required ethanol concentration threshold for its successful recovery from fermentation mixtures, or efficiently high productivities that would make scale-up of the processes economically viable. This may be due, for example, to the limited conversion potential of the carbohydrate content of some substrates, such as lignocellulosic substrates with high lignin content [8], due to limitations of substrate conversion due to the low potential of biocatalysts in terms of ethanol yields, and/or tolerance [9] or the presence of inhibitory to the fermentation process components [10]. In this light, the present work has focused on the exploitation of a food waste that is generated at consumer level i.e. a restaurant food waste, the exploitation of which copes with the circular economy goals and, which also has an extremely high carbohydrates content in the form of starch and lacks possible toxic compounds since it was initially meant to be used for human consumption.

According to the source and composition of the food wastes to be exploited, different processes have to be applied for their efficient utilization towards bioethanol, such as fractionation, liquefaction, mild, moderate or more severe pretreatment, and hydrololysis. In the case of starchy food wastes, such as the food waste that was selected in the current study, an initial step of hydrolysis of the starch is required prior to fermentation since most biocatalysts do not require the potential to produce the required amylolytic enzymes. The conventional way to break down starch into smaller oligosaccharides and glucose is liquefaction followed by enzymatic saccharification, which includes its hydrolysis by a-amylases i.e. thermostable endoglucanases that hydrolyze the internal α-1, 4-glycosidic bonds of starch and glucoamylase that may further hydrolyze the remaining oligosaccharides to glucose [11]. During liquefaction process, aquatic suspensions of starch forms a gel (gelatinization) upon heating at about 70 °C, which is eventually liquefied to produce dextrins at a high temperature and low pH environment; a process that is reported to facilitate enzymatic hydrolysis of starch [12]. In the case though that a starchy food waste originates from unconsumed cooked food, its starch content is expected to have been gelatinized to some extent due to the thermal processing of cooking, possibly making thus the need of pretreatment and/or hydrolysis less severe. Into this context, among the priority goals of the current study was to investigate the effect of thermochemical and enzymatic pretreatment of the starchy food waste selected, applied solely and in combination, on the saccharification efficiency and the subsequent fermentation of slurries and hydrolysates via novel yeast strain, aiming to identify the minimal requirements of the process.

However, as was mentioned above, finding efficient biocatalysts is also of great importance for achieving sustainable ethanol production. As such, the current study has a dual purpose, aiming not only at determining the optimal sacharification process of the food waste, but also at identifying fermentative microorganisms of high potential. A great number of microorganisms, mainly yeasts, can be utilized for bioethanol production from food wastes, the selection of which should be based on criteria such as exhibiting high ethanol yield, enhanced ethanol tolerance, high ethanol production rates, capability of exploiting simple, inexpensive media and resistance to high substrate concentration and possible inhibitors generated during pretreatment [13]. Searching for new microbial strains with high ethanologenic potential among various environments is indeed constant in the research community, aiming to identify, eventually, ethanol hyper-producers that combine as many of the above desirable properties as possible. In the current study, with the aim of discovering a possibly hyper-fermenting yeast, the isolation of numerous strains inhabiting various natural food product as well as food wastes was conducted, leading -upon the initial screening of their fermentation capacity- to the selection of three promising stains that were shown to belong to the species *Kluyveromyces marxianus* and *Pichia kudriavzevii*. Both species are established C5, non-conventional yeasts that have great potential as biocatalysts for ethanol production from various wastes including lignocellulosic ones, such as citrus peels [14], sugarcane bagasse [15], sunflower meal [16] and pomegranate peels [17], as well as hydrolysates of starchy ones, such as taro waste [18] and cassava starch [19] hydrolysates. To our knowledge though, neither of the wild strains of the selected species has been so far evaluated for bioethanol production from whole pretreated starchy food wastes.

2. Materials and methods

2.1. Food waste

The starchy biowaste was a mixture of meal left-overs that were collected from restaurants of Asir region, Saudi Arabia and contained mainly cooked rice. Immediately upon collection the biowaste was transferred to the laboratory, it was dried at 50–55 °C for 24 h and subsequently it was air dried, grinded at particle size of ≤2.5 mm, homogenized and packed in polypropylene bags in batches of 2 kg until use. Prior to pretreatment and fermentation experiments, the air dried waste, namely WR (wasted rice) was further grinded using a laboratory stainless steel mill (A11 Basic, IKA, Germany) to a particle size of ≤0.5 mm.

2.2. Isolation and selection of yeast strains

Initially 34 yeast strains were isolated via the dilution plate method from various fruits, vegetables,food products and foood wastes from Asir region, Saudi Arabia and were purified as described by Hashem et al. [20]. Initially, all isolates were screened in terms of possible amylolytic activity, which was assessed via the iodine test. Subsequently, their fermentation potential using different sugars i.e. the monosaccharides glucose and fructose and the disaccharides sucrose and maltose, was assessed. Fermentation tests were conducted in sealed Erlenmeyer flasks (250 mL) batch cultures in triplicate with 10 g/L of each sugar as the sole carbon source, supplemented with 1 g/L yeast extract. Cultures were incubated at 30 °C and 150 rpm and after 72 h the fermentation potential was estimated by quantifying the concentration of ethanol and the residual carbon source concentrations and estimating the achieved ethanol yields and fermentation efficiencies (*FE*) according to Eqs. (2) and (3) (par. 2.5.3). The origin, starch hydrolysis results and alcoholic fermentation potential of the isolates are shown in Table 1. Although non exhibiting starch hydrolysis capacity, the strains KKU12, KKU14 and KKU25 were selected for further experimentation based on the fermentation potential of glucose (ethanol yield ≥90% of the maximum theoretical). Strains KKU14 and KKU25 also excibited maltose

Table 1
Isolaton source, starch hydrolysis rcapacity, and ethanol production potential of the yeast isolates used in the current srudy.

Isolate	**Origin**	Starch hydrolysis	Fermentation efficiency (*FE*)			
			Glucose	Fructose	Sucrose	Maltose
KKU12	Dates "Barhi"	nd	VH	M	VH	nd
KKU14	Dates "Sefri"	nd	VH	VH	W	VW
KKU25	Wasted Rice	nd	VH	VH	H	VW

nd = not detected.
VW<30%.
W = 30%–50% of theoretical yield of ethanol.
M = 50% - 80% of theoretical yield of ethanol.
H = 80%–90% of theoretical yield of ethanol.
VH ≥ 90% of theoretical yield of ethanol.

fermentation capacity, which though was very weak (ethanol yield <30% of the maximum theoretical).

2.3. Molecular identification and phylogenetic analysis

For the selected strains, total DNA extraction was performed, using 10^6 cells on the average, via the DNeasy PowerSoil Kit (QIAGEN) following the manufacturer's protocol. The extracted DNA samples were forwarded to University of Patras for PCR amplification of the D1/D2 domain of 26S rDNA regions using the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′), and NL4 (5′-GGTCCGTGTTT CAAGACGG-3′) [21] PCRs were carried out in 15 μL volumes (1X PCR Master Mix: KAPA2G FAST Multiplex PCR Kit and 1 U of Hot start Taq DNA Polymerase), 1 μL of each primer (10 μM) and 1 μL DNA template, filled to 15 μL with Milli-Q Water). The thermocycling program included an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30sec, annealing at 52 °C for 1 min and extension at 72 °C for 90 s, with a final extension step at 72 °C for 10 min. The PCR results were analysed by horizontal electrophoresis in 1% agarose gel stained with GelRed® (Biotium, USA), after which they were inspected under UV light and photographed. PCR products were purified using commercially available spin columns (Macherey-Nagel). The PCRs yielded a product of approximately 600 bps. Sequencing was conducted on an AB3700 capillary sequencer (Macrogen Europe, the Netherlands) using the primers of the amplification procedure. The resulted chromatograms were manually inspected and the corrected sequences were subjected to multiple alignment with CLUSTAL-W v1.4 [22]. The ambiguous regions were removed using Gblocks' 0.91b default parameters [23]. Maximum likelihood (ML) analysis was conducted at PhyML with default parameters. Confidence in the nodes was assessed by 1000 bootstrap [24].

2.4. Pretreatment of WR

Three pretreatment approaches of the WR were investigated i.e. the enzymatic pretreatment via commercial amylolytic enzymes, the thermochemical pretreatment via HCl and the combined two-step chemical-enzymatic pretreatment. In all cases three solid loadings of the waste were tested i.e. 10% TS WR (w/v), 20% TS WR (w/v) and 30% TS WR (w/v). Enzymatic pretreatment of WR was performed in duplicates at pH 4.8 and 50 °C using a fungal α-amylase, FA (α-amylase from *Aspergillus oryzae*, Sigma-Aldrich) and amyloglucosidase, A, (amyloglucosidase from *Aspergillus niger,* Sigma-Aldrich) at enzymatic loadings 50 FA U/g starch and 25 AU g starch, respectively. For the pH adjustment, 0.1 M sodium acetate buffer was used. To avoid bacterial contamination, 2‰ sodium azide was added to the suspension. Thermochemical pretreatment was conducted using 1g HCl/100 g TS WR at 121 °C for 20 min. After cooling, the pH was adjusted to 4.8 using 6 N NaOH. The combined pretreatment included two sequential steps, i.e. thermochemical pretreatment as described above and then enzymatic hydrolysis of the residual starch with of 50 FA U/g starch and 25 AU g starch. The efficiency of each pretreatment approach was assessed by estimating the saccharification efficiency, *SE*, using Eq. 1

$$SE\,(\%) = \frac{Final\,concentration\,of\,sugars - Initial\,concentration\,of\,sugars\,(g/L)}{Initial\,concentration\,of\,starch\,(g/L)} \quad (1)$$

2.5. Bioethanol production

2.5.1. Precultures preparation and inoculation

Precultures were prepared by transferring a full bacteriological loop of yeast colonies from slant cultures (YMA medium) that were stored at 4 °C, to 100 ml YMB medium in Erlenmeyer flask (250 ml) under aseptic conditions. The flasks were capped with hydrophobic cotton and incubated at 30 °C and 150rp overnight, until reaching $OD600_{nm}$ ~1.6. For the inoculation, an estimated volume of preculture corresponding to 10% of the culture volume was centrifuged at 4100 rpm for 15 min and the yeast pellet was re-suspended in a salt solution of KH_2PO_4, $MgCl_2.6H_2O$ and $(NH4)_2SO_4$ at final concentrations 1 g/L, 2 g/L and 3 g/L each for cultures with substrates derived from 10% TS WR (w/v), 20% TS WR (w/v) and 30% TS WR (w/v) solids loading, respectively, whereas no adjustment of the pH was performed.

2.5.2. Fermentation experiments

All fermentation tests were performed in duplicate with monocultures of the isolates KKU12, KKU14 and KKU25, in batch mode, at 30 °C and constant agitation at 150 rpm. Serum vials of 160 mL total volume and 100 ml working volume were used, sealed with rubber stoppers, which were punctured with sterilized needles with adjusted 0.22 μm membrane filters to allow for CO_2 venting and maintenance of sterile conditions. Two types of experiments were conducted, i.e. using a) the whole hydrolysed slurry of the pretreated waste and b) the hydrolysates upon removal of solids. Whole slurry fermentation tests were conducted with enzymatically, thermochemically and combined pretreated WR at solids loading 10% TS WR (w/v) and 30% TS WR (w/v) and the ethanol production efficiency was assessed after 72 h of fermentation, by quantifying the concentrations of ethanol and residual sugars. For the fermentation tests with hydrolysates, WR at solids loading 10% TS WR (w/v), 20% TS WR (w/v) and 30% TS WR (w/v) were subjected to chemical/enzymatic combined pretreatment and after which the remaining solids were removed via initial centrifugation (4100 rpm, 20 min) and subsequent filtration (GF/F, Whatman) under aseptic conditions. The microbial growth, in terms of OD_{600nm}, pH drop and consumption patterns of sugars and ethanol were followed versus time for 72 h, whereas the final concentration of microbial biomass was also quantified gravimetrically at the end of the fermentation.

To assess the efficiency of alcoholic fermentation, bioethanol yield in terms of sugars uptake, $Y_{E/S}$, fermentation efficiency, *FE*, and feedstock bioconversion, $Y_{E/Waste}$, were calculated according to the following equations:

$$Y_{E/S}\,(g/g) = \frac{Bioethanol\,concentration\,(g/L)}{Consumed\,carbohydrates\,(g/L)} \quad (2)$$

$$FE\,(\%) = \frac{Y_{E/S}}{Y_{E/S}\,max} \quad (3)$$

where $Y_{E/S}$ is the estimated ethanol yield from the consumed carbohydrates, and $Y_{E/S}max$ is the maximum theoretical ethanol yield, 0.51 g ethanol/consumed hexoses, according to the Guy-Loussac equation.

$$Y_{E/waste}\,(g/kg\,TS\,WR) = \frac{Bioethanol\,concentration\,(g/L)}{WR\,loading\,(kg\,TS\,WR/L)} \quad (4)$$

2.6. Analytical methods

Total solids (TS), total suspended solids (TSS), humidity, volatile solids (VS), volatile suspended solids (VSS), ash, total chemical oxygen demand (t-COD) and Total Kjeldahl Nitrogen (TKN) were quantified according to Standard Methods [25]. Crude protein content was determined by multiplying TKN by a factor of 6.25 [26]. Sugars and total carbohydrates were quantified according to DuBois et al. [27]. Reducing sugars were quantified according to Miller [28]. Starch content was determined using total the Megazyme starch assay kit. Lipids and oils content were estimated in a Soxlet apparatus (SER 148, VELP). Ethanol was quantified via HPLC-RI as described by Ben Atitallah et al. [29].

2.7. Statistical analysis

The statistical analysis of the obtained data was conducted with the use of the SPSS Inc.17 software package. After checking for homogeneity of the variance (Levene's test of equality of error variances), the significant differences among each treatment were assessed non-parametrically, using the Mann Whitney u test ($p < 0.05$, ANOVA).

3. Results

3.1. Characterisation of WR

The composition of the WR is presented in Table 2. It is may be assumed that WR is a promising feedstock for bioethanol production since it has an ~85% carbohydrate content, on dry basis, for which however pretreatment is essential for the hydrolysis of starch. The carbon to nitrogen ratio of the waste is 382.61 ± 35.30 g t-COD/g TKN, which is quite low compared to other food wastes such as kichen biowaste [30] and restaurant food wastes [31], with the nitrogen coming primarily from the rice proteins. As such, the addition of external nitrogen source is essential for its exploitation as substrate for bioethanol production to ensure sufficient microbial activity.

3.2. Identification of yeast strains

The yeast identification procedure was based on the sequencing of PCR amplified 600 bps D1/D2 region of the yeast 26S ribosomal DNA as previously proposed by Hashem et al. [21] and Kurtzman [32]. The particular region can distinguish between the generality of yeast species known and thus, allows for the classification and phylogenetic identification of unknown isolates. The resulted sequences were compared by BLAST search (National Center for Biotechnology Information Basic Local Alignment Search Tool NCBI BLAST; http://www.ncbi.nlm.nih.gov/) to the D1/D2 regions of all submitted yeast species in GenBank database, aiming to accurate and rapid identification. The scores obtained were expressed as percent of the genetic identity. The obtained sequences were deposited to Genbank (Table 3). Maximum Likelihood phylogenetic analysis of the nucleotide sequences along with 13 sequences retrieved from Genbank showed a clear grouping of the analysed samples herein ie. KKU12, KKU14 and KKU25 with their conspecific and/or closest taxonomic relatives, verifying their correct identification (Fig. 1).

Table 2
Chemical composition of the starchy food waste.

Parameter	Value
TS, %	92.59 ± 0.06
VS, % TS	95.87 ± 0.02
Ash, % TS	4.13 ± 0.02
Sugars, %	7.42 ± 0.32
Starch, %	69.70 ± 0.02
t-COD, %	98.89 ± 2.13
TKN, %	0.26 ± 0.02
Proteins, %	1.63 ± 0.13
Lipids and oils, %	7.78 ± 0.04
pH[1]	4.7 ± 0.01

Table 3
Molecular identification of yeast samples. The percentage of similarity and best match were determined using BLASTN against the NCBI non-redundant database.

Sample ID	Best hit on GENBANK (Accession number)	Similarity (%)	Genbank Accession Number
KKU12	*Kluyveromyces marxianus* isolate V3-19 (OP364978.1)	97.14	OP740683
KKU14	*Pichia kudriavzevii* strain YF1702 (MN886501.1)	99.00	OP740684
KKU25	*Kluyveromyces marxianus* strain TTG-428 (MT334456.1)	99.79	OP740685

The isolates KKU12 and KKU25 were identified as *Kluyveromyces marxianus* whereas KK14 as *Pichia kudriavzevii*. Both *K. marxianus*, and *P. kudriavzevii* are non-conventional species, non-pathogenic for humans and animals, and have been proposed for alcoholic fermentation of residual biomass and waste based feedstocks for transportation bioethanol production [13].

3.3. Effect of pretreatment/hydrolysis on the saccharification of WR

The pretreatment of WR aimed at the saccharification of the waste in order to enhance its subsequent fermentation efficiency towards ethanol. As such the pretreatment methods that were assessed were a) the enzymatic pretreatment via commercial amylolytic enzymes, b) the thermochemical pretreatment via HCl and c) the two stepped thermochemical-enzymatic pretreatment, using in all cases three solid loadings WR i.e. 10% TS WR (w/v), 20% TS WR (w/v) and 30% TS WR (w/v). As shown in Fig. 2, the higher solids loading resulting to the higher liberation of total soluble sugars and reducing sugars for all pretreatment handlings, whereas the enzymatic and combined chemical/enzymatic pretreatment led to much enhanced saccharification efficiencies compared to the chemical pretreatment. It can be assumed, thus, that applying the specific thermochemical pretreatment solely cannot be a proposed method for the efficient exploitation of the waste towards ethanol, since a considerable amount carbohydrates are not hydrolysed and thus will remain un-exploitable by the yeasts. In a previous study with similar food waste that was subjected to chemical pretreatment with HCl under more severe conditions [33], complete starch hydrolysis was achieved, but the saccharification yield was lower than expected, indicating that degradation of the liberated sugars occurred. For that reason milder conditions were selected in this study, which though not fully hydrolysing starch, seem to facilitate to some

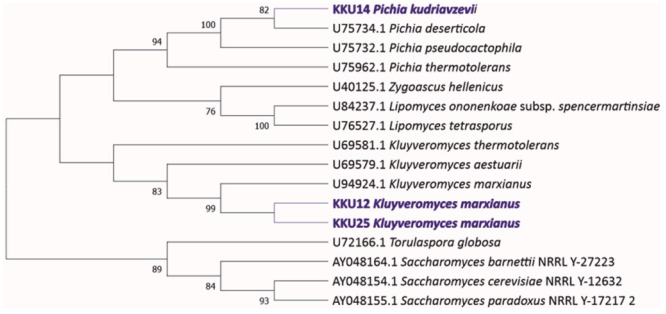

Fig. 1. Maximum likelihood (ML) tree based on the D1/D2 region of the 26S ribosomal DNA for the three samples of this study highlighted in bold blue (KKU12, KKU14, KK25) and 13 yeast sequences downloaded from GenBank with their accession numbers. Bootstrap support values over 75% are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

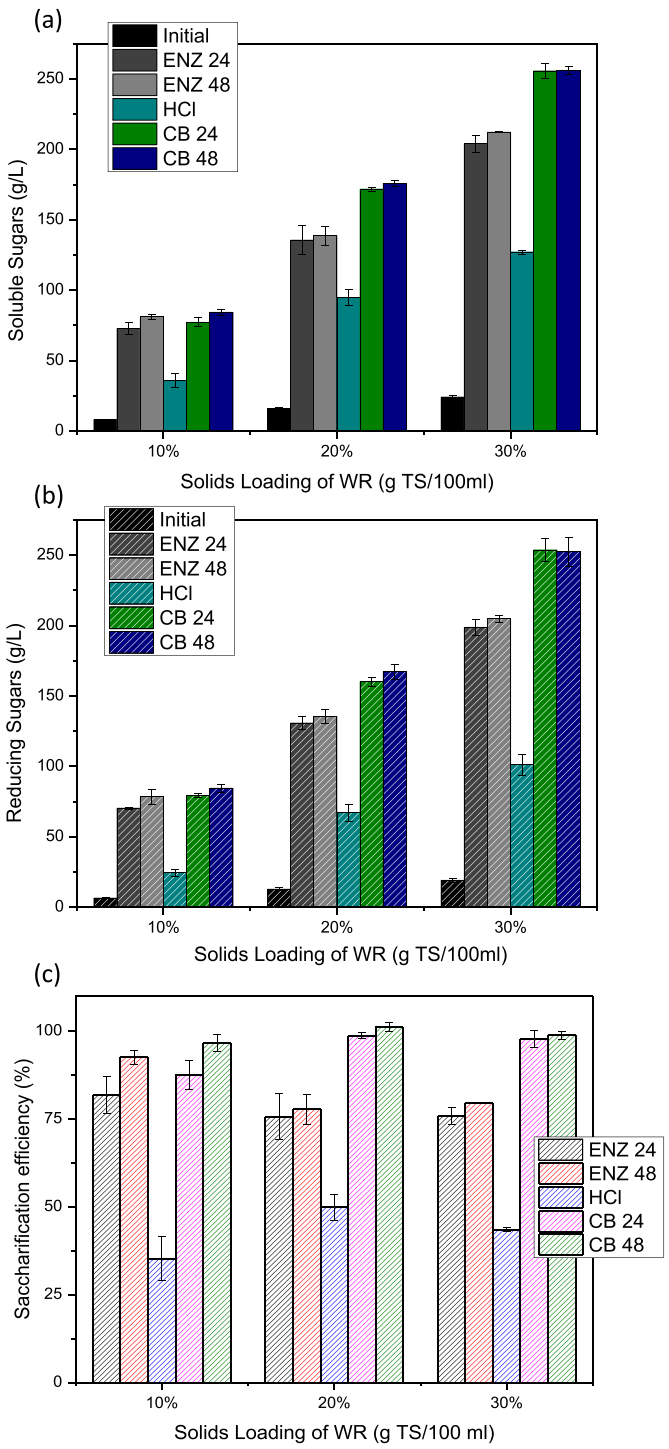

Fig. 2. Effect of enzymatic, thermochemical and combined pretreatment of 10%, 20% and 30% aquatic suspensions of WR TS on the liberation of soluble sugars (a) reducing sugars (b), and saccharification efficiency (c). Initial, concentration of sugars before pretreatment; ENZ 24, 24 h of enzymatic hydrolysis with 50 U AG/g starch; ENZ 48, 48 h of enzymatic hydrolysis with 50 U AG/g starch; HCl, hydrolysis with 1g HCl/100 g TS WR, at 121 °C for 20 min; CB 24, chemical hydrolysis followed by 24 h of enzymatic hydrolysis with 50 U A/g residual starch; CB 48, chemical hydrolysis followed by 48 h of enzymatic hydrolysis with 50 U A/g residual starch. Experimental points represent the mean value of two replicate handlings and three technical replicates from each ±SD (N ≥ 6).

extent the enzymatic hydrolysis, since as it can be observed in Fig. 2a, the combined pretreatment leads to higher liberation of sugars for loading 20% and 30%.

As it regards the effect of different pretreatments on the liberation of reducing sugars, comparison of Fig. 1a and b reveals that in the case of enzymatic and combined pretreatment the soluble sugars are almost all reducing, as also reported in previous studies concerning the enzymatic saccharification of other types of starchy [34] as well as lignocellulosic/starchy biowastes like kitchen biowaste [30] and hydrolysed domestic food waste [35]. This is not the case however for the thermochemical pretreatment, for which the concentration soluble sugars seem to be considerable higher than the concentration of reducing sugars. For the direct comparison of the values the ratio of reducing to soluble sugars was estimated for all cases, and the values are presented in Table 4, from which is clear that the values for the enzymatic and combined hydrolysis of the same solids loading do not have any significant statistically difference. On the contrary, all those values are statistically much higher than the ones estimated from the concentrations of sugars after solely thermochemical pretreatment. This can attributed to the different mechanism of enzymatic and thermochemical pretreatment. Enzymatic pretreatment was performed by the synergetic action of α-amylase and amyloglucosidase. The saccharification mechanism of those enzymes involve the hydrolysis of the 1,4-α-glycosidic bonds of starch to oligosaccharides of three or more molecules of glucose by α-amylase and subsequent hydrolysis of oligosaccharides to glucose monomers by amyloglucosidase [11]. On the contrary the effect of thermo-acid hydrolysis of starch via hydrochloric acid is expected to lead random breakdown of the 1,4-α-glycosidic bonds via the protonation of the anomeric oxygen leads to the formation of lower molecular weight polymers or oligomers [36] i.e. dextrins i.e. mixture of oligosaccahrides that are not reducing.

For the further better evaluation of the efficiency of each process in terms of the saccharification of the waste due to its hydrolysis, the parameter of saccharification efficiency *SE*, was estimated in each case using Eq. (1). The results are presented graphically in Fig. 2c. As shown *SE* is in all cases much higher for the enzymatic and combined pretreatment than for thermochemical pretreatment reaching, in the case of combined pretreatment almost complete saccharification of the waste. Interestingly enough, it seems that the effectiveness of enzymatic pretreatment seem to be depended on the solid loading and seems to be more effective for the lower loading i.e. 10% than for the higher ones. Nevertheless, as shown from previous studies the saccharification of food wastes mainly depends on the accessibility of complex carbohydrates to enzymatic attract (e.g. presence of lignin) and the enzymatic loading and as such keeping the ratio of enzymatic to solids loading stable the *SE* is not expected to be affected as previously reported for the fermentation of enzymatically pretreated food waste slurries in SSF [30] and SHF [35] mode with up to 40% loading of solids as well as for the fermentation of enzymatically hydrolysed household food wastes with 45% solids loading [37]. The negative effect of solids loading observed in the present study can probably thus be attributed to the difficulty in

Table 4
Ratio of concentrations of reducing to soluble carbohydrates that were liberated after the enzymatic, chemical and combined pre-treatment of WR aquatic suspensions 10% TS WR (w/v), 20% TS WR (w/v) and 30% TS WR (w/v). Experimental points represent the mean value of duplicate handlings and two technical replicates from each ±SD (N ≥ 4).

Pretreatment	h	Reducing to soluble carbohydrates (%)		
		10% TS	20% TS	30% TS
Enzymatic	24	98.97 ± 1.29	98.16 ± 3.14	98.11 ± 1.44
	48	97.57 ± 2.36	99.94 ± 0.63	97.62 ± 2.05
Combined	24	99.84 ± 2.03	96.23 ± 1.99	100.63 ± 1.79
	48	98.04 ± 1.98	98.15 ± 2.15	99.15 ± 2.96
Chemical	–	69.99 ± 7.94	79.03 ± 3.23	71.99 ± 5.17

the mixing of the slurries for higher loadings which was much thicker than for the 10% loading. This assumption is further supported by the results of combined pretreatment which exhibit the same *SE* for all three enzymatic loadings. During combined pretreatment the initial liquefaction of WR due to chemical hydrolysis of starch resulted to less thick mixtures the subsequent enzymatic hydrolysis of which was facilitated, even for the highest loading of solids. As such, preceding thermochemical pretreatment prior to the enzymatic one, provides dual advantage to the process since, not only the action of the enzymes is facilitated, but also a lower enzymatic load is required per initial amount of WR, since part of the starch is dissolved and hydrolysed before the addition of enzymes.

3.4. Bioethanol production from WR substrates

3.4.1. Fermentation of the whole pretreated WR slurries

In order to assess the effect of pretreatment on the ethanol production efficiency of the waste, fermentation tests were performed, with the whole pretreated WR slurries using the three isolates of the non-conventional yeast strains. In Fig. 3 the ethanol titers and the estimated carbohydrates consumption after 72 h of fermentation are illustrated for the lowest and highest solids loadings that were tested during pretreatment, i.e. 10% TS WR (w/v) and 30% TS WR (w/v). As shown, all strains led to similar final ethanol concentrations with no statistical significantly differences for the enzymatic and combined pretreatment for both solids loadings tested. The maximum achieved values were obtained from *P. kudriavzevii* and were 42.19 ± 0.53 g/L and 83.30 ± 7.51 g/L, for the solids loading 10% and 30%, respectively; whereas the same strain also exhibited the highest accumulation presentence of carbohydrates in 72 h. Comparison of the performances among yeasts can only be made, though, upon estimation of their *FEs*. As it regards the comparative overall consumption of carbohydrates after 72 h of fermentation for the different handling, in the cases of the enzymatic and combined pretreatment it was considerably higher for the lower solids loading reaching up to 95%, whereas for WR solids loading of 30% TS (w/v), it ranged from 64.38 ± 2.48% to 80.70 ± 4.39%. This lower uptake capacity of available sugars for the higher solids loading may be accounted to different reasons such as, the cease of the metabolic activity of the strains due to pH, substrate inhibition or product inhibition to the high accumulation of ethanol in the fermentation broth as reported by Thatipamala et al. [38] and Zhang et al. [39]. In terms of the sustainability of the process, the feedstock's insufficient utilization for the solids loading of 30% TS waste may be disadvantageous. Still, the solids loading of 10% TS waste may not be efficient for providing the ethanol concentrations that are required for successful distillation from the fermentation broth, which is reported to be > 40 g/L [40] since the achieved ethanol titers are at the edge or lower than this threshold. As it regards the thermochemical pretreatment of the waste it was proven to be insufficient for subsequent fermentation since both carbohydrates consumption and achieved ethanol yields were extremely low compared to the other pretreatments.

To further, access the ethanologenic capacity of the yeasts from the pretreated wastes, the fermentation efficiency, *FE* was estimated via Eq. (2) and Eq. (3). As shown in Fig. 4a the fermentation of enzymatically and combined pretreated WR slurries led to considerably higher *FEs* compared to the thermochemically pretreated waste. Indeed the chemical pretreatment, although leading to the liberation of considerable amount of free sugars was proven to produce an insufficient substrate for further fermentation due to its quite low assimilation and also fermentation capacity by the yeasts. It may be assumed that the selective conditions of thermochemical pretreatment are not severe enough to warranty the efficient saccharification of the complex carbohydrates of the dried WR to monosaccharides and disaccharides, such as glucose and maltose, but to oligosaccharides which may not be fermentable by *K. marxianus and P. kudriavzevii*. This assumption is also supported by theestimation of the ratio of reducing to total soluble carbohydrates that

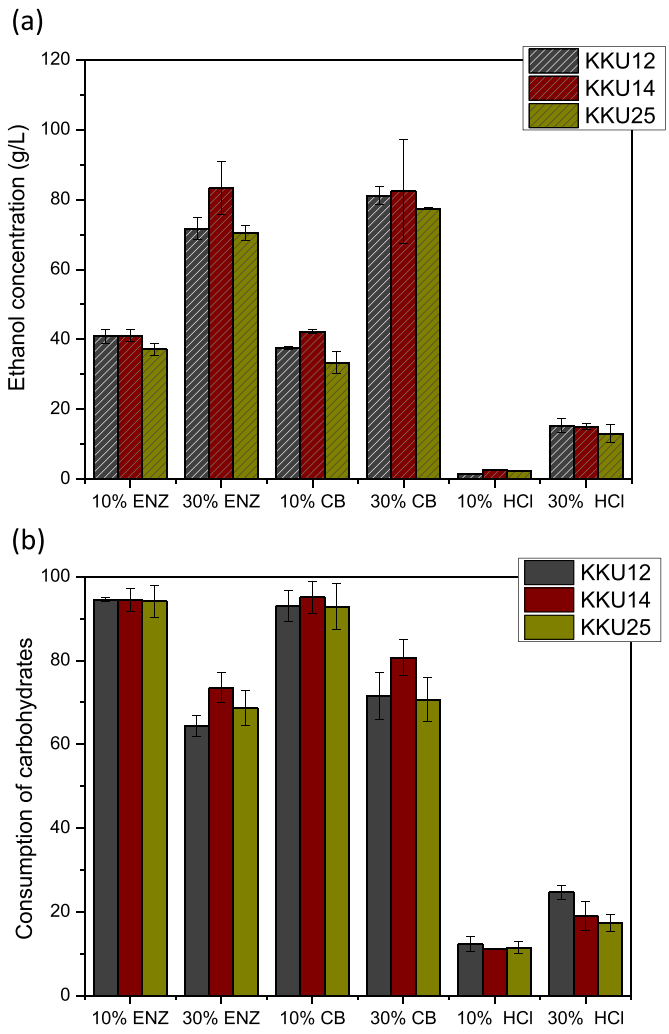

Fig. 3. Ethanol concentrations (a) and carbohydrates consumption (b) from the alcoholic fermentation of enzymatically, thermochemically and combined chemically-enzymatically pretreated WR at initial solids loadings 10% and 30% after 72 h of incubation via the yeasts *Kluyveromyces marxianus*, V3-19 (KKU12), *Pichia kudriavzevii*, YF1702 (KKU14) and *K. marxianus* isolate, TTG-428 (KKU25). ENZ 24, 24 h of enzymatic hydrolysis with 50 U AG/g starch; ENZ 48, 48 h of enzymatic hydrolysis with 50 U AG/g starch; HCl, hydrolysis with 1g HCl/100 g TS WR, at 121 °C for 20 min; CB 24, chemical hydrolysis followed by 24 h of enzymatic hydrolysis with 50 U A/g residual starch; CB 48, chemical hydrolysis followed by 48 h of enzymatic hydrolysis with 50 U A/g residual starch. Experimental points represent the mean value of a duplicate culture and three technical replicates from each ±SD (N ≥ 6).

was estimated for the thermochemical treatment of the waste, which had an average value for the 3 solids loadings studied of 73.67 ± 7.62. This value shows that indeed, during the chemical hydrolysis of starch, approximately 1/3 of the soluble carbohydrates have not undergone complete hydrolysis, i.e. they are oligosaccharides. In previous studies in which a similar waste was assessed as substrate for ethanol production the ethanol yields were quite high [33], but in that study the biowaste was subjected to acid pretreatment with HCl in more severe conditions and without being priory dried. With regard to the enzymatic and combined pretreatment it was shown that KKU12, *Kluyveromyces marxianus*, V3-19 exhibited the highest *FEs* for all three solids loadings tested, which exceeded 90% of the theoretical maximum. Interestingly enough, isolate KKU25 which was identified to belong to the same species with isolate KKU12, led to relative lower *FEs,* with values of approximately 79% of the theoretical maximum and similar to the *FEs* reached by isolate KKU14, *Pichia kudriavzevii*, YF1702. It should be

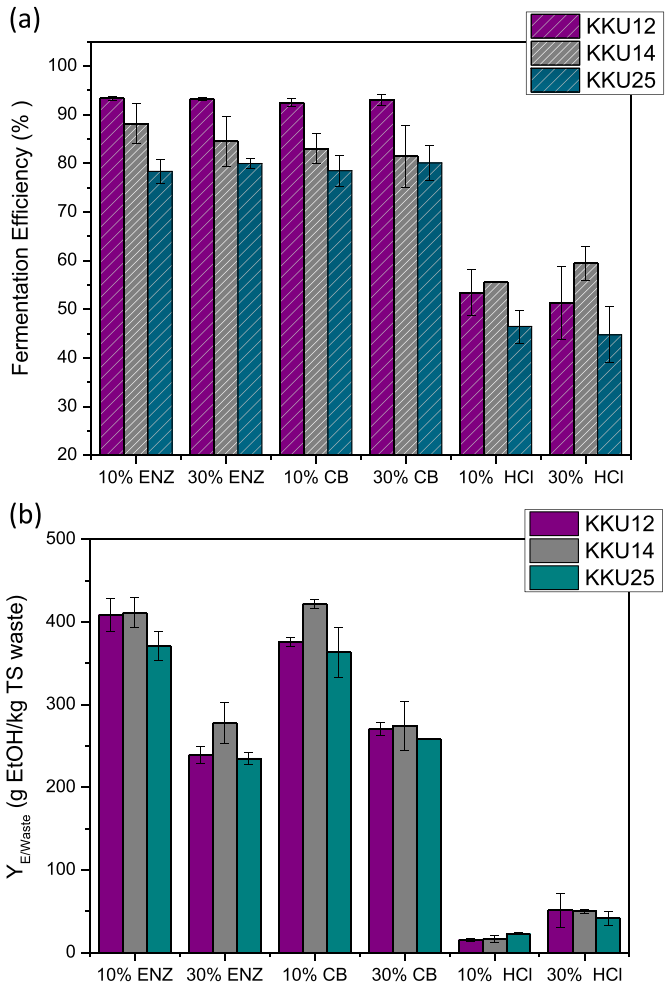

Fig. 4. Estimated fermentation efficiency (a) and ethanol yield from initial waste during the alcoholic fermentation of enzymatically, chemically and combined chemically-enzymatically pretreated WR at initial solids loadings 10% and 30% after 72 h of incubation via the yeasts *Kluyveromyces marxianus*, V3-19 (KKU12), *Pichia kudriavzevii*, YF1702 (KKU14) and *K. marxianus* isolate, TTG-428 (KKU25). ENZ 24, 24 h of enzymatic hydrolysis with 50 U AG/g starch; ENZ 48, 48 h of enzymatic hydrolysis with 50 U AG/g starch; HCl, hydrolysis with 1g HCl/100 g TS WR, at 121 °C for 20 min; CB 24, chemical hydrolysis followed by 24 h of enzymatic hydrolysis with 50 U A/g residual starch; CB 48, chemical hydrolysis followed by 48 h of enzymatic hydrolysis with 50 U A/g residual starch. Experimental points represent the mean value of a duplicate culture and three technical replicates from each ±SD (N ≥ 6).

noted though that the solids loading did not seem to affect *FE* for neither of the strains studied. The ethanol yields form the initial feedstock, $Y_{E/WASTE}$, were also estimated for all pretreatment handlings and are presented in Fig. 4b. As expected, $Y_{E/WASTE}$ was effected considerably by the solids loading, being higher for all biocatalysts tested for the lowest solids loading i.e. 10% TS WR (w/v), due to the considerably higher bioconversion of carbohydrates.

3.4.2. Fermentation of hydrolysates of combined chemically/enzymatically pretreated WR

Based on the results presented above, the combined chemical/enzymatic pretreatment was considered as the most favorable in terms of both saccharification and also fermentation, and it was selected for further experimentation using the WR hydrolysates as fermentation substrate. The hydrolysates obtained from combined pretreated WR at solids loading 10% TS (w/v), 20% TS (w/v) and 30% TS (w/v) were tested in order to investigate the kinetics of the yeasts and possibly clarify potential limitations of the process. In Fig. 5 the kinetics of the microbial biomass increase and the pH change during the 72 h of fermentation for the three yeast strains are illustrated. It is noteworthy that the isolates KKU12 and KKU25, which belong to the same species, exhibited almost identical microbial growth, with a greater increase in biomass for the higher substrate concentrations and a maximum increase on the 24 h, while for the isolate KKU14, the microbial growth does not seem to be affected by the substrate concentration. As far as the pH is concerned, its change is similar for the three yeast strains, presenting in all cases a significant drop from 4.7 to approximately 3.2, during the first 24 h of fermentation, followed by an increasing trend. The initial pH decrease that was observed is typically reported during alcoholic fermentation of various substrates and is most usually correlated to the assimilation of ammonium by the yeasts during which H^+ are released [41]. It is also noteworthy that for the lower solids loading, the drop in pH is greater for all cases, something that can be attributed to the faster ability to internalize pH at the highest concentrations of waste, as is also observed for other similar food wastes [30]. The biphasic evolution though is not as common, and though it has not been observed during the fermentation of other food wastes such as kitchen food wastes via *Saccharomyces cerevisiae* and *P. stipites* [30], date palm sap fermented via *P. anomala* [29] it is reported to occur in some cases [42]. Indeed, according to Akin et al. [42] who observed such a two phase pH evolution during grape must fermentation by *S. cerevisiae*, the rise of pH after the initial drop was attributed to the ethanol concentration increase in the medium during the same period. This is not though sufficient explanation for the case of the fermentation of WR hydrolysates of the current study, since the pH increase was not only noticed during ethanol titers built up, but even after the highest concentration of ethanol was achieved at 24 h of fermentation in the case of 10% TS WR (w/v) solids loading as illustrated in Fig. 6 (graphs b, d, f). A possible explanation could be the accumulation of some intermediate metabolites such as citric, malic and succinic acids, which may show increased concentrations during the main fermentation phase, while are subsequently consumed resulting though to a negligible increase of the ethanol titers [43].

Taking a closer look at the evolution of ethanol in comparison to the consumption of sugars (Fig. 6), it appears that its production actually ceases at the same time as the assimilation of sugars stops, i.e. at 30 h and 48 h for cultures with hydrolysates coming from pretreated WR of 10% TS (w/v) and 20% TS (w/v) solids loading, respectively, time points at which complete substrate depletion is observed. For the hydrolysates obtained from pretreated WR 30% TS (w/v) solids loading, the ethanol production for *P. kudriavzevii* stops at about 48 h due to the inability of the yeast to further consume the substrate, despite the fact that the latter is not yet exhausted. Both strains of *K. marxianis,* on the contrary, seem to be able to continue the fermentation even after 72 h of incubation, though with slower rates than *P. kudriavzevii.* The estimated values regarding the maximum consumption of sugars , $Y_{E/S}$, *FE*, and $Y_{E/WASTE}$, after 72 h of fermentation are presented in Table 5. The obtained results in terms of the *FEs* and $Y_{E/WASTE}$ are comparable to those obtained from the fermentation of pretreated slurries, whereas the consumption of sugars seem to be slightly higher in all cases.

It should be noted though, that the estimation of economic viability of the proposed methodology is the most critical parameter that will determine its sustainability, and subsequently its applicability for scaling up. In order to draw solid conclusions regarding the economic viability of the process, all the parameters of the upstream and downstream process of ethanol production should be evaluated, including the cost of collecting, drying and maintaining the waste until its use, the energy requirements of the bioreactors, the cost of input materials, the energy requirements and the degree of efficiency of the ethanol distillation process from the fermentation mixtures etc., in relation to the recovered energy from the combustion of produced ethanol [44]. For the assessment of the above, the collection of additional information and data from larger-scale experiments is required as previously performed for similar processes regarding ethanol production from enzymatically

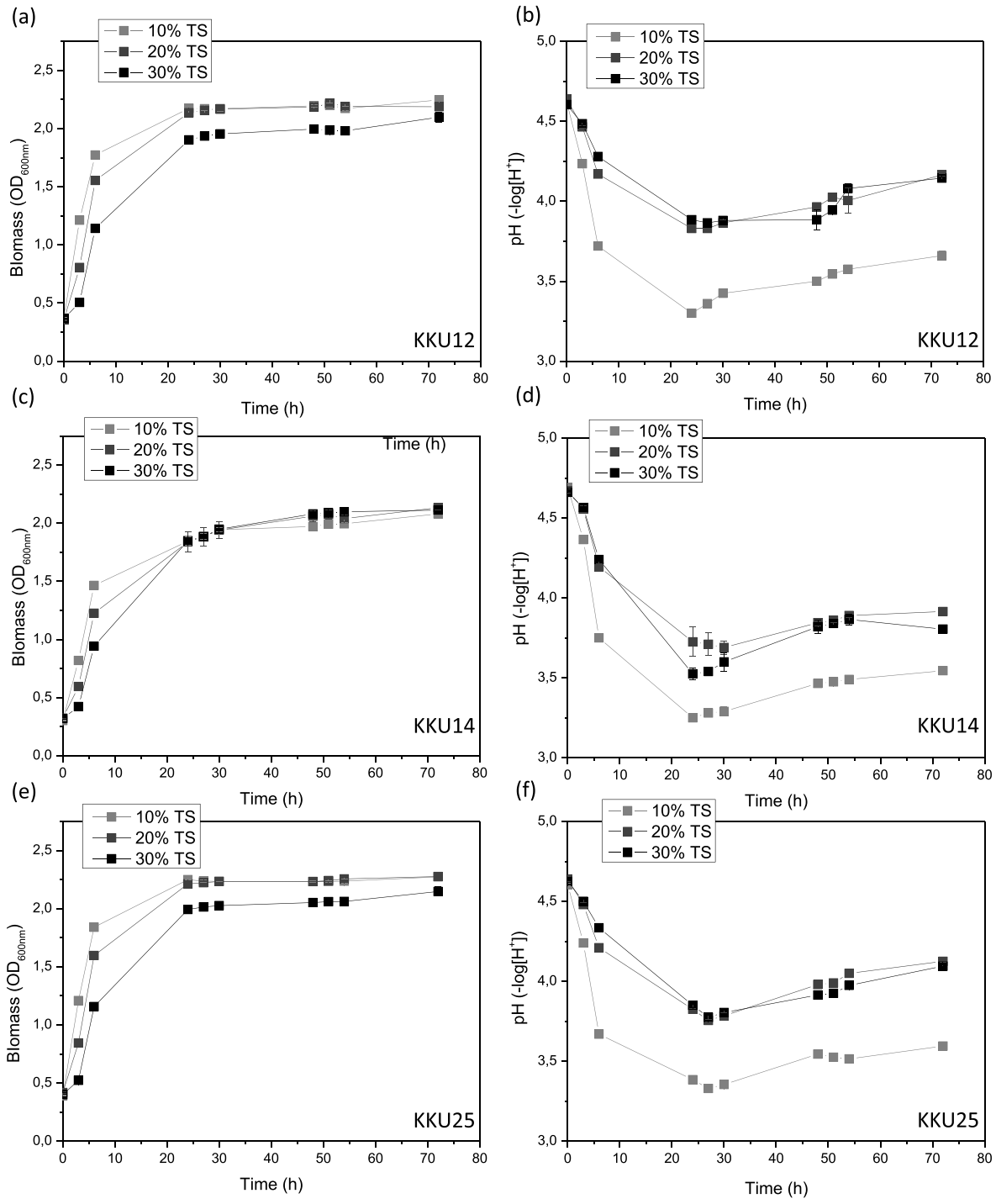

Fig. 5. Profiling of biomass production (a, c, e) and pH change (b, d, f) during the alcoholic fermentation of hydrolysates from pretreated WR (chemical hydrolysis with 1g HCl/100g TS WR, at 121 °C for 20 min followed by 48 h of enzymatic hydrolysis with 50 U A/g residual starch) at initial solids loadings 10%, 20% and 30% via the yeasts *Kluyveromyces marxianus*, V3-19 (KKU12), *Pichia kudriavzevii*, YF1702 (KKU14) and *K. marxianus* isolate, TTG-428 (KKU25). Experimental points represent the mean value of duplicate culture and two technical replicates from each ±SD (N ≥ 4).

hydrolysed food wastes [45] and ethanol and biodiesel coproduction from industrial hemp [46]. However, the possible maximum energy recovery from the waste through the combustion of the produced ethanol from the laboratory-scale experiments of the current study can be estimated via the calculation of its energy content and through which some first conclusion regarding the energy efficiency of the process, may be drawn. In order to calculate the stored energy of the produced ethanol generated from the different fermentation experiments with the hydrolysates the energy density (ED) of ethanol which was assumed to be 26.4 kJ/g [47]. The estimated values of the maximum possible recovered energy via ethanol from initial feedstock, ER_{max} are presented in Table 5. By the evaluation of the results it can be assumed that the fermentation with either of the yeast strains leads to the comparable energy production for the same solids loading, with the solids loadings of 10% and 20% TS WR (w/v) yielding to similar ER_{max} values, which are though statistically higher than those obtained from experiments with loading 30% TS WR (w/v). As though, analysed above the true energy gain could only be estimated if all the parameters of the overall

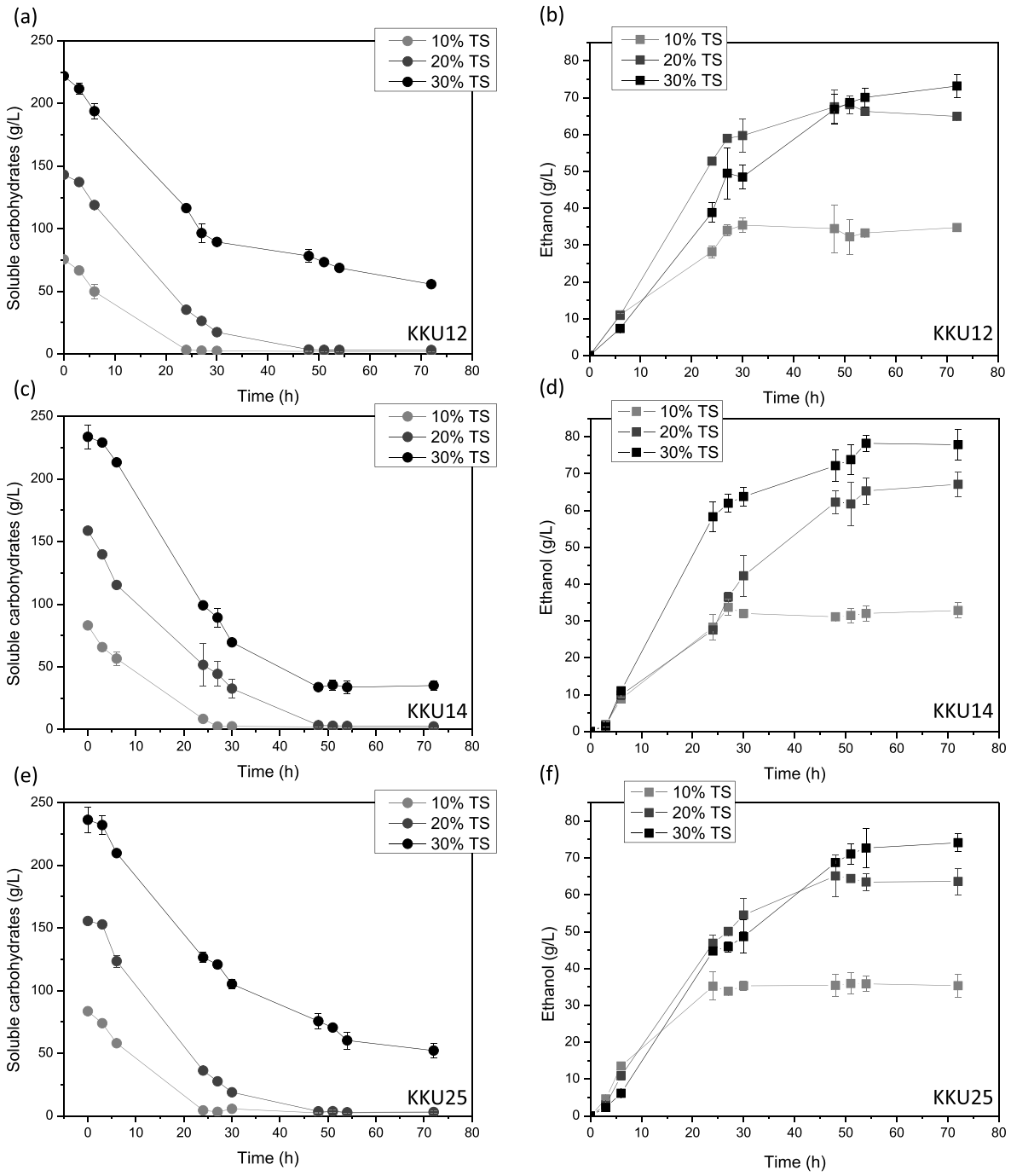

Fig. 6. Profiling of ethanol production (a, c, e) and soluble carbohydrates consumption (b, d, f) during the alcoholic fermentation of hydrolysates from pretreated WR (chemical hydrolysis with 1g HCl/100g TS WR, at 121 °C for 20 min followed by 48 h of enzymatic hydrolysis with 50 U A/g residual starch) at initial solids loadings 10%, 20% and 30% via the yeasts *Kluyveromyces marxianus*, V3-19 (KKU12), *Pichia kudriavzevii*, YF1702 (KKU14) and *K. marxianus* isolate, TTG-428 (KKU25). Experimental points represent the mean value of duplicate cultures and two technical replicates from each ±SD (N ≥ 4).

process are taken into account.

4. Conclusions

Studying the effect of different pretreatment methods on waste saccharification and ethanol production demonstrated that mild thermochemical hydrolysis is not sufficient to be applied alone. However, it was shown to provide significant advantages when combined with the enzymatic process, by increasing the saccharification efficiency and contributing also to the sustainability of the process since smaller amounts of enzymes are required to treat the same initial amount of waste. The fermentation experiments with the isolated yeasts showed that *K. marxianus* outperforms *P. kudriavzevii* in terms of the ethanol production capacity from both the whole pretreated slurries and the hydrolysates of the pretreated waste, whereas the removal of solids seemed to slightly increase the accumulation capacity of the subsrate for all solids loadings tested. Among the different solids loading of WR that were studied, 20% TS WR (w/v) proved to be optimal for the biotechnological exploitation of waste, leading to the complete utilization of the carbohydrate content, high yields and concentrations of ethanol (>60 g/L) and relatively fast completion of the fermentation process (~48 h). The fermentations with lower solids loading, 10% TS WR (w/v), did not

Table 5
Estimated maximum consumption of sugars, ethanol yield per consumed sugars, $Y_{E/S}$, fermentation efficiencies, *FE*, ethanol yield from initial feedstock, $Y_{E/WASTE}$ and, maximum possible recovered energy via ethanol from initial feedstock, ER_{max}, at 72 h fermentation of hydrolysates coming from combined chemically-enzymatically pretreated WR at initial solids loadings 10%, 20% and 30% TS WR (w/v).

	Solids loading	*K. marxianus*, V3-19 (KKU12)	*P. kudriavzevii*, YF1702 (KKU14)	*K. marxianus*, TTG-428 (KKU25)
Consumption of sugars, %	10%	98.53 ± 0.08	99.04 ± 0.05	98.53 ± 1.26
	20%	98.76 ± 0.04	98.94 ± 0.07	98.99 ± 0.12
	30%	72.64 ± 0.79	84.98 ± 2.09	76.95 ± 3.30
$Y_{E/S}$, g/g	10%	0.47 ± 0.02	0.40 ± 0.03	0.44 ± 0.04
	20%	0.46 ± 0.01	0.43 ± 0.02	0.42 ± 0.03
	30%	0.45 ± 0.02	0.39 ± 0.03	0.43 ± 0.04
***FE*, %**	10%	92.56 ± 2.95	78.99 ± 5.76	85.90 ± 8.06
	20%	90.98 ± 1.70	84.25 ± 4.81	81.83 ± 4.96
	30%	88.99 ± 3.12	77.26 ± 6.17	83.46 ± 8.51
$Y_{E/WASTE}$, g/kg TS WR	10%	347.49 ± 0.99	320.45 ± 20.93	358.60 ± 30.69
	20%	324.58 ± 3.78	326.53 ± 17.04	317.38 ± 18.03
	30%	243.84 ± 7.12	260.71 ± 13.83	242.35 ± 8.04
ER_{max} MJ/kg SWR	10%	9.17 ± 0.03	8.46 ± 0.55	9.47 ± 0.81
	20%	8.57 ± 0.10	8.62 ± 0.45	8.38 ± 0.48
	30%	6.44 ± 0.19	6.88 ± 0.37	6.40 ± 0.21

result to the required ethanol concentration of 40 g/L, which is the limit for the successful distillation of ethanol from the fermentation mixture, while for the maximum loading tested, 30% TS WR (w/v) incomplete utilization of the waste was observed.

Authors' contributions

Conceptualization: I.N., M.H; Methodology: I.N.; G.A., S.A.A., Y.S. M.; Formal analysis and investigation: M.A, M.K., I.N.; Writing - original draft preparation: I.N., K.M, S.A.A., Y.S.M.; Writing - review and editing: I.N.; G.A., G.L.; Visualization: I.N., M.K.; Funding acquisition: I.N., M.H.; Resources: M.H., G.L.; Supervision: I.N., M.H., G.L.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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