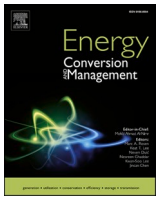
Energy Conversion and Management 302 (2024) 118105

Contents lists available at ScienceDirect

Energy Conversion and Management

journal homepage: www.elsevier.com/locate/enconman



Research Paper

Consolidated bioprocessing of the organic fraction of municipal solid waste into bioethanol

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A B S T R A C T

Organic fraction of municipal solid waste (OFMSW) has the potential to sustain large-scale biofuel production. So far, OFMSW is mainly converted into biogas by anaerobic digestion (AD), and limited research is available on its use to produce bioethanol. This paper reports, for the first time, the conversion of starch-rich OFMSW to bioethanol by using a novel yeast co-secreting both glucoamylase and alpha-amylase enzymes. As such, OFMSW can be converted to bioethanol without adding costly enzymes following a consolidated bioprocessing (CBP) approach. The OFMSW, sampled at an industrial AD plant, was processed to bioethanol with an outstanding yield, approaching 100 % of the theoretical maximum. Moreover, the co-conversion of OFSMW with starch-rice waste, namely discolored rice (DR) available in large quantities close to the AD plant, was performed to test the feasibility of valorizing different waste substrates simultaneously. The ethanol levels reached 60 g/L, indicating that both the developed process and yeast strain have important features towards ethanol production from organic waste streams.

1. Introduction

Recycle, reuse, and reduce have emerged as high-priority plans due to strict waste disposal regulations, limitation of resources, effects of global warming, and environmental concerns. This strategy can be an attractive solution to the current municipal solid waste management practices, which use landfilling as a predominant method, irrespective of the country's financial state. A large share (42 to 75 %) of municipal solid waste comprises organic fractions [1]. This organic fraction of municipal solid waste (OFMSW) has the potential to be converted into biofuels [2]. The green paper issued by the European Commission on the management of biowaste defines OFMSW as biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers, and retail premises, as well as comparable waste from food processing plants [3]. A major fraction of biowaste comprises food waste (FW), which contains raw or cooked food items and includes food materials scraped at any step between "farm and fork". Generally, FW related to households is generated before, during, or after food preparation, including vegetable peels, meat trimmings, rotten or excess components and prepared food [4]. Energy production from OFMSW could stand as a technical and economically viable alternative to biowaste management since the process of ethanol production is already industrially applied, and the OFMSW is free of cost.

The European Union (EU) generates around 140 teragrams (Tg) of FW [5], 42 % of which is contributed by the domestic section. Currently, OFMSW is managed by composting, AD, incineration, landfilling and its use for feeding animals [6,7]. Out of these methods, worldwide, most FW goes to landfills and incineration, while a small portion is utilized for composting and AD (sustainable management). Importantly, negative outcomes from landfilling and incineration include groundwater contamination or the emission of toxic gases and dioxins [8]. European legislation also aims to minimize landfilling practices in member states [9].

In Europe, a sustainably managed portion of OFMSW is used mostly for AD, wherein the product obtained is biomethane. Although largely applied as biofuel, methane is rarely used in heavy commercial vehicles. Some studies also showed the production of biohydrogen from OFMSW by dark fermentation, but there is a need for strict control of the

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https://doi.org/10.1016/j.enconman.2024.118105

Received 30 October 2023; Received in revised form 29 December 2023; Accepted 12 January 2024

Available online 19 January 2024

chemical composition of OFMSW [10,11], which is quite difficult at a very large scale. On the contrary, bioethanol could be a better alternative as biofuel from OFMSW, which can be used for blends (E10 or E20) in existing gasoline [12] or flexi-fuel (E100) vehicles [13]. Moreover, bioethanol is a precursor for high-value chemicals [14]. Previous studies focused mostly on the bioethanol potential of single and main components of OFMSW, such as kitchen organic waste (KOW), green organic waste (GOW), and paper and cardboard (PCW). The theoretical yield estimated from these fractions were 363 mL/dkg (kilogram dry weight), 420 mL/dkg and 505 mL/dkg, respectively, but only after the adoption of additional steps of chemical and physical pre-treatment as well as hydrolysis [15]. The OFMSW treated with sulfuric acid at higher temperatures could also be saccharified with cellulolytic enzymes to reach ethanol concentrations of 246 mL/dkg of OFMSW [16]. Another attempt using hydrothermal pre-treatment and amylolytic and cellulolytic enzymes could yield up to 191 g of ethanol per dkg of OFMSW [17]. Verhe et al. [18] used PCW, a cellulose-rich fraction of OFMSW, to produce a final ethanol concentration of 66 g/L at a substrate loading of 40 % (w/w), but this was only after acidic pre-treatment and costly enzymatic saccharification of the PCW.

Starch and cellulose are the most important components for energy production from OFMSW using a biotechnological approach [19]. Mahmoodi et al. [17] used amylolytic and cellulolytic enzymes to achieve high glucose yields of 520 g/dkg of OFMSW, followed by sequential bioethanol and methane production. Cellulose is thus considered a major source of sugars to produce ethanol from OFMSW. By pre-treating OFMSW with cellulases, 66 g/L ethanol was produced in 57 h using a 2 m^3 fermenter [18].

Along with OFMSW, agro-industrial waste streams obtained from rice mills [20–22] can be adopted to potentially enrich the carbohydrate content of OFMSW. In this research, indeed, the close proximity of both an OFMSW-treating plant and a rice mill was exploited towards bioethanol production. DR, a starchy waste from the rice milling industry, was therefore selected as a promising substrate co-processed with OFSMW into ethanol. DR being a starchy rice waste stream, usually goes to animal feed. While with its reasonably high starch content and 7.5 Tg (Teragram) of availability worldwide, it could be utilized for production of biofuels. If this amount would be used for bioethanol, its biofuel potential would be of almost 3 Tg [23].

This study specifically focused on the one-step conversion of OFMSW to ethanol in a CBP approach using the amylolytic ER T12.7 strain (based on the industrial Ethanol Red^{TM} yeast) [24]. In the CBP approach, the amylolytic enzyme production, saccharification, and ethanol production occurs in a single fermenter reducing the cost of fermentation and making the process industrially feasible [25,26]. Specific efforts were spent on testing the bread and pasta (BP) fraction of OFSMW, which represents a particular waste stream mostly found in Europe [27,28], and a different composition of OFMSW to mimic the large seasonal variability already reported in the literature [9]. Furthermore, this work evaluated the potential to supplement OFMSW with DR to improve ethanol yields and showcase the advantage of simultaneous conversion of multiple waste streams to ethanol using recombinant industrial yeast strains.

2. Methods and materials

2.1. Yeast strains and growth conditions

Two *Saccharomyces cerevisiae* strains, the industrial yeast EthanoRed^{TM} (ER V1) and the recombinant amylolytic CBP yeast ER T12.7, previously developed by Cripwell et al. [24] on a ERV1-chassis by simultaneously expressing the native α-amylase (*temA*) and the codon-optimized glucoamylase gene (*temG_Opt*) of *Talaromyces emersonii* were adopted in this study. Strains were maintained in 20 % glycerol stocks at −80 °C and routinely plated on YPD agar (g/L: yeast extract 10, peptone 20, glucose 20, agar 15) and incubated at 30 °C for 48 h. Pre-inoculums were prepared by inoculating a single colony in YPD broth and culturing the strains for 72 h. All the media were sterilized by autoclaving at 121 °C for 20 min before plating.

2.2. Characterization of OFMSW and discolored rice

The sample of OFMSW was collected in June 2021 from the waste reception area of an AD plant of organic waste in Este, Italy. The OFMSW delivered at the plant was source-segregated at a household level, and the collection area involved a population of approximately 150,000 inhabitants. About 100 kg of OFMSW was manually sorted and divided in the following fractions: fruit and vegetable (FV); meat, fish and cheese (MFC); BP; shells and bones (SB), paper, rejected materials (R) and undersieve 20 mm (U). Plastics, plastic bags, metals and glass were classified as rejected materials. The results of the manual sorting procedure are reported in Table 1.

Using the sorted fractions, a composition of organic waste was prepared by maintaining the same proportion of the single fractions as given in Table 1 without the R fraction. After mixing and grinding this composition, a slurry was obtained from the same AD facility, referred to as OFMSW (Fig. 1A). Additionally, the BP fraction was collected separately to simulate seasonal variation in the starch fraction, as described by Alibardi et al. [9]. The OFMSW and BP samples were stored at −20 °C until further use. The rice-milling waste stream, DR was obtained from a milling plant near Este, dried in a forced-air oven at 60 °C for 48 h, milled in a hammer mill and then sieved through a 1.25 mm screen. The chemical composition of OFMSW, BP and DR was determined according to international standards [29] and is reported in Fig. 1B.

2.3. Fermentation experiments

In general, OFMSW is quite viscous and has a lot of particulate matter that also includes partially milled solids like leaves, seeds, rinds of fruits, etc. This particulate matter makes the slurry non-homogenized and difficult to mix. Therefore, before the recombinant strain could be evaluated, a suitable substrate loading of the OFMSW needed to be determined. This was achieved using small-scale fermentation settings; different substrate loadings were tested (5, 7.5, 10, 12.5, 15 % dry w/v), and the substrate loading of 7.5 % dry w/v (dw/v) was found to be efficient for mixing by using magnetic stirrers (data not shown). This loading aligns well with the values usually adopted for full-scale AD applications [7,20,30,31].

The *S. cerevisiae* strains were inoculated in 200 mL YPD culture medium in 500 mL Erlenmeyer flasks and incubated on a rotary shaker (30 °C) at 150 rpm for 72 h. Small-scale fermentation experiments were conducted in 120-mL serum bottles containing 100 mL of fermentation medium. Once autoclaved (121 °C, 15 min), the different substrates were used singly or in combinations with different substrate loadings.

Briefly, 7.5 % dw/v (dry w/v) was adopted for OFMSW, BP and enriched OFMSW (OFMSW supplemented with BP to simulate the winter OFMSW composition, where BP can account for up to 15 % of the wet OFMSW [9]). A loading of 10 % dw/v was adopted for DR, while 17.5 % dw/v was used when combining 10 % dw/v of DR and 7.5 % dw/v of the enriched OFMSW. A 7.5 % dw/v was determined as the optimum

Table 1
Fractions of OFMSW: manual sorting and segregation.

Fraction	Weight (kg)	Percentage
Fruit and vegetables (FV)	53.4	55.5
Meat, fish, cheese (MFC)	3.8	3.9
Bread and pasta (BP)	5.2	5.4
Undersieve (20 mm) (U)	13.2	13.7
Paper (P)	10.4	10.8
Shells and bones (SB)	1.2	1.2
Rejected materials (R)	7	7.3
Total	96.3	100

(b)

Feedstock	Starch	Cellulose	Hemicellulose	Lignin	Protein	Lipid	Ash
				(% dw)			
OFMSW	3.53±6.2	10.19±5.9	7.27±8.2	5.91±7.4	25.05±7.2	2.85±8.1	31.44±4.8
Enriched OFMSW[a]	10.65±5.9	8.55±7.2	6.47±3.9	5.00±6.3	22.89±8.1	3.19±3.5	28.23±6.9
BP	39.62±8.6	0.43±7.2	9.74±4.8	0.91±7.5	15.44±7.1	14.20±8.2	2.69±4.6
DR	84.60±8.4	0.10±8.6	0.90±9.1	-	8.0±6.8	-	0.50±3.8

[a]OFMSW was enriched with 15% (w/w) BP to increase its starch content to levels typical of OFMSW collected in winter

Fig. 1. Origin and composition of the bioethanol feedstocks used in this study. (a) Composition of OFMSW and enriched OFMSW and origin of discolored rice (DR). (b) Chemical composition of OFMSW, Enriched OFMSW, bread and pasta (BP) and DR.

loading after several trials were conducted; this was completed to reduce the substrate's viscosity, which hampers the fermentation rheology. The fermentation experiments were carried out for 72 h for all the substrates except DR and combination of enriched OFMSW and DR wherein the time was prolonged for 96 h.

All the experiments were carried out in triplicate, and bottles were inoculated with 10 % (v/v) pre-inoculum. A needle was inserted through the rubber stopper of fermentation bottles for CO_2 removal, from the start of fermentation. The fermentation experiments were performed under oxygen-limited conditions. Samples (2 mL), taken daily throughout the fermentation, were kept at − 20 °C for future high-performance liquid chromatography (HPLC) quantification of glucose, maltose, ethanol, glycerol, acetic acid, and other volatile fatty acids (VFAs).

2.4. Analytical methods, calculations and statistical analysis

Fermentation samples were thawed and centrifuged at 11000 × g for 10 min and filtered through a 0.22 μm filter before HPLC analysis, which was performed using a Shimadzu Nexera HPLC system equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The chromatographic resolution was achieved using a Phenomenex Rezex ROA-Organic Acid H+ (8 %) column (300 mm × 7.8 mm). The column temperature was set at 60 °C, and the analysis was performed at a flow rate of 0.6 mL/min using isocratic elution, with 5 mM H_2SO_4 as the mobile phase [32]. Ultra violet (UV) detector was used for VFAs analysis The concentrations were calculated by plotting calibration curves of external standards.

The ethanol yield, or the amount of ethanol produced from starch available in the substrate (g/g), was determined considering the quantity of starch available during the fermentation and compared to the maximum theoretical yield of 0.56 g of ethanol/g of available starch. The volumetric productivity (Q) was computed as grams of produced ethanol per liter of fermentation medium per hour (g/L/h), and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity exhibited by the *S. cerevisiae* strains. Statistical analyses were performed using the GraphPad Prism 5 package (GraphPad Software, Inc., San Diego, California). Descriptive statistics, mean values and standard deviations were computed. Data were analyzed with two-way factorial ANOVA (Analysis of Variance) using the Duncan test.

3. Results and discussion

3.1. OFMSW sampling and composition

A total of 96.3 Kg of OFMSW was collected in June 2021 from a full-scale AD plant and sorted manually (Table 1). Within the sample, 89.3 Kg was compostable materials, while 7 Kg was composed of plastic bags, metals, rubber, etc. Considering the biodegradable fraction, FV amounted to 55.5 % (w/w), while starchy components such as BP amounted to only 5.4 % (w/w) of the total collected sample. Considering all the fermentable components, the MFC represented the smallest fraction of the total OFMSW sample (Table 1). The U fraction was similar to the content already reported in the literature [11], where U ranges are between 18 and 22 % [9].

A similar OFMSW composition was reported in the case of food-waste collected in summer [9]. The same Authors indeed deeply investigated the composition of OFMSW over time and found that, as an example, the BP contribution of OFMSW varied between 7.7 and 1.3 % (w/w) for May and June, respectively [9]. Data collected by Hanc et al. [33] from urban settlements presented 58.2 % (w/w) FV fraction in OFMSW, and similar numbers (around 43 % w/w) were previously reported [11]. Hence, it is evident that the FV fraction constitutes a major contribution in OFMSW. Overall, it is also important to note that the composition of OFMSW from different countries showed diverse compositions depending on feeding habits, ways of segregation, and seasons [34].

Chemical analysis of OFMSW confirmed that the occurrence of macromolecules was strictly linked to the shares of OFMSW fractions (Fig. 1). The OFMSW collected and analyzed in this study showed the presence of 25 % protein, as also reported by Magdalena et al. [35], while much lower (about 8 %) protein components were described in two different reports [16,17]. The range of the protein component in three different mixtures tested by Alibardi et al. [9] was 15–17 %. The highest fermentable component per dry weight was protein in selected fractions. For example, the separately collected and analyzed MFC and vegetable fractions showed 52 and 11 % proteins, respectively, while the fruit fraction only contained 3 % [10]. A noteworthy and recent review compiled a big number of OFMSW chemical compositions and resulted in the following range (% Total solids): starch (11.7–56.5), cellulose (3.2–49.0), hemicellulose (1.8–16.0), lignin (1.8–29.1), protein (6.8–25.8), lipid (5.6–24.7) [34]. Lower concentrations of cellulose (10.19 %) and hemicellulose (7.27 %) were detected in the OFMSW (Fig. 1), as the FV fraction was the highest (Table 1) compared to the other fractions. Starch was the least abundant polysaccharide present in the tested OFMSW obtained in this study, although this fraction is strongly influenced by the season during which it was sampled. Interestingly, almost 37 % of dw was made of non-fermentable fractions such as lignin and ash (Fig. 1B). Overall, at least 49 % dw of the sampled OFMSW can be exploited to obtain biofuels.

As expected, the composition of the BP fraction was hugely different from OFMSW (Fig. 1B). The most abundant polysaccharide was starch, with much lower amounts of hemicellulose and a very limited quantity of cellulose. Protein composition in the BP fraction contributed 15.44 % dw, in agreement with other BP fractions reported by Alibardi et al. [10]. As predicted, DR was characterized by the highest starch content and limited amounts of hemicellulose and cellulose, with values similar to those recently reported [23,36]. Thus making DR an attractive substarte to target using an amylolytic yeast.

3.2. Fermentation of OFMSW

Ideally, substrates used for bioethanol production would be fermented without a need for any exogenous enzyme addition via a CBP approach [25,37–39]. The same should also apply in the case of processing OFMSW. Nevertheless, so far, CBP approaches have not been adopted for the conversion of OFMSW to bioethanol whereas only separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) setups with [16,17] or without [35] OFMSW pre-treatments were indeed adopted and showed limited efficiency. This paper specifically focused on a CBP approach to process OFMSW, relying on the amylolytic ER T12.7 strain recently reported for its improved ethanol productivity during starch-to-ethanol fermentations. As an alternative feedstock, OFMSW can be rich in starch, varying from 2 to 56.5 % dw [34]. Moreover, significant seasonal fluctuations were detected, with winter having the highest values and summer having the lowest [9]. Therefore, using the recombinant amylolytic strain ER12.7, which produces extracellular amylases, would facilitate higher concentrations of fermentable sugars from the starch fraction, thus improving the productivity of ethanol production in commercial setups.

The recombinant ER T12.7 strain was then evaluated for the one-step production of ethanol from 7.5 % dw of OFMSW and the BP fraction (Fig. 2 and Table 2). The ER V1 strain was included as a non-recombinant benchmark for the fermentation of OFMSW.

From OFMSW, the recombinant yeast produced up to 6.4 g/L of ethanol with complete starch depletion and the available simple sugars (Fig. 2). This was significantly better than the fermenting performance of ER V1; the parental yeast only produced 3.9 g/L ethanol from the monosaccharides in the OFMSW. As reported in Table 2, ethanol productivity of the CBP yeast was almost 1.6-fold that of the parental with Q_{max} values even higher (1.9-fold), further supporting the significant improvement achieved by adopting the recombinant strain for processing OFSMW to ethanol.

In the case of BP (7.5 % dw/v), the recombinant ER T12.7 yeast readily processed starch into ethanol with levels of about 25 g/L observed after 48 h of fermentation (Fig. 2) with complete hydrolysis and consumption of the starch available in the feedstock (Table 3). On the contrary, the benchmark parental ER V1 strain only produced up to 6.7 g/L ethanol, consuming all the free monosaccharides available in the feedstock, but not converting the starch component to ethanol. The recombinant strain once again outperformed the parental yeast in terms of ethanol productivities: both final (0.32 g/h/L) and maximum volumetric (0.81 g/h/L) parameters were 3.5-fold higher than those of the benchmark yeast with a sharp increase compared to the performances obtained from OFMSW (Table 2).

Considering the data of Figs. 1, 2 and Tables 2 and 3, starch content

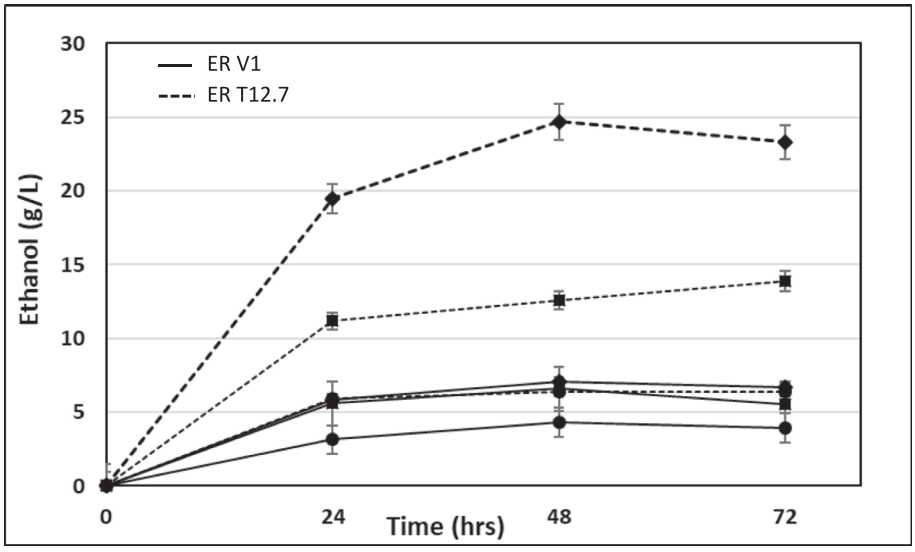

Fig. 2. Ethanol production during the fermentation of OFMSW, enriched OFMSW, and BP by *S. cerevisiae* ER T12.7 and ER V1. BP- Bread and pasta, OFMSW (●), enriched OFMSW (■), BP (◆). Continuous lines and dashed lines represent ethanol production by the parental (ER V1) and recombinant (ER T12.7) strain, respectively. The experiments were performed using 7.5% dw for each substrate in triplicate, and error bars represent the standard deviation from the means of replicates.

Table 2
Conversion of OFMSW, BP and DR to ethanol and other byproducts, separately or in combination, using *S. cerevisiae* parental strain ER V1 and its recombinant ER T12.7, after 72 or 96 h.

Time (h)	72						96			
Product (g/L)	OFMSW		BP		Enriched OFMSW [a]		DR		Enriched OFMSW + DR	
	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7
Glucose	0.07 ± 0.02	0.38 ± 0.09	0	0.12 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.30 ± 0.02	0.27 ± 0.02	0.18 ± 0.01	2.39 ± 0.16
Maltose	0.07 ± 0.01	0.24 ± 0.05	0.11 ± 0.02	0.65 ± 0.01	0.12 ± 0.01	0.78 ± 0.04	0.08 ± 0.005	0.87 ± 0.05	0.22 ± 0.01	0.64 ± 0.04
Glycerol	0.38 ± 0.03	0.49 ± 0.02	0.20 ± 0.18	2.62 ± 0.04	0.92 ± 0.06	1.07 ± 0.07	1.21 ± 0.07	4.02 ± 0.23	0.97 ± 0.07	4.85 ± 0.31
Ethanol	3.95 ± 0.15	6.39 ± 0.45	6.68 ± 0.44	23.30 ± 0.55	5.58 ± 0.31	11.28 ± 0.76	7.10 ± 0.47	53.18 ± 2.56	12.05 ± 0.47	66.22 ± 3.43
Ethanol yield [a]		100 ± 7		100 ± 1		100 ± 1		97 ± 3		96 ± 3
***Q* (g/L/h)**	0.05	0.09	0.09	0.32	0.08	0.19	0.07	0.55	0.13	0.69
***Q_{max}* (g/L/h)[b]**	0.13	0.25	0.24	0.81	0.23	0.47	0.17	1.22	0.27	1.51

BP- bread and pasta, DR- discolored rice, Q- ethanol productivity, Q_{max}- maximum productivity, [a]- Ethanol yield (% of the theoretical yield) was calculated as the amount of ethanol produced per gram of available glucose. To calculate the yield of ethanol from starch, the ethanol production by non-amylolytic ER V1 was subtracted from that of ER T12.7, [b]- Q_{max} was detected after 24 h, The experiments were performed in triplicate and the standard deviation obtained from the means of replicates.

Table 3
Initial and final concentrations of glucose and starch detected in the substrates fermented by the parental yeast ER V1 or the recombinant strain ER T12.7.

Feedstock	ER V1				ER T12.7			
	Glucose (g/L)		Starch (g/L)		Glucose (g/L)		Starch (g/L)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
OFMSW	7.78 ± 0.61	0.07 ± 0.02	4.35 ± 0.21	3.69 ± 0.80	7.68 ± 0.43	0.38 ± 0.09	4.22 ± 0.25	–
Enriched OFMSW	10.98 ± 0.42	0.19 ± 0.01	10.18 ± 0.43	9.51 ± 0.35	10.81 ± 0.34	0.18 ± 0.01	10.33 ± 0.51	–
BP	13.10 ± 0.57	–	29.67 ± 0.62	28.80 ± 0.51	13.42 ± 0.51	0.12 ± 0.01	29.89 ± 0.57	–
DR	14.01 ± 0.28	0.30 ± 0.02	84.83 ± 1.59	83.49 ± 0.65	14.23 ± 0.21	0.27 ± 0.02	83.23 ± 1.17	–
Enriched OFMSW + DR	23.71 ± 0.53	0.18 ± 0.01	100.77 ± 3.12	99.09 ± 1.79	24.01 ± 0.33	2.39 ± 0.16	99.98 ± 2.48	–

-, not detected.

was the limiting factor for ER T12.7 in terms of ethanol production. Although, the higher starch content found in BP greatly improved ethanol production by the recombinant yeast. To access the promise of ER T12.7 for the conversion of OFMSW samples typical of winter seasons, where BP shares can account for up to 8–15 % of wet organic waste [9,40], the OFMSW sampled at the waste management plant (referred to as OFMSW) was specifically supplemented with 15 % (w/w) of the BP fraction. This new composition was defined as enriched OFMSW. As such, the resulting feedstock was fortified in its starch content with slightly lower quantities of cellulose, protein and ash (Fig. 1B).

As reported in Fig. 2, the recombinant ER T12.7 strain took advantage of the increased starch availability in the enriched OFMSW substrate with final ethanol production of about 13.9 g/L after 72 h, which was 2.5-fold higher than the parental *S. cerevisiae* ER V1 strain. The final productivity of the recombinant yeast was 0.19 g/L/h, and Q_{max} approached 0.47 g/L/h after 24 h of fermentation. Both values were again much higher than those detected for the parental yeast, indicating that the recombinant strain has great promise for converting different seasonal compositions of OFMSW into ethanol.

The ER T12.7 strain consistently produced higher levels of ethanol during CBP on all tested substrates compared to those produced by the parental yeast (Fig. 2). The low residual glucose (Table 3) and maltose concentrations (Table 2) in the fermentation broth indicated a rapid sugar uptake by the recombinant strain. Moreover, limited glycerol concentrations were detected, suggesting that the carbon metabolism was mainly directed to ethanol production (Table 2).The exceptional performance of the recombinant ER T12.7 strain is especially promising, considering the high content of VFA available in the various tested systems. These inhibitory concentrations (1 and 4 g/L of formic and acetic acid, respectively) were previously reported in recently published studies [41] as hampering the growth and fermenting activities of many *S. cerevisiae* strains during the processing of OFMSW [42] and other pre-treated lignocellulosic materials [23,43,44]. When the fermentation setups containing OFMSW were analyzed for VFAs content, high shares of acetic, propionic, and butyric acid were observed (Fig. 3). Acetic acid had the highest concentration. On the contrary, the experimental set up with only BP showed very low VFAs (acetic, propionic and heptanoic acid were 0.54, 0.16, 0.11 g/L, respectively). Such low levels can be ascribed to starch being one of the most recalcitrance polymers in OFSMW, and the microbial conversion of starchy components in VFAs can require additional time. Considering the high content of VFAs in the OFMSW fermentation bottles, the ethanol levels achieved in this proof-of-concept study on OFMSW fermentations could be further enhanced by using higher substrate loadings and upscaled experiments.

Low production levels of bioethanol have been reported without treating OFMSW [45,46]. Even after pre-treating OFMSW and following a hydrolysis step, the ethanol levels were still limited to 8.32 g/L with a final volumetric productivity of only 0.17 g/L/h [47]. Slightly higher ethanol levels and fermentation performance were obtained by adopting the parental yeast ER V1 on OFMSW, but still required hydrothermal pre-treatment and excessive amounts of costly exogenous cellulases and amylases [48]. Ethanol levels similar to those obtained in this work (up to 23.3 g/L) were obtained from the hydrolysate of kitchen waste only after acidic pre-treatment (sulfuric acid, 60 °C, 3 h) and/or enzymatic hydrolysis with both expensive commercial amylolytic and cellulolytic enzymes [49].

Generally, the volumetric productivity of ethanol for both ER T12.7 and the parental ER V1, reached its maximum at 24 h of fermentation (Table 2). As far as OFMSW enriched with BP is concerned, the analysis of this raw material was important to be able to evaluate the different yields obtained based on the seasonal changes; in fact, values have been obtained which are higher than those of OFMSW and lower than BP

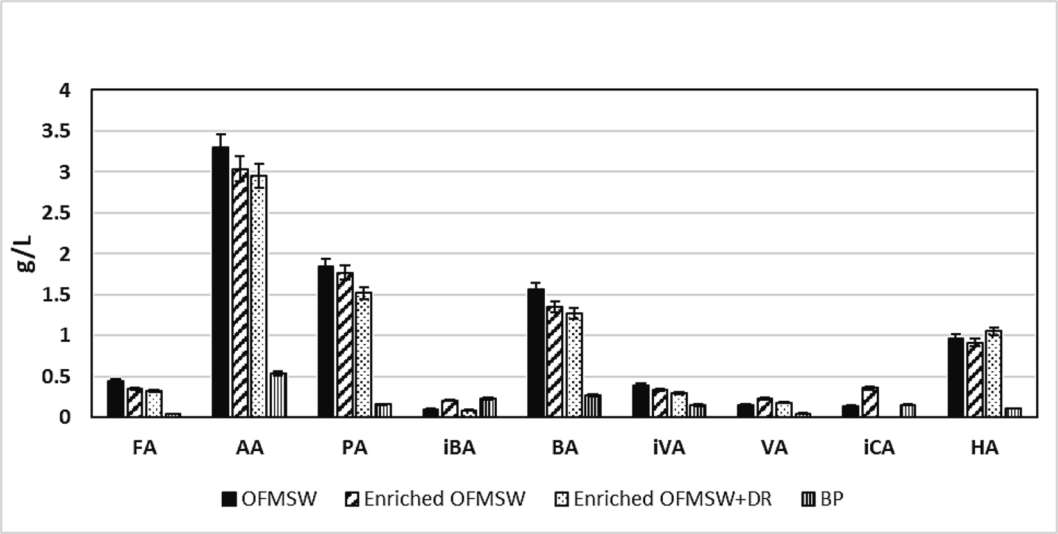

Fig. 3. Initial concentration of VFAs at different fermentation set ups. FA-formic acid, AA-acetic acid, PA-propionic acid, iBA-isobutyric acid, BA-butyric acid, iVA-isovaleric acid, VA-valeric acid, iCA-isocaproic acid, HA-heptanoic acid. The experiments were performed in triplicate and error bars represent the standard deviation from the means of replicates.

(Fig. 1). The recombinant strain also achieved high fermentative ability when BP was used as substrate: from the 29.7 g/L available starch in the BP fraction, 16.6 g/L ethanol (after subtracting ethanol produced by ER V1) was produced, corresponding to 100 % of the theoretical yield (Table 2).

3.3. Co-processing of DR and OFMSW during CBP

The OFMSW was efficiently converted into ethanol by the *S. cerevisiae* ER T12.7 strain. Nevertheless, the final ethanol concentrations are not yet suitable for any industrial development of the technology. To further improve the overall process viability, the OFMSW composition suggested the need for a CBP yeast capable of hydrolyzing cellulose and, possibly, hemicellulose, which should both form a significant fraction of OFMSW worldwide [34]. Further development of CBP yeast strains is required to co-express cellulases and amylases. This would enable more of the available carbon in OFMSW to be exploited for bioethanol production. Alternatively, ethanol levels can be readily enhanced by adopting the efficient amylolytic CBP yeast in the co-processing of OFMSW and a starch-rich byproduct.

Since there is a rice-milling plant near the MSW management plant, DR, a byproduct of the rice milling process, was selected as a suitable feedstock to supplement OFMSW. Furthermore, it is well suited for this purpose owing to its high starch content (Fig. 1B) and its outstanding global bioethanol production potential of 2.9 Tg from the annual 7.5 Tg of DR produced. In this study, DR was subsequently adopted to enhance the starch content of OFMSW to convert a mixture of two waste substrates into ethanol, which could make bioethanol production more economically viable.

The recombinant yeast strain was tested for the first time on the rice byproduct and displayed great promise from 10 % (dw/v) of DR (Fig. 4, Table 2). The highest ethanol levels were detected after 72 h (53.6 g/L),

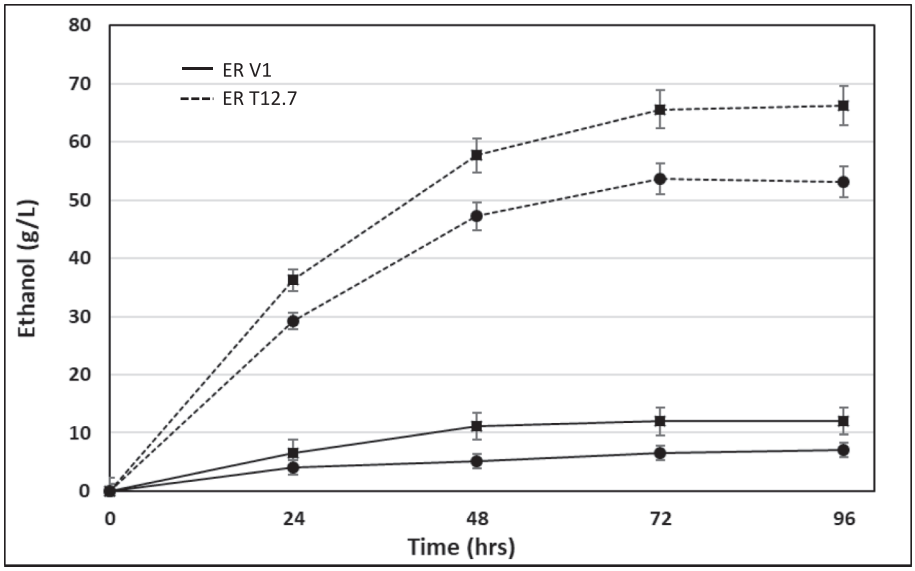

Fig. 4. Ethanol production during fermentation of DR (discolored rice, 10 % dw), enriched OFMSW + DR (17.5 % dw) by *S. cerevisiae* ER T12.7 and ER V1. DR (●), Enriched OFMSW + DR (■). Continuous lines and dashed lines represent ethanol concentrations by parental (ER V1) and recombinant (ER T12.7) strains, respectively. The experiments were performed in triplicate, and error bars represent the standard deviation from the means of replicates.

demonstrating complete starch consumption and an outstanding starch-to-ethanol yield of 99 % of the theoretical maximum. The CBP strain exhibited ethanol productivity of great interest [24] with Q_{max} of 1.22 g/L/h (Table 2) at an industrially accepted level. On the contrary, the parental *S. cerevisiae* ER V1 produced limited amounts of ethanol from the simple sugars available in the broth, and the resulting ethanol performances were very limited. The Q_{max} and final productivity were found to be at least 2-fold less than those of the recombinant yeast and the difference may reach up to 5-folds depending on substrates. Overall, the fermenting ability of the ER T12.7 strain is a hallmark once compared with the literature. DR was previously processed into ethanol at higher substrate loading with an ethanol yield of 88 and 91 % by the *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] strains, respectively, co-expressing other glucoamylase (*TLG1*) and alpha-amylase (*SFA1*) genes [36]. Both strains displayed productivity values similar to those achieved by ER T12.7.

It was evident that substrate loading essentially plays an important role in altering fermentation productivity. As an example, when ER T12, the parental strain to ER T12.7, and M2n T1 were used for broken rice (BR) fermentation where the substrate loading was 20 % dw, the productivities were 0.97 and 0.82 g/L/h after 96 h [50]. In the current study with DR, a 10 % dw substrate loading could reach up to 0.55 g/L/h (Table 2). Different productivities were obtained depending on the substrate. As an example, productivities with ER T12 and ER T12.7 using rice bran (20 % dw), potato waste (10 % dw), and potato peel (10 % dw) could reach up to 0.99, 0.55, 0.31 g/L/h and 1.15, 0.68, 0.38 g/L/h respectively during starch fermentation [24]. This suggests that doubling substrate loading may double the productivity during ethanol fermentation if both rheology and substrate mixing is maintained as optimal. In the case of BR, higher productivities (>1.00 g/L/h) could only be achieved by supplementation with 10 % commercial amylase cocktail [50]. Evidently, substrate loading and substrate variation have a huge effect on altering the productivity in conversion of starch to ethanol using amylolytic recombinant strain of yeast.

The co-processing of both feedstocks, OFMSW and DR, into ethanol was also assessed. The substrate loading of DR was specifically adopted to ensure good mixing conditions once combined with 7.5 % dw/v enriched OFMSW. From 17.5 % dw/v substrate loading, 66.2 g/L ethanol was achieved after 96 h of fermentation. Most of the starch available was consumed within 48 h (data not shown), resulting in a noteworthy Q_{max} of 1.51 g/L/h after 24 h (Table 2), which was even higher than that detected in the sole DR fermentation. This value is very promising as 1 g/L/h is the industrial requirement for ethanol strains [43,51]. Once again, the parental strain confirmed its poor fermenting abilities, with up to 12 g/L ethanol produced most likely from the simple sugars freely available. As such, the recombinant engineered strain demonstrated nearly 3- and 5-times higher ethanol levels and productivity values compared to the parental (Table 2).

Combining both waste substrates was pivotal to boosting ethanol performances and final titers, which was above the industrial threshold of 60 g/L [43]. Moreover, this is the first report on co-processing two waste streams with diverse origin and highly heterogenous compositions into bioethanol. Although further efforts in process optimization and integration are ongoing for the industrial application of ER T12.7 on such feedstocks, this approach will pave the way for future exploitation of different byproducts with various compositions and origins into bioethanol. Moreover, after ethanol production, large part of OFMSW feedstock in the form of carbohydrates, proteins and lipid still remains unused. After distillation of ethanol, this fraction can be further processed for anaerobic digestion which will in turn help to offset the cost of bioethanol production increasing the process sustainability and industrial feasibility [35,52].

Overall, the promising fermenting yield obtained so far at lab-scale should be further assessed during upscaling to test both industrial fitness and saccharifying activity of the recombinant yeast at industrial level. Repeated fermentations at higher scale are likely to further enhance the robustness of the recombinant yeast and, together with substrate loading optimization, should be considered as key experiments towards the large-scale application of this CBP yeast.

4. Conclusion

This paper clearly demonstrates that OFMSW can be beneficially converted into bioethanol by employing a highly efficient amylolytic CBP yeast strain for the near-complete conversion of the starch content in OFMSW. This is the first proof of concept that the co-conversion of DR and OFMSW to bioethanol can greatly help in agro-industrial and domestic waste management with the production of value-added biofuel. Further investigations are needed to increase substrate loading and to make the process continuous, while improving the rheology of the system to increase the final ethanol titers towards industrial development. The co-conversion of OFSMW and DR revealed a promising strategy to achieve higher ethanol levels while reducing the cost of fermentation. Increased ethanol yields make this approach more suitable for industrial application and open novel research routes towards co-processing different industrial byproducts into valuable compounds such as ethanol. Ultimately, this approach could pave the way towards a biorefinery concept to obtain biofuels and other value-added chemicals from mixed organic waste streams from different industries.

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

All the data necessary to reproduce the results is shared in the manuscript

Acknowledgements

This work was funded by Università degli Studi di Padova [BIRD187814/18, BIRD210708/21, DOR1928058/19, DOR2084579/20, DOR208705/20, DOR2107797/21, DOR2114239/21] and by the bilateral joint research project between Italy and South Africa [grants ZA18MO04 and 113134, respectively]. Authors are grateful to S.E.S.A. S.p.A- for providing OFMSW samples.

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