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Ethanol Production from *Schinus molle* Essential Oil Extraction Residues

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Abstract

The present study determines the best conditions for the fermentation of *Schinus molle* drupes by the combination of different types of hydrolysis with the search for an adequate yeast strain. *Schinus molle* seed residues from an essential oil extraction plant (EOEP) have a high potential for ethanol production. Native yeast strains were isolated from the residues and were used to ferment the lignocellulosic residues, along with baker's yeast (*Saccharomyces cerevisiae*) at 30 °C and pH 5.5 for comparison. Morphological and biochemical characterizations were carried out on the isolated yeast strains. Thermogravimetric and high-performance liquid chromatography analyses were done on the *S. molle* seeds (fresh and residue) to determine the ethanol production potential. The followed methodology included increasing the sugar content by hydrolysis with chemical (sulphuric acid, acetic acid, and sodium hydroxide), physical (thermal, vacuum, and ultrasound), and enzymatic treatments (amyloglucosidase and α-amylase). Once the optimum combination of yeast-hydrolysis was determined, a comparison of the greenhouse gas emissions between the original and proposed processes was done. The fermentation of the residues might replace methane from uncontrolled decomposition and reduce the solid residues in 50%/day, hence the EOEP global warming potential is reduced by 47%. The yearly income was estimated to increase by USD 2592.50 from 6302.6 L of ethanol produced from the residues.

Graphic Abstract
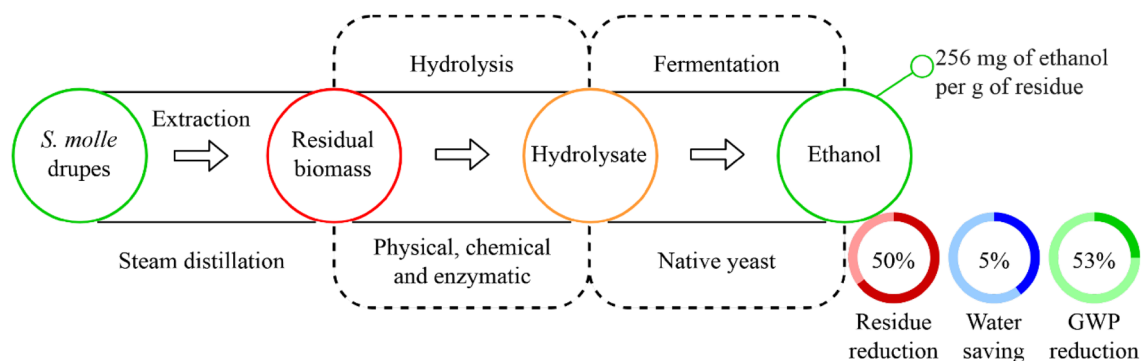
Keywords *Schinus molle* · Essential oil extraction · Ethanol · Native yeast · Waste treatment

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Statement of Novelty

Our research group identified the problem of accumulation of waste from the essential oil extraction units, since the essential oil yields are often low, the produced residue increases considerably. The current research proposes the

decrease of the generated waste by producing ethanol. In order to achieve the ethanol production, several hydrolytic pre-treatments and fermentation conditions were used for a sector that is steadily growing for advancing second generation fuels. An approach in the treatment of lignocellulosic residues is proposed regarding the global warming potential, mass indicators, and additional means of income.

Introduction

Schinus molle is a tree belonging to the *Schinus* genus within the Anacardiaceae family, is prevalent in the semi-valley areas where the ground is moist but well drained [1], and the settled trees are drought resistant with soil tolerances as saline soils. The *S. molle* species are worldwide abundant as they are found as native species as well as anthropogenically inserted. The invasive species compendium from the Centre of Agriculture and Biosciences International (CABI) reports the growth of the plant in at least 40 countries, where the latitude and altitude vary from 25°N to −35°S and 400 to 4200 m, respectively [2]. The principal use of the plant is from the seeds, from which the essential oil is extracted. The dry mature drupes of the species are reported to have an average size of 6.35 mm [1] with an average fatty acid concentration of 5.35 wt% and essential oils concentration of 1.15 wt% [3]. A qualitative description of the drupes' sweetness by Goldstein and Coleman [4] was found but no further quantification of monosaccharides or polysaccharides has been found.

In Bolivia, the main uses of the plant are between ornamental and essential oil extraction. The essential oil extraction starts from the harvesting of the fruits, which are in racemes of drupes with sizes of 4.11 ± 0.44 mm of diameter. Fruits are mechanically peeled and milled until approximately 99.4% are in a size particle of ≤ 850 μm. Once the particle size is fixed, the matter is placed in the distillation tank. Essential oils are extracted by steam distillation, where the essential oils are extracted from the freshly milled seeds. At the end of the process, the distillate is taken to a settler for phase separation while the residual biomass is discarded. In order to produce more than 100 L of essential oil per month, a plant would process 1430 kg of seed feedstock with 5.82% of essential oil content. After the extraction has concluded, the residual biomass is pumped out to the residue pool, while the essential oil is driven to a refining process. The residual biomass treatment consists in pumping the biomass sludge towards a landfill-like pool where it is left accumulating until it is retrieved by third parties for its use as land additive for various local crops.

The high residue output contrasts with the current need for more environment-friendly processes, calling to a proper management of residual biomass. The yearly production of

essential oil at the scale of the processing plant described above derives in estimated 30,000 kg of CO_2 produced from combustion and 1694 kg of greenhouse effect gasses (GHG) by the disposed residual biomass (19,500 kg approx.). In addition to the high output of CO_2 delivered to the atmosphere by the boiler, the production of methane gas add-up to the GHG that increase the negative effects caused by the process residues [5]. The actual management of these residues has a negative impact in the atmosphere, as well as on the land and water. Accounting the negative effects on the land, water, and surrounding vegetation are the biomass degradation leachates which cause eutrophication, e.g., increasing its biochemical oxygen demand (BOD) among other biochemical indicators [6]. On the evidence of the impacts of the produced residue, it is important to have an alternative to reduce the environmental impact. Hence, cleaner energy alternatives are being developed day by day and the available technology makes the lignocellulosic materials [7] to be suitable candidates for the production of second generation biofuels, e.g., ethanol from wood residues. The production of second generation ethanol has been studied from olive oil residues [8], spent coffee residues [9], rice straw [10], pineapple leaf and waste [11], wheat straw [12], sweet sorghum stalks [13], corn stover, lignocellulosic feedstocks [14], and even industrial orange residues [15].

The implementation of second generation biofuel processes within established processes is a proven strategy to increase the feedstock exploitation and decrease the residues [16]. The residues of the contemplated process constituted by the *S. molle* seeds, which at sight resemble a core heavily protected by a packed shell of hardwood [3]. Thus, the content of holocellulose would be higher than other second generation feedstock [17]. The microorganism activity found in the residue pool by the presence of bubbles and temperature higher than the ambient temperature proves that the nutrient levels are enough for the microorganism activity. The present study proposes the valorisation of the *S. molle* residues through a biotechnological mind-set, in addition to reduce the environmental impact of the essential oil extraction process (EOEP).

Materials and Methods

Materials

The *Schinus molle* mature drupes were collected from regional providers (farmers) in Cochabamba, Bolivia. The drupes were peeled and the resulting seeds were crushed in a plaque mill to have a particle size < 850 μm. The samples were stored at 4 °C in a sealed vessel and named "fresh sample" (FS) until further use. After the EOEP, the bagasse formed from the crushed seeds and water vapour

was collected. The bagasse was then dried for 48 h at 60 °C in shallow trays avoiding any thermally activated reaction. After the bagasse was completely dried, it was ground to a size particle of < 500 μm. The biomass was stored in a sealed vessel and named "residue sample" (RS) until it was used in the experiment. The characterization of the biomass moisture, volatile matter, ash content, and fixed carbon was done via a Netzsch STA 449F3 thermogravimetric analyses (TGA) equipment.

The reagents used for the chemical hydrolysis were concentrated sulphuric acid (95-98% reagent grade, Sigma-Aldrich), sodium hydroxide (≥ 97.0% ACS reagent, Sigma-Aldrich), hydrochloric acid (35–37% AR, Sigma-Aldrich), α-amylase from *A. oryzae* (> 800 FAU/g, Fungamyl), amyloglucosidase from *A. niger* (> 260 U/mL, Sigma-Aldrich), and deionized water (DIW). The neutralization of the solutions was done with 1 M solutions of HCl and NaOH. The evaluation of each hydrolysate monosaccharide content was performed with a Merck-Hitachi LaChrom HPLC system coupled with a D-7200 auto sampler. The column installed is Aminex HPX-87C 300×7.8 mm, with degassed DIW as the mobile phase. The injection method was in loop mode with a volume of 20 μL, at a constant temperature of 80 °C. The volumetric flow was constant at 0.6 mL/min. The detector used in the system was an IR 16× detector. The calibration curves were done with cellobiose, glucose, xylose, galactose, arabinose, and mannose. After the hydrolysis, each sample was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through 0.45 μm PTFE syringe filters. The next step was to dilute the samples in a 1:10 volumetric ratio with DIW to be placed in the HPLC auto sampler sample tray.

The fermentation process required 5 g suspended biomass in 35 mL of DIW with the addition of calcium hydroxide (> 95%, Sigma Aldrich) with the pH fixed at 5.5. The ethanol content after the fermentation was measured via GC analyses. The equipment used was an Agilent GC 6890 using HP-FFAP polyethylene glycol capillary column (30 m × 530 μm × I.D. 1 μm) at an oven temperature of 215 °C with helium as the carrier phase at a volumetric flow of 7.2 mL/min and a split ratio of 80:1. The equipment has a flame ionization detector (FID) working at 300 °C. Calibration curve was determined with 0.1, 1.0, 2.0, 3.0, and 10.0 vol%. The sample preparation for the GC analysis consisted in centrifugation of the samples to obtain only the supernatant, which was filtered through a 0.45 μm PTFE syringe filter and placed in the analysis tray.

Characterization of the *S. molle* Feedstock

The FS and RS samples were analysed by thermogravimetric analysis based on modified standards for moisture, ash content (ASTM E1755-01), volatile matter (ASTM E872-82),

and proximal fixed carbon determinations. The modification was performed in terms of the initial mass, which was reduced to fit the equipment capacity. The moisture content was determined placing a crucible with 30 mg of the samples (FS and RS) and carrying out the temperature increase up to 105 °C for 24 h. Once it was proven that the mass remained constant, the weight of the samples was recorded and used for the further calculations. Afterwards, the samples were taken to the TGA equipment to undergo through the ash determination program as described in Table 1 under synthetic air atmosphere (N_2/O_2). The volatile matter procedure is also listed in the Table 1, except that the atmosphere was N_2 only.

The fixed carbon was determined by Eq. 1, where m_{FC} stands for fixed carbon mass percentage, m_M stands for moisture, m_{VM} stands for volatile matter, and m_A stands for ash content.

$$m_{FC} = 100 - \left(m_M + m_{VM} + m_A \right). \tag{1}$$

Main Monosaccharides and Klason Lignin Determination

The monosaccharide profile of the *S. molle* seeds was determined with the standard SCAN-CM 71:09. The standard focuses in the determination of the content of five monosaccharides including glucose, mannose, galactose, arabinose, and xylose. Additionally, the method is complemented by Klason lignin determination by a gravimetric method. The extractives were calculated by proximity from the difference between the total biomass and the determined monosaccharides, Klason lignin, and ashes.

Hydrolysis of *S. molle* Biomass

The chemical hydrolyses were done with sulphuric acid, acetic acid and sodium hydroxide separately against a blank with water only. Additionally, since the standard for sugar quantification uses a vacuum followed by thermal treatment such treatments were tested with the chemicals afore mentioned. The hydrolysis started with 200 mg of RS sample

Table 1 TGA temperature program for *S. molle* biomass characterization

Step	Rate (°C/min)	Time (min)	Temperature (°C)
1	5	–	220
2	–	30	220
3	5	–	550
4	–	120	550
5	5	–	900
6	–	7	900

Table 2 Chemical hydrolysis experiments

Hydrolysis	Compound	Vacuum	Autoclave
1	H_2O	+	+
2	H_2O	+	−
3	H_2O	−	+
4	H_2SO_4	+	+
5	H_2SO_4	+	−
6	H_2SO_4	−	+
7	NaOH	+	+
8	NaOH	+	−
9	NaOH	−	+
10	CH_3COOH	+	+
11	CH_3COOH	+	−
12	CH_3COOH	−	+

Table 3 Enzymatic hydrolysis experiments

Enzyme	Temperature (°C)	pH	Volume (μL)
Amyloglucosidase	70	5.5	427
Amyloglucosidase	70	5.5	1700
α-Amylase	55	4	10
α-Amylase	55	4	40

with the addition of 3 mL of the hydrolytic compound at a concentration of 12.1 M following the treatments detailed in Table 2.

After the hydrolysis was carried out, the mixture was levelled up to 35 mL of DIW to a pH of 7 with 1 M of HCl and 1 M of NaOH and filtered through 0.45 μm PTFE syringe filter to fix the solutions at the HPLC operating conditions. A diaphragm pump with 9.0 mbar of vacuum capacity was used for the vacuum treatments, while an autoclave that reach a temperature of 121 °C with a pressure of 1.2 bar was used for the thermal treatments.

An additional set of experiments were performed using ultrasound as it was reported that the pressure differences and the production of free radicals enhance the hydrolysis of lignocellulosic biomass [18, 19]. The ultrasound was applied using a Sonics VC505 equipment with a 11 mm probe with 20 kHz at 40% of power in pulses of 1–2, i.e. 1 s of ultrasound pulse every 2 s of pause. The samples were prepared the same way as before, 3 mL of the 12 M H_2SO_4 added to 200 mg of RS with ultrasonic treatment for 1 h inside a water bath. The same experiments were repeated with 12.1 M NaOH and 12 M CH_3COOH and water only samples.

The hydrolysis experiments were completed adding hydrolytic enzymes, such as α-amylase and amyloglucosidase. Since the commercially available enzymes have high activity and concentration, 5 g of biomass were treated with enzymes. The preparation consisted in suspending 5 g of the RS biomass in 35 mL of DIW, supplemented with 20 mg of $Ca(OH)_2$ to ensure the bimetallic presence for the enzyme activity. The optimal hydrolysis conditions of each enzyme were used in the treatment of the *S. molle* residual biomass (RS). The pH and temperature were adjusted accordingly to each enzyme optimum as listed in Table 3. The volume of used enzyme was calculated considering the enzymatic activity of each enzyme, 260 UI/mL and 800 FAU/g for amyloglucosidase and α-amylase, respectively. Additionally,

a 4× enzyme excess was planned to assess the maximum hydrolysable matter in RS.

After the enzymatic hydrolysis ended, the enzymes were denaturalized by thermal treatment in a boiling water bath. The samples were fixed to a pH of 7 and filtered through a 0.45 μm PTFE syringe filter for its analysis in the HPLC equipment.

Saccharomyces cerevisiae and Native Yeast Strains

The first strain was obtained from the local supplier as bakers' yeast *Saccharomyces cerevisiae*. The isolation procedure started by taking samples from the sludge at the end of the EOEP, specifically from the land-fill like disposal area, which expels a characteristic odour of biomass decomposition with the presence of bubbles on the surface. Such samples were added to a laboratory flask with YPD liquid culture media. The native yeast strains grew on YPD culture broth, i.e., yeast extract (1 wt%), peptone (2 wt%), and dextrose (5 wt%) suspended in DIW. In a second round of isolation procedure, the YPD culture broth was supplemented with 5 vol% of ethanol. Further maintenance of the strains was carried by the use of solid culture media in petri dishes with the YPD culture media supplemented with 2 wt% of agar. Once the strains were isolated the identification and characterization were done. The morphological characterization was performed, including Gram test and yeast strain colony characterization. On the other hand, the biochemical characterization was done via bioMérieux API 20E and Remel RapID yeast plus identification kits.

Fermentation Base Conditions

Yeast strains were grown in YPD liquid culture medium to determine their growth curve. The culture medium was adjusted to pH 5.5 at 24 °C with 200 rpm of agitation. The growth rates were determined by the optical density in aliquots of 1.5 mL that were taken from the 4th hour continuously every 4 h until 24 h were completed. Afterwards, the yeast was inoculated to the fermentation of the *S. molle* residues. The starting fermentation conditions were established using commercial yeast and the isolated yeast as described in Table 4. A Biochrom LibraS22 UV–Vis spectrophotometer

Table 4 Fermentation base conditions

Conditions	Unit	Value
S. molle matter	g	5
Yeast	mg/g molle	0.35
Distilled H_2O	mL	35
$Ca(OH)_2$	mg	20
Time	h	24
Temperature	°C	30

was used to measure the optical density of the samples at 600 nm of wavelength. The fermentable matter (FS or RS), yeast content, distilled water, $Ca(OH)_2$, and temperature were constant in this experiments.

As the enzymatic treatment requires the addition of calcium, 20 mg of $Ca(OH)_2$ was added to all the experiments in order to null its effect. The reactor temperature was kept as reported by Tehrani et al. [9]. The reaction time was the first variable to study, where the aliquots were taken at 6, 12, 18, 24 and 36 h. Due to the possible stringency within the *S. molle* seeds' components, the yeast amount reached 70 mg of yeast per gram of biomass. Additionally, the prepared culture broth was not autoclaved to maintain environmental conditions, i.e., the process require less energy. The initial measurement of the fermentation was evaluated with HPLC, having a comprehensible monosaccharide initial profile for each experiment. The results of the fermentation, monosaccharide and ethanol content, were measured by GC-FID and HPLC, respectively.

Fermentation of the Hydrolysates with Nutritional Additives

The fermentations were carried out using the hydrolysate (highest monosaccharide concentration) with the different yeasts to determine the highest ethanol production and later fermentation processes with the produced hydrolysates were performed. The conditions that exhibited the best ethanol production were supplemented by the addition of nitrogen sources as peptone and yeast extract at concentrations of 1 wt% and 2 wt%, respectively. The data gathered until this point will be used for the improvement of the environmental impact of the EOEP.

Impact on Resource Productivity and Material Reuse

A mass balance analysis was carried out to measure the improvement of the process environmental impact, and it can be measured by several indicators as found in the literature. They include the e-factor, process mass intensity (PMI), mass productivity (MP), and reaction mass efficiency (RME) [20–22]. The e-factor, as well as the PMI, MP, and RME are based on the mass ratios, which are known variables in this work [23]. Additionally, the balances used for the environmental impact are a step closer to a life cycle assessment (LCA) [24–26]. The analysis is delimited to an EOEP with a processing capacity of 100 kg of *S. molle* seeds and includes a mass balance, as well as the process gas emissions on the established essential oil extraction process. The mass balances including the greenhouse gases were calculated for a facile comparison between the established process and the process coupled with the ethanol production from the residue. Furthermore, the yearly income increase from the produced ethanol was calculated based on the international FOB prices described by the U.S. Grains Council [27].

Results and Discussion

Analysis of the *S. molle*

Fresh seeds (FS) and residue seeds (RS) properties such as mass moisture, volatile matter, and ash content in a dry basis via TGA analyses are in Table 5. The major differences between the *S. molle* seeds, fresh and used, lie in the percentage of fixed carbon. When comparing with wood chips, forest residue, and sweet sorghum [28], the majority of the biomass is volatile matter, whereas in the *S. molle* samples resulted 10% less volatile mater, specifically in the FS sample.

The difference might be originated from the fact that different parts of one plant have different major components directly related to its physiological functions. In this case, the drupes and seeds have other role in the plant development. The water content (moisture) was different between FS and RS samples, and it will affect the resulting profile increasing the dry base content of volatile matter in RS. The high percentage of volatile matter present in RS, suggests the possibility to find RS a good feedstock for gasification.

Table 5 Biomass thermogravimetric analyses in dry basis compared to Ref. [28]

Property	FS	RS	Forest residue	Wood chips	Sweet sorghum	Wheat straw
Moisture (%)	16.6	7.6	6.3	3.9	7.0	10.3
Volatile matter in air (%)	61.9	74.0	74.1–79.3	80.0	77.2	77.7
Ash (%)	9.1	5.4	1.3–4.1	0.6	4.7	4.7
Fixed carbon (%)	29.6	20.6	19.4–21.9	19.4	18.1	17.6

However, a close similarity was found when the RS samples are compared to the other lignocellulosic biomass in terms of fixed carbon. RS fixed carbon content is comparable to that of olive kernel (17.2%) [29]. The higher content of fixed carbon in the FS samples suggest that might have higher heating value for additional applications. The content of ashes is higher in the FS and RS samples, suggesting the presence of higher mineral content than that of the forest residues, wood chips, and sweet sorghum. The higher content of minerals can be advantageous for the fermentation, reducing supplements added into the fermentation broth. The content of monosaccharides and Klason lignin were determined to share the biomass percentages presented in Fig. 1.

The sugar content analyses revealed that the glucose content is 16% for both FS and RS samples. Where the major component is observed to be the extractives, which for this study were calculated by the difference between the total biomass and the determined monosaccharide, Klason lignin and ash content. As the fermentation potential of the residues will be based on the carbon and nitrogen sources availability, and it is important to clarify that the extractives include the soluble lignin, proteins, and other soluble components as a whole [30]. The glucose content shows that the ethanol production from *S. molle* biomass is feasible (16 wt%). The theoretical ethanol production based on the glucose content only would reach 82 mg of ethanol per gram of *S. molle* seeds. Additionally, if all the monosaccharides are considered suitable to produce ethanol, such production could reach a theoretical 122.7 mg of ethanol per gram of *S. molle* seeds. On the other hand, the dissolved lignin within the extractives might hinder the ethanol production due to microorganism inhibition [31, 32]. The lignin also hinders the enzymatic activity by its competitive inhibition role [33]. Therefore, the lignin derivatives—aliphatic carboxylic acids, furan aldehydes, and acetic acid, among others—effects might be reflected in a lower ethanol yield depending on the yeast and enzyme resistances. These compounds are present as by-products of the common pre-treatments (acid, enzymatic, and hydrothermal pre-treatments) [34]. In the extractives share of the *S. molle* biomass, terpenes are the

majoritarian components of the extracted essential oils. The concentration of terpenes in the FS samples not only might hinder the fermentation due to their reported high concentration of 1.5 g/L [35] but could also limit the growth of other microorganisms due to the anti-microbial properties of the terpenes [36]. Additionally, the terpenes are also by-products of the fermentation and the yeast strains might be accustomed to their presence.

Biomass Hydrolysis

The chemical hydrolyses resulted in a detection of a maximum amount of 14.36 g/L of monosaccharides where 92% is identified as glucose detected after the treatment with 3 mL of a solution of 12.1 M H_2SO_4. The set of results presented in Fig. 2 show also that the most monosaccharides are released using H_2SO_4 when compared to NaOH and CH_3COOH. Because of the absence of vacuum treatment in the H_2SO_4 hydrolysis had a higher glucose release, the use of a 12.1 M solution of H_2SO_4 with no treatment at all was considered (absence of vacuum and thermal treatments). Such experiment led to a value of 9.76 ± 0.41 g/L of glucose showing that the thermal method by autoclave is still the leading treatment.
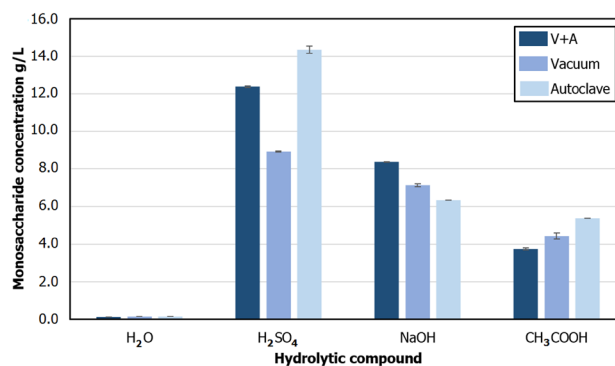
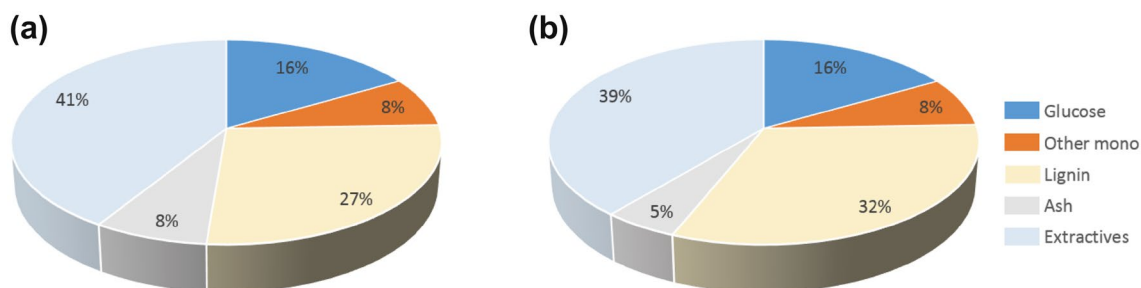**Fig. 2** Monosaccharide evaluation of RS hydrolyses

Fig. 1 Monosaccharide content analyses of the samples **a** FS and **b** RS

The experiments with the ultrasonic treatment showed the results presented in Fig. 3. By the use of ultrasonic treatment, the maximum concentration of liberated monosaccharides reached 15.6 ± 0.18 g/L. The H_2SO_4 hydrolytic effect was followed by the NaOH and CH_3COOH in a decreasing trend. Another important observation is that the experiments with vacuum, steam explosion, and ultrasound showed the water-soluble monosaccharides is as low as 0.12 g/L.

The enzymatic hydrolyses released the monosaccharide concentrations showed in Fig. 4. In the enzymatic hydrolysis, it was determined that the amyloglucosidase has a higher activity, with the implication that the RS biomass has 1,4- and 1,6- links in carbohydrates resulting in the release of α-limit dextrin, maltotriose, maltose, and isomaltose. However, the resulting monosaccharide concentration from the α-amylase is relatively lower, meaning that the surface of RS has a lower amount of amylose and amylopectin, i.e., lower 1,4-α-glucosidic linkages to hydrolyse (maltose).

The carbohydrates present in the *S. molle* seeds are comprised by different kinds of polysaccharides plus a high amount of lignin, that might have a hindering effect on the enzymes' hydrolytic activity. The development stage where the seeds have more essential oil might also be determining for the carbohydrate composition [37], but to date no specific report on carbohydrates of *S. molle* seeds was found. Additionally, the blank had an apparent high concentration of monosaccharides due to the soluble matter under the pH and temperature conditions of the enzymatic treatments.

Saccharomyces cerevisiae and Isolation of Native Yeast Strains

The isolation procedure led to two yeast strains. One yeast strain grew on the YPD alone culture media, while the other yeast strain grew on the 5 wt% ethanol YPD and they were named Y1 and Y2, respectively. Both yeast strains resulted positive for Gram stain tests and the morphological characterization is listed in Table 6.
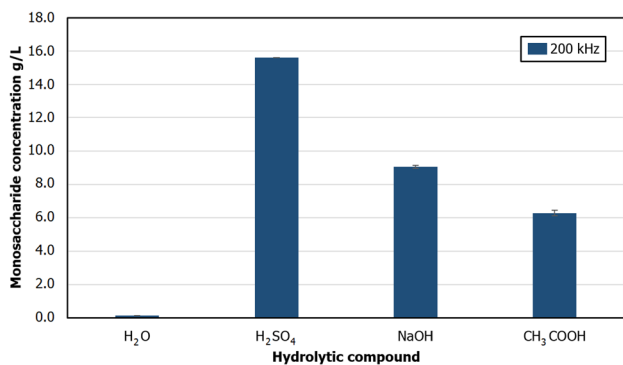

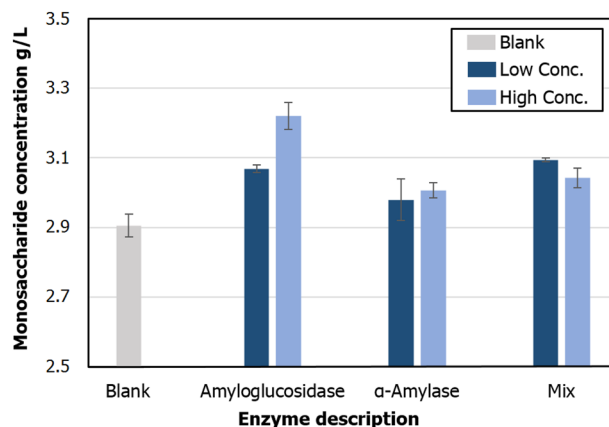**Fig. 3** Monosaccharide evaluation of RS ultrasonic aided hydrolyses

Fig. 4 Monosaccharide evaluation of RS enzymatic hydrolyses

Further characterization using the analytical profile index (API 20E) resulted in the characteristics described in Table 7. Among the results, the tests were positive for all the monosaccharides and polysaccharides included in the API 20E kit (glucose, sucrose, rhamnose, melibiose, and arabinose). The fermentation or oxidation were all positive as listed in the Table 7. The only enzyme production for detection of both yeast strains was for gelatinase. Enzyme tests for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and urease, resulted negative for both strains. The analysis of the RapID yeast plus kit indicated that the yeast strain Y1 has biochemical properties similar to *Candida apicola* with a 94% of positive hits in the test. The biochemical properties of Y2 had 83% of similarity to the species *S. cerevisiae*. However, the yeast identification has to be confirmed with further analysis, e.g., 18S rRNA and protein-coding gene analyses [38].

Fermentation Evaluation

On the contrary of the commercial bakers' yeast *S. cerevisiae*, which is readily usable, the isolated native yeast strains must be cultivated to be inoculated at their exponential growth for their optimal performance in the fermentation.

Table 6 Morphologic features of the isolated yeasts

Characteristic	Y1	Y2
Gram test	+	+
Appearance	Circular	Circular
Margin	Smooth	Smooth
Elevation	Convex	Convex
Texture	Creamy	Creamy
Pigmentation	Seashell white	Seashell white
Optical property	Opaque	Opaque

Table 7 API 20E and RapID yeast plus characterization profiles

Test	API 20E		RapID	
	Y1	Y2	Y1	Y2
β-Galactosidase	+	+	ND	ND
Gelatinase	+	+	ND	ND
Glucose	+	+	+	+
Sucrose	+	+	+	+
Melibiose	+	+	ND	ND
Arabinose	+	+	ND	ND
Maltose	ND	ND	–	–
Trehalose	ND	ND	–	–
Rafinose	ND	ND	+	+
Histidine β-naphthylamide	ND	ND	+	–
Leucyl-glycine β-naphthylamide	ND	ND	+	–
ρ-Nitrophenyl-α,D-glucoside	ND	ND	–	+
ρ-Nitrophenyl-α,D-galactoside	ND	ND	–	+

ND not determined

Accordingly, the growth curves in Fig. 5 indicate that the exponential growth for both yeast strains start at the 7th hour until the 16th hour, and both reach quiescence state approximately at the 17th hour. The established times for the aliquot extraction might have played a role in the determination of the growth curve for Y2. The growth curve for Y2 seems to be still increasing at the last point. However, due to the sampling times the maximum might have passed undetected between 16 and 20 h. Further fermentation experiments were done inoculating the yeast at its exponential growth state, i.e., at the 10th hour.

The fermentations that were carried out with *S. cerevisiae*, Y1, and Y2 in their exponential growth state resulted in the ethanol production levels depicted in Fig. 6. The ethanol
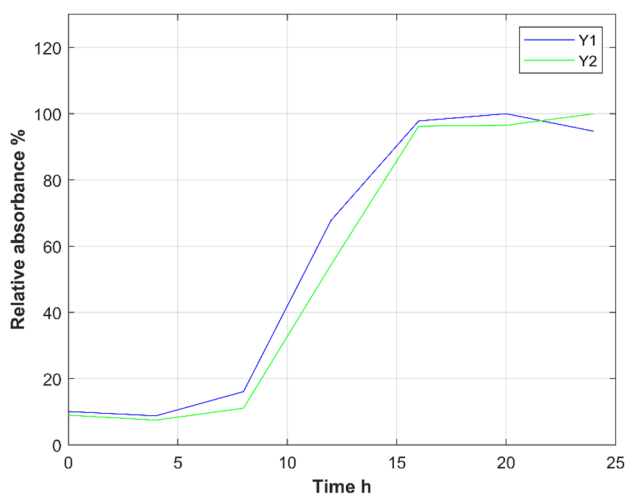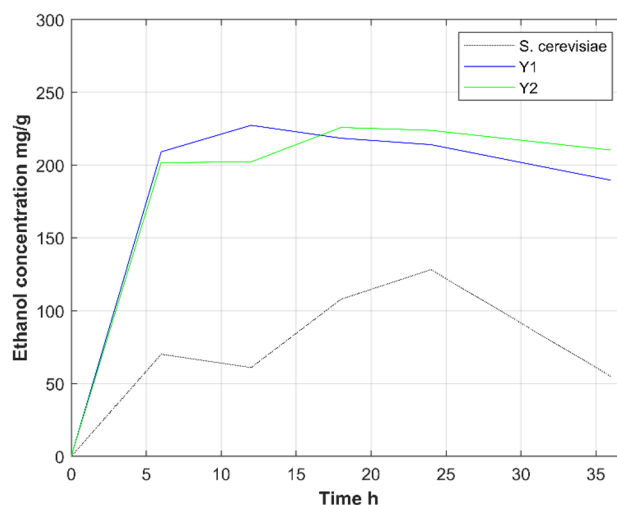
Fig. 6 Initial fermentation process

production is higher using both native yeast than using the *S. cerevisiae*. The ethanol production increased exponentially within the 6th hour, while the maximum was achieved at 12th and 16th hours, with Y1 and Y2, respectively. Additionally, the ethanol content is detected to reduce only 11% and 6% in the case of Y1 and Y2, while the decrease of ethanol content in the case of the *S. cerevisiae* fermentation process reaches a lowest of 57%. Such decrease of the ethanol production is related to the acetic fermentation which is produced by the oxidation of ethanol called acetogenesis [39].

The data depicted in Fig. 6 shows that the ethanol content is stable from the 5th hour until the 24th hour, and the *S. cerevisiae* has a continuous ethanol increase until that time, as a consequence the fermentation base conditions for FS and RS were kept for a 24 h fermentation time. The ethanol production of both biomass samples (FS and RS) resulted in the ethanol content values depicted in Fig. 7. The ethanol production for the fermentation evaluation shows that the yeast *S. cerevisiae* has the peak ethanol production within the fresh seeds samples (FS). The first possible explanation is that FS has a higher content of soluble sugar to be a facile carbon source available for the yeast biological processes, while the *S. molle* seeds residue has its soluble extractives stripped off by the EOEP. The second explanation is that the total content of the *S. molle* seeds might have controlled the growth of other bacteria keeping the fermentation by *S. cerevisiae* only. The difference on ethanol production by *S. cerevisiae* might also be used to estimate the carbon source availability, where a difference of 10 mg/g in ethanol production can be estimated to be caused by the reduction of 4 wt% of the fermentable carbon source. Such difference might be caused by the loss of soluble fermentable matter during the essential oil extraction.
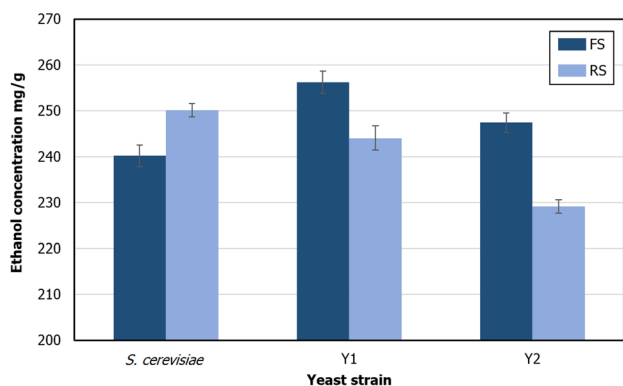

Fig. 5 Growth rate curves for the isolated yeast strains

Fig. 7 Fermentation of FS and RS with the different yeast strains

The native yeasts Y1 and Y2 fermentations with the *S. molle* seeds residue (RS) had better results. Such difference might rely on the microorganisms adaptability to develop capacities that make them adapt to the surrounding environment [40, 41]. Such mechanisms are even improved under long term nutrient stress [42], thus, improving the microorganism exploitation of the available nutrients. The ethanol production of the yeast Y1 is the highest of all with 256.3 mg/g.

Fermentation with the Hydrolysates

Since the H_2SO_4 hydrolytic method resulted in highest monosaccharide release, as Fig. 8 indicates, the production of ethanol reached a maximum of 88.36 mg/g of *S. molle* residue (RS). However, by the use of a strong acid such as H_2SO_4 might lead to the production of high concentrations of SO_4^{-2} salts, that plus the by-products formed from lignin resulted in a yeast inhibition [43]. Furthermore, it is also important to highlight the increase of the ethanol production by the addition of yeast extract and peptone to the hydrolysate before the fermentation began. Such addition showed
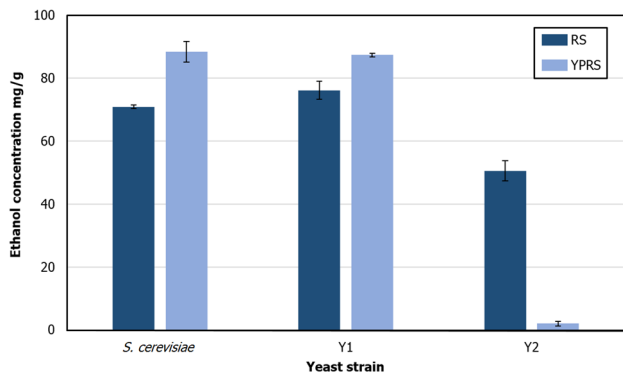
that a lack of nitrogen-based nutrients might be hindering the ethanol production by the different yeast. The nitrogen sources, as well as carbon sources, play an important role in the longevity of the microorganisms affecting the overall production of ethanol [44].

The fermentation of the enzyme-driven hydrolysates was done afterwards with the results showed in Fig. 9. The combination of α-amylase and amyloglucosidase (Mix) with the isolated yeast Y2 resulted in the superior ethanol production. The ethanol production of *S. cerevisiae* displayed that the enzymatic treatment with α-amylase releases more fermentable matter (monosaccharides) than amyloglucosidase. Such result implies that the *S. molle* residual biomass (RS) might have more polymers composed by 1,4- and 1,6-α linkages as in maltotriose, and amylopectin and dextran than higher oligosaccharides [45]. Additionally, the combination of enzymes resulted in a production of 102 mg/g with an amount of residual monosaccharides of 0.01 g/L. Likewise, the fermentation by the yeast Y1 showed that the combination of enzymes was advantageous for higher ethanol production, even if the obtained ethanol concentration was considerably lower (88 mg/g) than the fermentation with *S. cerevisiae*. The ethanol production with the yeast Y2 was higher than the previous production processes. The effect of α-amylase increased the ethanol production to 88 mg/g, while the effect of amyloglucosidase kept the same level of ethanol production as with the *S. cerevisiae* (74 mg/g), and the combination of both enzymes (Mix) showed an ethanol production of 111 mg/g. The isolated yeast Y2 was identified to have a close similarity to *S. cerevisiae*, accordingly it is possible that both yeast strains have a less assimilation capabilities of some components released by the amyloglucosidase from the RS biomass. However, the combination of both enzymes led to the most advantageous monosaccharide content which in turn produced the highest concentration of ethanol as a result of a synergistic activity of the enzymes
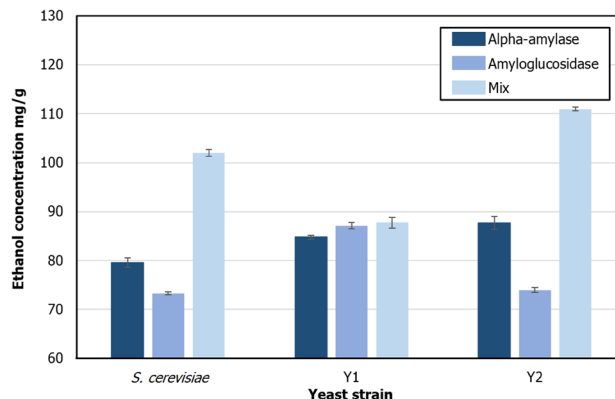

Fig. 8 Fermentations with the H_2SO_4 hydrolysate with different yeast strains

**Fig. 9** Fermentations with the enzyme hydrolysates and different yeast strains

[46]. The result might be explained by the selectiveness of the enzymes that combined release more fermentable matter from the residual biomass from *S. molle* (RS).

Impact of the Fermentation in the Process

Two mass balances were done to compare the impact between the present EOEP process and the proposed implementation. The first mass balance is depicted inside the blue-dashed box in Fig. 10, where the essential oil extraction process was evaluated as one operational unit. The assumptions include the consideration of optimal combustion of natural gas and air to CO_2 and H_2O. The combustion feeds the boiler to produce steam, and only the material entering and leaving the process were considered for the purpose of this study. The peeling and milling operations normally consume electrical energy and were thus discarded for the calculations of the gas emissions. The *S. molle* residual bagasse was determined to be constituted by 18 wt% of *S. molle* seed residues (biomass) and 82 wt% of H_2O. An estimation of the greenhouse gases emitted from the uncontrolled decomposition of such mixture reached 6.49 ± 1.21 kg of CH_4 per dry kg of biomass per day [47]. The drupe peels and seed loss through the processes were estimated to reach 5.9 kg/day and were no further considered for any reusability. The water feed is considered as one input line of 700 kg/day. The outputs included water with the bagasse, as well as water from the combustion, water loss by the process inadequacies, and water separated from the essential oil.

The green-dotted box in Fig. 10 shows the system boundaries considered for the mass balance of the extraction process coupled with the fermentation of residual seeds process. The fermentation process was assumed to behave like a fed-batch process, where the yeast and bagasse are fed to the system as they come from the EOEP, and the ethanol is separated as it is produced. The importance of the improvement of the water recovery is evident, the fermentation process requires 618 kg of H_2O, which could be recovered water instead of adding potable water. The ethanol production was assumed to reach the best production conversion reached in this study (256 mg/g) and the biomass in the bagasse was also reduced reaching a 47 wt% of reduction. The CO_2 production was also calculated based on the ethanol production, reaching 44.19 kg/day, which apparently is higher than the production of gases from the uncontrolled residue decomposition. However, the estimated GHG emitted from the uncontrolled decomposition (including 6.49 kg of CH_4 per day) but the CO_2 produced by fermentation has an infrared activity of 21 times lower than that of the CH_4 on a 100-year time horizon [48]. According to the IPCC fifth assessment report (AR5), 2013 on the global warming potential (GWP), CH_4 has a factor of 34 over a unit of CO_2 for the 100-year time horizon [49]. As a result, the implementation of the residue fermentation could reduce 47% of GWP. The results on the comparison of the processes are listed in Table 8 by the indicators e-factor, process mass intensity (PMI), mass productivity (MP), and reaction mass efficiency (RME).

Even though the indicators listed in Table 8 are commonly used for synthesis and catalytic synthesis processes,
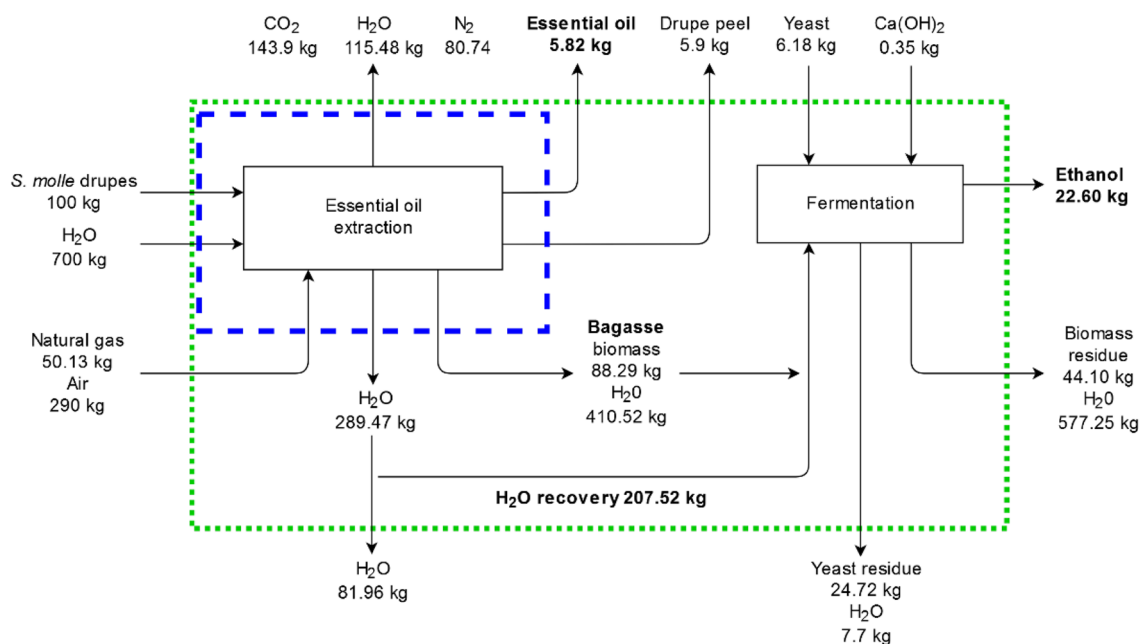
Fig. 10 Mass balances of the essential oil extraction and fermentation processes. (Color figure online)

Table 8 Mass indicators for the comparison of the environmental impact improvement

Indicator	Definition	Extraction	Extraction and fermentation
E-factor	$\frac{Product\ kg}{Total\ waste\ kg^{a}}$	48.07	9.54
Process mass intensity (PMI)	$\frac{Total\ mass\ used\ kg}{Mass\ of\ total\ product\ kg}$	145.98	37.44
Mass productivity (MP)	$\frac{1}{PMI} \times 100$	0.68	2.67
Reaction mass efficiency (RME)	$\frac{Mass\ of\ total\ product\ kg}{Total\ mass\ used\ kg}$	0.007	0.027

[a]Total waste does not include water

such indicators show clearly the improvement of the EOEP by the coupling with a fermentation process. The e-factor decreases five times, while the PMI decreases approximately four times. On the other hand, the MP increases up to 2.67 and the RME considerably increases from 0.007 to 0.027.

Based on the ethanol production established in the mass balance presented in Fig. 10, it might be possible to produce 22.6 L of ethanol per day. According to the U.S. Grains Council, 2019 the Houston "Free on board" (FOB) average price of ethanol from January-2018 to January-2019 ranged at US$ 0.39 per litre, while the price in FOB Santos, and FOB Gulf were US$ 0.476, and US$ 0.368 per litre, respectively [27]. Considering the differences that the ethanol price might have depending on the region, it was calculated that a yearly production of 6302.6 L of ethanol could lead to an income of US$ 2592.50.

Conclusions

Schinus molle seed residues are suitable for a more sustainable essential oil extraction process and ethanol production. The biotechnological approach of isolating yeast from the residual disposal place reduces the local environmental consequences due to the insertion of foreign microorganisms into the local ecosystem. In this study the isolated native yeasts perform better than the commercial *S. cerevisiae* in different conditions. Whereas the isolated yeast Y1 had the best ethanol production in the bare RS fermentation and H_2SO_4 hydrolysate fermentations, the isolated yeast Y2 had a higher ethanol production in the enzymatic hydrolysate fermentation. The implementation of innovative technology approaches is advisable due to the increased potential of ethanol production, as determined by the effect of ultrasound in the hydrolysis treatments that showed comparable monosaccharide release to more energy intensive treatments such as vacuum and thermal treatments. The addition of the fermentation of *S. molle* seed residues might lead to an improvement of the impact of the extraction process. However, a study on the energy demand, the energy efficiency, and the energy demand of the proposed fermentation process could enlighten better the improvement. The potential of ethanol production from *S. molle* residues shows that the essential oil extraction process could be a more benign process as indicated by the adaptation of the quantitative metrics of e-factor, PMI, MP, and RME.

Compliance with Ethical Standards

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