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Bioethanol production from edible insect excreta: a case study on frass from house crickets

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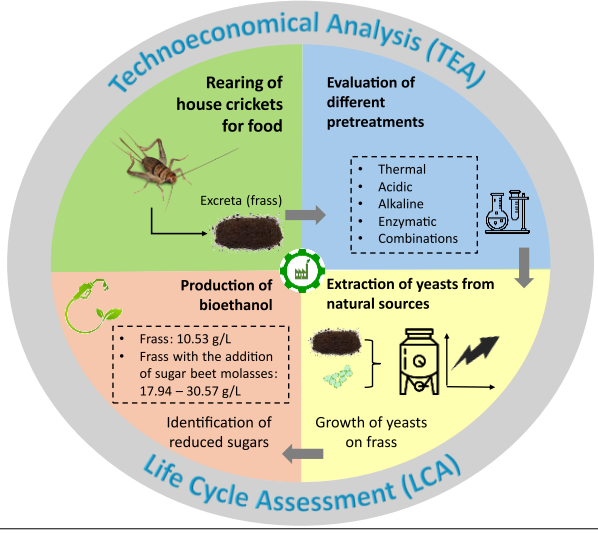
HIGHLIGHTS

- Excreta from house crickets can be used for the production of bioethanol.
- Enzymatic, acidic, and alkaline hydrolysis of frass were investigated.
- The production of sugar and free amino proteins reached 30 and 5 g/L, respectively.
- Various yeast strains can grow on hydrolyzed frass.
- A combined substrate of frass with molasses led to an ethanol yield of 30 g/L.

GRAPHICAL ABSTRACT



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ABSTRACT

House crickets are among the most promising edible insect species for inclusion in future agri-food systems. Their appeal stems from environmentally sustainable rearing practices, a high nutritional value, and a long history of traditional use as food. Additionally, their rearing produces a byproduct known as frass, which holds potential as a valuable biomaterial. The utilization of house cricket frass as a substrate for bioethanol production was explored. Frass was digested with cellulases at 10% enzyme/dry matter of substrate, 50°C, pH=5, 48 h. This hydrolysis was combined with different treatments, like acidic (1% sulfuric acid) and alkaline (1% sodium hydroxide), and compared to protease treatment (50°C, pH=6.8, 24 h). The production of sugar and free amino proteins reached 30 and 5 g/L, respectively. Several yeast strains, isolated and identified from various organic waste sources, were tested. The fermentation was performed with *Saccharomyces cerevisiae* for 48 h with frass hydrolysate, pretreated with sulfuric acid, and digested with proteases and cellulases. The addition of molasses at 0–60 g/L was considered. Sugar consumption exceeded 80%, with ethanol concentrations reaching 12.56 g/L without molasses and 30.57 g/L with the addition of molasses. Cricket frass was utilized as a substrate for bioethanol production, and the process was significantly enhanced by supplementing it with sugar beet molasses.

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Contents

Abbreviations

P	Bioethanol concentration (g/L)
P_0	Bioethanol concentration at the beginning of the fermentation (g/L)
P_f	Bioethanol concentration at the end of the fermentation (g/L)
P_m	Potential maximum bioethanol concentration (g/L)
$P_{i,p}$	Predicted bioethanol concentration at time i (g/L)
$P_{i,e}$	Experimental bioethanol concentration at time i (g/L)
$r_{p,m}$	Maximum bioethanol production rate (g/L/h)
t_L	Lag phase (h)
t	Fermentation time (h)
$Y_{p/s}$	Bioethanol yield over consumed total sugars (g/g)
S_0	Total sugar concentration at the beginning of the fermentation (g/L)
S_f	Total sugar concentration at the end of the fermentation (g/L)
K	Percentage of consumed total sugars (%)
E	Required specific energy to distill the bioethanol ($MJ/kg_{ethanol}$)

1. Introduction

Edible insects will play a significant role in the future agri-food systems because they are a nutritional and environmentally friendly alternative to conventional livestock (Rumpold and Schlüter, 2013; Lange and Nakamura, 2021). Edible insects are expected to support the construction of resilient regional agri-food systems (Queiroz et al., 2021) while addressing environmental problems of agriculture by reducing the use of feed resources and water, utilization of organic sidestreams and reduction of greenhouse gas emissions (Van Huis and Oonincx, 2017; Lange and Nakamura, 2021).

The rearing of edible insects generates a variety of organic wastes, e.g., feces (commonly known as *frass*), exuviae, and uneaten food (Ojha et al., 2020). In the context of insect rearing or farming, frass can include parts of shed exoskeleton and uneaten feed apart from feces (Barragán-Fonseca et al., 2022). Frass is generated in large amounts, up to thousands of tonnes per year (Food and Feed, 2019), and 40 times larger than the produced animal biomass (Poveda, 2021). The insect frass's main utilization method is as fertilizer (Poveda, 2021; Barragán-Fonseca et al., 2022). However, frass can also be utilized as feed for fish (Yildirim-Aksoy et al., 2020; Arabzadeh et al., 2022), as substrate for the production of biogas (Wedwitschka et al., 2023) and as substrate for the production of biochar with a high adsorption capacity of anionic dyes and heavy metals (Yang et al., 2019a and b).

Biofuels, including bioethanol, biogas, and biomethane, are crucial alternatives to fossil fuels, which are significant contributors to global warming (Kaup and Selbmann, 2013). Despite this, many countries remain reliant on fossil fuels (Pollmann et al., 2019). Bioethanol can be derived from various sources, including edible feedstocks like sugarcane and corn, as well as non-edible feedstocks and marine resources, primarily algae (Aghaei et al., 2022). The primary requirement for bioethanol production is a raw material rich in sugars, starch, or lignocellulose (Bušić et al., 2018). As manure is lignocellulosic biomass (Rezania et al., 2020), its potential for bioethanol production has been investigated using waste from various animals, including cattle (Vancov et al., 2015), horses (Lee et al., 2021), pigs, and poultry (Bona et al., 2018).

The process of producing bioethanol from lignocellulosic biomass consists of chemical (e.g., thermal, acidic, and alkaline) and enzymatic (e.g., proteases, cellulases, and hemicellulases) pretreatment methods to hydrolyze cellulose to hexoses and hemicellulose to pentoses and to reduce lignin (Rezania et al., 2020). The conversion of lignocellulose to free fermentable sugars determines the efficiency of bioethanol production (Binod et al., 2011). Afterward, sugar monomers are converted to ethanol with fermentation with yeasts (Rezania et al., 2020). Recent advancements in bioethanol production tend to focus on improving processes like the pretreatment of biomass, the fermentation of pentoses, which are less

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fermentable than glucose, the simultaneous saccharification and fermentation, the co-fermentation, the downstream processing, the combination of the processes mentioned above and the utilization of the byproduct (Carrillo-Nieves et al., 2019; Chauhan et al., 2023). Considering the complexities of the "food-feed-fuel competition" (Fradj et al., 2016), a synergistic framework has been proposed to address this issue. This framework prioritizes food production, directs non-edible fractions to animal feed, and finally allocates residual materials for biofuel production (Muscat et al., 2020). This approach aims to maximize resource efficiency while minimizing conflicts between these critical sectors. Even though bioethanol from non-edible sources, e.g., lignocellulosic biomass, requires pretreatment methods, it does not compete with food and feed requirements, and its production is seen as sustainable (Melendez et al., 2022) with generally low environmental impact, as in the case of cow manure (de Azevedo et al., 2017).

Within this framework, insect frass was also explored as a substrate for bioethanol production. House crickets (*Acheta domesticus*) were chosen for this study due to several advantages: they are already consumed in certain parts of the world (Magara et al., 2021), have been approved as a novel food in the European Union (EFSA Panel on Nutrition et al., 2021), and are currently used as a food ingredient (Rossi et al., 2021). These attributes make house crickets a promising candidate for sustainable bioethanol production. The goal of the present study was to explore the cricket frass as a potential feedstock for bioethanol production and prove its suitability using a conventional process of separate fermentation and hydrolysis. The production of biofuels using fractions of insect farming as feedstock has been somewhat explored in the past. Insect lipids can be used for the production of biodiesel (Nguyen et al., 2019), and the frass from various species has been used for the production of biogas. **Table 1** summarizes some recent studies that focused on the production of biofuels from insect-derived feedstocks. The present study is the first one to discuss the production of bioethanol using an insect-derived fraction as feedstock and the first one to present the production of bioethanol from frass and biofuels from the rearing of house crickets. House crickets have been overlooked for the production of biofuels in comparison to other species, even though they find such broad applications as food and feed.

2. Materials and Methods

All chemicals described in the manuscript were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) unless stated otherwise.

2.1. Frass collection

Adult house crickets were obtained from Tropic Shop (Nordhorn, Germany) and housed in 22 L polypropylene boxes placed inside a climatic chamber (Polyklima, Freising, Germany). The chamber was maintained at controlled conditions of 32°C, 70% relative humidity, and an 8-hour photoperiod per day to ensure optimal environmental conditions for the crickets. The adult crickets were given commercial cricket feed (Tropic Shop, Nordhorn, Germany) and a mixture of sand and coconut fiber (20:1) as an egg-laying substrate that was kept wet at all times. The adult crickets were left to reproduce for 1 week and then were inactivated by freezing at -20°C. The eggs hatched, and the newborn crickets were reared in the same conditions until adulthood. The frass was collected with a brush throughout the whole life cycle of the crickets and was used for further analysis. Frass from edible insects, including cricket species, have been shown to contain crude fibers and proteins (Bulak et al., 2020), making the material appropriate for fermentation. The presence of cellulose, hemicellulose, and lignin in the house cricket frass was confirmed in preliminary tests (**Table S1**).

2.2. Hydrolysis of frass

2.2.1 Methods for hydrolysis

Various hydrolysis methods were evaluated for their applicability to cricket frass. Enzyme selection was guided by the frass composition, as detailed in **Table S1**. Given that the frass was rich in cellulose, hemicellulose, and proteins, the chosen enzymes were required to exhibit both proteolytic and cellulolytic activities to break down these components effectively. The selected enzyme (Cellic CTec3, Novozymes, Bagsvard, Denmark) is appropriate for this use. The parameters and conditions of each hydrolysis were based on the required conditions for optimum enzymatic activity according to the manufacturer's instructions (Novozymes, Bagsvard, Denmark). The amount of enzyme added was the maximum amount of enzyme that could be added, according to the manufacturer. Each hydrolysis was performed with 5.85 g of frass, which corresponds to 5 g of dry weight (85.47%) and 50 mL of the total volume of the liquid phase, leading to a 10% solid content. The following treatments were performed:

- Thermal: Frass was mixed with 50 mL of water, and the mixture was thermally treated at 121 °C for 15 min with an autoclave (3870ELV, Tuttnauer Co. Ltd., USA).
- Thermal/Cellulase: Frass was mixed with 45 mL of water, and the mixture was thermally treated at 121 °C for 15 min. Afterward, the pH was adjusted to 5 with a pH meter (3219 WTW, Fischer Scientific GmbH, New Hampshire, USA) using HCl, 6N. Then, an aliquot of 0.5 mL of a solution containing the commercial cellulase Cellic CTec3 (Novozymes, Bagsvard, Denmark), corresponding to 10% enzyme/g dry weight of the substrate, was added. Afterward, the mixture was homogenized at 50°C for 48 h inside a shaking incubator (GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) under agitation at 150 rpm.
- Thermal/Acidic/Cellulase: Acid hydrolysis was conducted using a 1% H_2SO_4 solution. Cricket frass was mixed with 44 mL of water and 1 mL of a 50% H_2SO_4 solution. The mixture underwent thermal treatment at 121°C for 15 minutes. Following this, the pH was adjusted to 5. Subsequently, 0.5 mL of Cellic CTec3 enzyme solution was added, corresponding to 10% enzyme per gram of dry substrate weight. The mixture was then homogenized and incubated in a shaking incubator at 50°C and 150 rpm for 48 hours.
- Thermal/Alkaline/Cellulase: An alkaline hydrolysis was performed with a 1% NaOH solution. In specific, frass was mixed with 42.5 mL of water and 2.5 mL of a 20% NaOH solution. After the thermal treatment (121 °C, 15 min) and pH adjustment (pH=5) of the mixture, an aliquot of 0.5 mL of the solution with Cellic CTec3 was added, corresponding to 10% enzyme/g dry weight of the substrate. The mixture was

Table 1.
Summary of recent studies focusing on the production of biofuels used as feedstock fractions from the rearing of edible insects.

Insect Species	Fraction	Type of Biofuel	Reference
Tenebrio molitor	Raw mealworm oil	Biodiesel	Siow et al. (2024)
Hermetia illucens larvae	Extracted lipids	Biodiesel	Ishak and Kamari (2019)
H. illucens larvae	Insect biomass was directly transesterified, and the Biodiesel was then isolated	Biodiesel	Nguyen et al. (2020)
H. illucens, *T. molitor*, various genera of *Gryllus* spp.	Frass	Biogas	Bulak et al. (2020)
Bombyx mori	Frass	Biogas	Łochyńska and Frankowski (2018)
Zophobas morio	Frass	Biogas	Bulak et al. (2023)
H. illucens	Frass	Biogas	Wedwitschka et al. (2023)
H. illucens	Frass	Biogas	Dong et al. (2024)
Acheta domesticus	Frass	Bioethanol	**Present Study**

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homogenized for 48 h at 50 °C and 150 rpm inside a shaking incubator.

- Thermal/Proteases: Frass was mixed with 49.9 mL of water, and the mixture was thermally treated (121 °C, 15 min). Afterwards, 0.05 mL of Neutrase (Neutrase 0.8 L, Novozymes, Bagsvard, Denmark) and 0.05 mL of Flavourzyme (Flavourzyme 1000 L, Novozymes, Bagsvard, Denmark) were added to the mixture. The pH of the mixture was measured and was found to be equal to 6.8. The mixture was homogenized for 24 h at 50 °C and 150 rpm inside a shaking incubator. The number of total proteases corresponded to 1% proteases/g of the dry weight of the substrate.

2.2.2. Analysis of hydrolysates

The hydrolysates were centrifuged at 4800 rpm for 15 min at room temperature, and the supernatants were collected. These supernatants were analyzed for soluble sugar content and free amino nitrogen (FAN). For the determination of soluble sugar content, the hydrolysates were diluted 1:200 with water. The sugar content was then measured using the phenol-sulfuric acid method. Specifically, 1 mL of the diluted hydrolysate was mixed with 5 mL of 97% sulfuric acid and 1 mL of a 5% phenol solution. The mixture was incubated at 27°C for 15 min in a thermoblock (Labnet International Inc., Edison, NJ, USA), after which the absorbance was measured at 490 nm. The results were expressed in mg/mL (DuBois et al., 1956).

For the analysis of FAN, the hydrolysates were diluted 1:100 with water. Then, 0.5 mL of the diluted samples were mixed with 0.25 mL of an aqueous ninhydrin reagent, which contained 0.5% (w/v) ninhydrin and 0.3% (w/v) fructose in a 720 mM phosphate buffer at pH 6.8. Water served as the blank control. The samples were incubated for 20 minutes at 95°C in a thermoblock and then cooled in a water bath. After cooling, 1.25 mL of a 0.2% (w/v) potassium iodate (KIO_3) solution, prepared with 40% ethanol as the solvent, was added to each sample. The samples were thoroughly vortexed, and the absorbance was measured at 575 nm. Results were expressed in g/L (Dimopoulos et al., 2020).

2.3. Yeast tests and identification

The hydrolysates, which were produced using the methods described in *section 2.3.1*, were tested for their suitability for yeast growth. Initially, the hydrolysates were centrifuged at 4800 rpm for 15 min. All hydrolysates were tested both with and without the addition of sugar beet molasses at a concentration of 40 g/L, which corresponds to 20 g/L of added sugar. Finally, the hydrolysates were filtered using a 0.2 μm pore size filter (Sartorius Minisart NML, Goettingen, Germany) to produce the medium. The tested yeast strains were isolated on YM agar from various sources, such as municipal solid waste, cricket frass, coconut milk, sugar beet juice, and fresh tomato pomace, except for *S. cerevisiae* that was obtained from DSMZ (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH Braunschweig, Germany).

All strains were identified using MALDI-ToF MS (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry). The yeasts were cultivated in an agar plate for 24 h, and then a single colony was placed on two spots of a target. Then, 1 μL of 70% formic acid was applied to the first spot and was allowed to dry. Afterward, 1 μL of the matrix was placed in each spot of the target and was allowed to dry. Then, the samples were analyzed with the MALDI-ToF-MS. The spectra estimation was performed with the linear mode in a mass range between 3000 and 20000 m/z. The MALDI-ToF could measure 4 targets simultaneously. For each target, the instrument performed an automatic calibration after the start of the automatic measurement. For this purpose, an *E. coli* strain was used (DSM 1116). The automatic calibration was performed immediately after saving the spectrum by comparing the detected masses of the *E. coli* spectrum with the *E. coli* reference masses. The measurement deviations were ± 5. After the automatic calibration, the system conducted an initial reference measurement using E. coli to ensure accuracy before proceeding with the sample measurements. Once all the samples were measured, a final reference measurement was performed to validate the system's performance and consistency. During the reference measurements, the instrument must detect the *E. coli* at <90%.

The reliability of the identification was based on the identification percentage as follows: <75% was considered not reliable, 75–79.9% meant probable genus identification, 80–89.9% meant highly probable genus identification, 90–99.8% meant secure genus identification and probable species identification and 99.9% meant highly probable species identification. The yeast tests were performed as follows: the strains were reactivated from cryoculture at -80 °C in 5 mL of tryptic soy broth (TSB) (Merck Millipore, Burlington, USA). An aliquot of 250 μL of the medium was put into the microtiter plate of Bioscreen C (Oy Growth Curves Ab Ltd, Turku, Finland), and then 20 μL of the preculture was added. The plates were placed in the bioscreen, and the optical density was recorded at 420–580 nm every 5 min for 24 h. Before each measurement, a shaking interval of 20 moves for 15 s was performed.

2.4. Production of bioethanol

2.4.1. Pretreatment of frass

An amount of 50.82 g of cricket frass, which corresponds to 85% dry weight) was mixed with 427 mL of water and 8.68 mL of a 50% H_2SO_4 solution. The mixture was thermally treated with the autoclave at 121 °C for 15 min. Afterward, the mixture was placed inside a glass vessel controlled by a control unit (Biostat B, B. Braun, Melsungen, Germany), and the pH and temperature of the mixture were adjusted to 5 and 50°C, respectively. An external thermostat (FBC620, Fisher Scientific, New Hampshire, USA) was used for temperature control. The pH was kept constant at pH=5 using a NaOH 20% solution. After the conditions of the enzymatic hydrolysis were set, 4.34 mL of the Cellic CTec3 (Novozymes, Bagsvard, Denmark) solution and 0.87 ml of Fermgen proteases (IFF Bioscience/ DuPont, Delaware, USA) were added to the mixture. The enzyme-to-substrate ratios were set at 10% cellulase and 2% protease per gram of dry substrate. The mixture was stirred with a bladed impeller at 200 rpm for 48 hours. Following this, the mixture was centrifuged at 4800 rpm for 15 min at room temperature. The resulting supernatant was collected, frozen, and stored for further use.

2.4.2. Fermentation substrates

The production of bioethanol was performed using 5 different substrates: plain frass hydrolysate, frass hydrolysate with the addition of 20 g/L sugar from sugar beet molasses, a solution of 20 g/L sugar from sugar beet molasses without frass, frass hydrolysate with the addition of 60 g/L sugar from sugar beet molasses and a solution of 60 g/L sugar from sugar beet molasses without frass.

The substrates were prepared by adding the appropriate amount of sugar beet molasses to water or hydrolysate. All substrates were thermally treated with the autoclave at 100 °C for 15 min and then cooled down to 30°C.

2.4.3. Fermentation

The yeast strain used for fermentation was *S. cerevisiae* (DSM 70468, Heerlen, Netherlands). An inoculum was prepared in tryptic soy broth (TSB) at 30°C for 24 hours, with a total volume of 60 mL, without shaking. Subsequently, 3.2 mL of the inoculum was mixed with 40 mL of substrate. The fermentation samples were incubated at 30°C for 48 h in a shaking incubator set at 50 rpm. During the fermentation, aliquots were taken at different time intervals and analyzed for their content of ethanol, lactic acid, glucose, disaccharides, xylose, and arabinose. The analysis was performed using high-performance liquid chromatography (HPLC) (Knauer, Berlin, Germany). It was coupled with a refractive index detector (RI-71, SHODEX, Yokohama, Japan) and equipped with a Eurokat H column (300mm × 8mm × 10μm; Knauer, Germany), eluted with 5 mM H_2SO_4 at 0.8 mL/min.

2.5. Evaluation of ethanol production

The generation of bioethanol during the fermentation of all different substrates was expressed mathematically as a function of the fermentation time using the modified Gompertz model (Equation 1) (Dodić et al., 2012; Khalseh, 2016; Moodley and Kana, 2019):

$$P = P_m \cdot \exp\left\{-\exp\left[\frac{r_{p,m}\cdot\exp(1)}{P_m}\right]\cdot(t_L - t) + 1\right\} \quad \text{Eq. 1}$$

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where P is the bioethanol concentration (g/L), P_m is the potential maximum bioethanol concentration (g/L), $r_{p,m}$ is the maximum bioethanol production rate (g/L/h) and t_L (h) is the lag phase, so the time between the beginning of the fermentation and the exponential growth of the yeasts.

The model was initially applied to data from each replication of each sample to assess repeatability. Subsequently, it was applied to the mean values of data obtained for different substrates to evaluate overall trends and performance. The applicability of the model was evaluated using the regression coefficient R^2, as well as the RMSE and the χ^2, which were calculated using **Equations 2** and **3**, respectively:

$$RMSE = \sqrt{\frac{\sum_{i=0}^{N}(P_{i,p}-P_{i,e})^2}{N}} \quad \text{Eq. 2}$$

$$\chi 2 = \frac{\sum_{i=0}^{N}(P_{i,p}-P_{i,e})^2}{N-n} \quad \text{Eq. 3}$$

where $P_{i,p}$ and $P_{i,e}$ are the calculated and experimental values of the bioethanol (g/L), N is the number of observations, and n is the number of parameters of the model. A good fitting of the experimental data to the model is indicated with high values of R^2 and low values of RMSE and χ^2.

The bioethanol yield over consumed total sugars ($Y_{p/s}$) was calculated using **Equation 4** (Dodić et al., 2012):

$$Y_{p/s} = (P_f - P_0)/(S_0 - S_f) \quad \text{Eq. 4}$$

where P_f and P_0 are the bioethanol concentrations (g/L) at the end and the beginning of the fermentation, respectively, and S_0 and S_f are the total sugar concentration (g/L) at the beginning and the end of the fermentation, respectively.

The percentage of consumed total sugars (K) was calculated using **Equation 5** (Dodić et al., 2012):

$$K = (1-S_f/S_0)\cdot 100\% \quad \text{Eq. 5}$$

where S_0 and S_f are the total sugar concentration (g/L) at the beginning and the end of the fermentation, respectively.

The required specific energy to distill the bioethanol (E) that was produced after 48 h of fermentation was calculated from **Equation 6**, which is the equation used to estimate the specific energy consumption in ethanol distilleries (Comelli et al., 2016):

$$E\ (MJ/kg_{ethanol}) = 23.025 \cdot P_f^{-0.8255} \quad \text{Eq. 6}$$

where P_f is the bioethanol concentration (g/L) at the end of the fermentation

2.6. Statistical analysis and error analysis

Statistical differences in the bioethanol yield after 48 h, the required specific energy to distill the produced ethanol, the bioethanol yield over consumed sugars, the percentage of consumed sugars, the potential maximum bioethanol yield, and the maximum bioethanol production rate were explored among the different fermentation substrates. These statistical differences among means of data obtained for samples subjected to different processes were explored using One-way analysis of variance (ANOVA) at a significance level of 0.05 with Duncan's test applied post hoc to separate means. The Shapiro-Wilk test and the Levene test were performed prior to the analysis to verify if the data followed normal distribution and homogeneity of variance, respectively. The analysis was performed with IBM SPSS Statistics 23 (IBM Corp., Armonk, N.Y., USA).

Each process and measurement was performed in triplicate to account for both systematic and random errors. The cricket frass, used in the form of dry powder, was stored at 25°C throughout the study. The crickets were reared in a controlled indoor farming system, minimizing variations in the frass composition. However, it is acknowledged that the composition of cricket frass can be influenced by factors such as diet, abiotic rearing conditions, and the age of the crickets (Psarianos et al., 2024). Hydrolysates were analyzed immediately after processing to determine their content of free sugars and free amino nitrogen (FAN). Results are expressed as mean ± SD, and error bars on the graphs represent the standard deviation of the means across replicates of the process.

2.7. Life cycle assessment

A comparative life cycle assessment (LCA) was conducted for the production of bioethanol with the different substrates. The LCA in environmental engineering involves four phases: goal definition, inventory analysis, impact analysis, and interpretation. The first phase defines objectives, the second collects data on energy and material usage, the third assesses emissions' impacts, and the final interpretation synthesizes findings. A cradle-to-gate analysis was conducted, focusing on production processes in Germany. The functional unit was defined as 1 kg of substrate, resources, or ethanol. The study evaluated the environmental impact of ethanol production in terms of kilograms, taking into account factors such as raw materials, water usage, infrastructure, and energy consumption. However, the analysis excluded the environmental effects of bioethanol distribution, use, or disposal, as these impacts are expected to be consistent across bioethanol produced from different substrates.

2.7.1. Inventory analysis

The LCI was created for lab-scale ethanol production from 5 different types of 40 g substrate and 40 g ethanol production for the conventional one. The process inventory included the main input/output data for the various stages of each process. The background footprint data of the products and processes in the LCI were obtained from SimaPro 7.2 (Pre Sustainability, Amersfoort, Netherlands). All input energies were theoretically calculated, and the calculations are provided in the Supplementary Material (**Table S2**).

The infrastructure described in *Sections 2.2*, *2.3*, and *2.4*, used during production, consumes energy. The energy requirements were calculated based on the duration of the process operation, with detailed information provided in **Table S3**.

The assumptions considered when compiling the LCI were as follows:

- As the experiments were conducted in Germany, all data, including the electricity, chemicals, and other secondary data inputs into the LCA, were based on German or European averages that are available in the database.
- The environmental impacts associated with infrastructure manufacturing, maintenance, and land area usage for the ethanol production process were ignored, considering that these factors were identical across all scenarios considered in the study.
- The study excluded the impacts associated with capital goods and infrastructure because of the absence of relevant datasets in SimaPro 7.2.
- Electricity generated from a mixed energy source (fossil and renewable energy) was assumed to supply all the processes.
- All steps involved in the production of bioethanol were taken into account.
- Assuming negligible energy losses, the heat generated during all processes was included in the calculations.

2.7.2. Life cycle impact assessment

The environmental impact assessment of bioethanol production was conducted using SimaPro 7.2 software with the ReCiPe 2016 methodology, evaluating both midpoint and endpoint indicators. Midpoint categories included land use, climate change, resource depletion, ecotoxicity, radiation, and air pollution, while endpoint indicators focused on human health, ecosystems, and resource conservation over a 100-year timeframe (Altuntas et al., 2013). The analysis, based on the ReCiPe Endpoint (H) V1.11/Europe ReCiPe H/H methodology, provided a comprehensive comparison of different substrates. The inventory referenced the distribution of 99.7% anhydrous ethanol, ensuring a detailed evaluation of the environmental footprint across various stages of production.

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2.8. Techno-economic analysis

In this study, the effects of the main raw materials used in production (biological waste, sugar cane, and biomaterials used in industrial ethanol production) were examined. Labor, maintenance, and other costs of the process were not considered. The key considerations in this analysis included feedstock costs, which significantly influence profitability and vary based on agricultural practices, transportation, and availability of materials such as rye, sugar beet, and biowaste. **Figure 1** shows the process flow, mass flow, and energy flow. The techno-economic assessment calculated the fundamental materials and operational expenses linked to the production process based on mass and energy balances. Literature and regional German vendors provided the values. The local energy and water institutions in Germany were considered as well, together with databases of German third-party providers. The original investment and operational/maintenance expenses of the equipment, apparatus, and other structures were presumed to be similar.

3. Results and Discussion

3.1. Frass hydrolysis

All pretreatment methods affected the cricket frass and generated sugars and FAN. **Figure 2a** shows the sugar concentration in every sample. The absence of enzymatic treatment led to the lowest sugar concentration of 14.37 g/L. The most efficient treatment was the one with cellulose in the acidic environment, which led to a sugar concentration of 37.03 g/L. Regarding FAN (**Fig. 2b**), the highest yield was obtained after treatment with proteases and was equal to 5 g/L. Pretreatment has been reported as one of the most important steps for the conversion of biomass to bioethanol, and it aims to expand the material surface area, dissolve hemicellulose and lignin, and reduce the biomass particle size (Kumari and Singh, 2018). Acid and alkaline hydrolysis are the most commonly applied methods (Rezania et al., 2020). The alkaline hydrolysis allows lignin removal without losses in reducing sugars (Kim et al., 2016) due to the breaking of alkyl-aryl linkages of lignin, which is facilitated under alkaline conditions (Xu et al., 2020). Additionally, the porosity of the biomass is improved during alkaline hydrolysis. This delignification has been reported to improve the enzymatic digestibility of the biomass (Bali et al., 2015; Rezania et al., 2020).

Acid hydrolysis is an effective method for degrading polysaccharides and breaking polysaccharide-lignin linkages, resulting in the release of sugar monomers (Gonzales et al., 2016). Its higher efficiency in sugar yield is attributed to its ability to degrade hemicellulose and disrupt polysaccharide-lignin linkages, enabling the recovery of most sugar monomers (Gonzales et al., 2016). This advantage makes acidic pretreatment a key strategy for sugar recovery (Rezania et al., 2020). On the other hand, biological hydrolysis, a significant pretreatment method for bioethanol production (Rezania et al., 2020), utilizes bacteria, fungi, or extracted enzymes to degrade polysaccharides into fermentable sugars. This approach also disrupts crystalline structures, breaks lignin seals, and minimizes carbohydrate losses (Wagner et al., 2018). Commonly performed with cellulases (Shrestha et al., 2017), biological hydrolysis is more energy-efficient, requires fewer harsh chemicals, and reduces the formation of

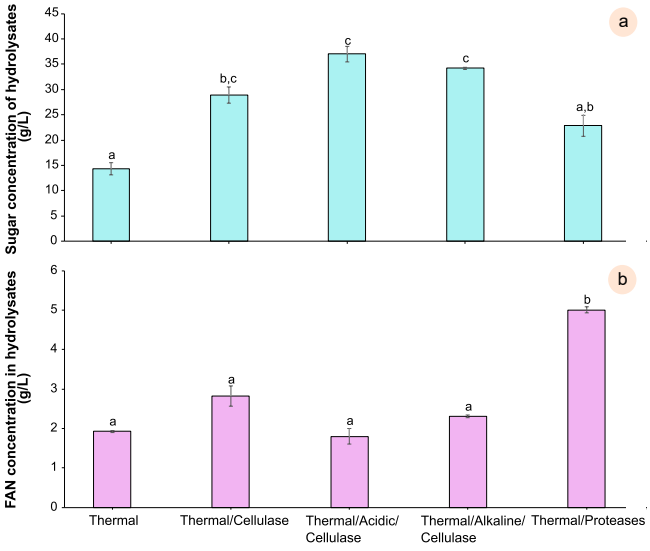

Fig. 2. Sugar concentration (g/L) (1a) and FAN concentration (g/L) (1b) in hydrolysates from frass that were subjected to different pretreatments. Error bars correspond to the standard deviation among means obtained from replicates of the same measurement. Superscript letters (a, b, c, and d) indicate significant differences ($p<0.05$) among the mean values obtained from replicates of the same measurement.

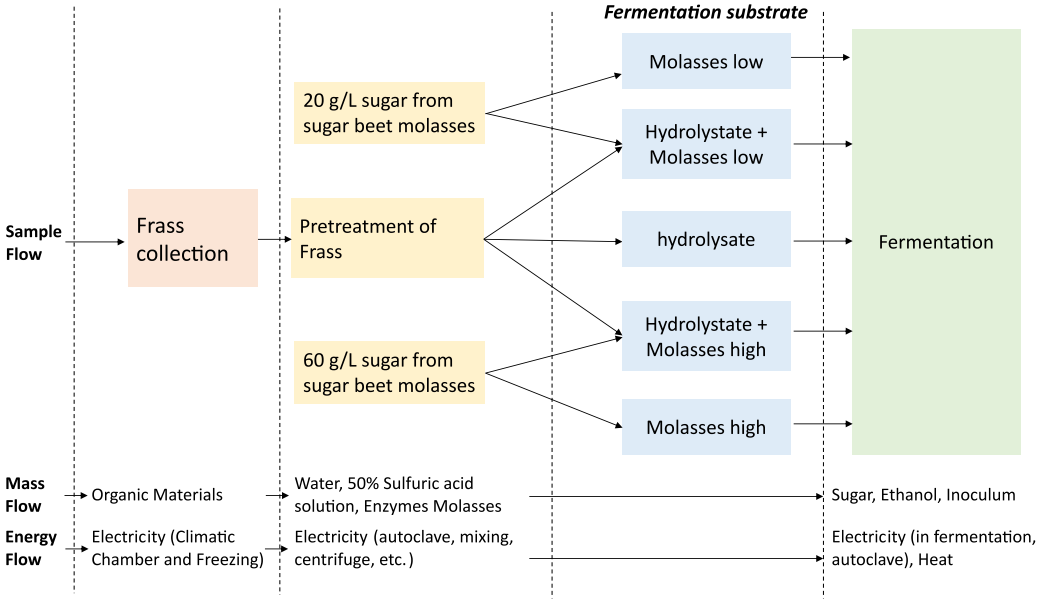

Fig. 1. Mass and Energy Flow for different ethanol production.

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inhibitory byproducts (Rezania et al., 2020). Recent advancements suggest that tailoring microbial consortia to specific biomass types can significantly enhance degradation efficiency (Vu et al., 2023).

The appropriate pretreatment for each biomass can vary. For instance, in the case of horse manure, an alkaline/enzymatic treatment was more efficient than the respective acidic because it led to higher concentrations of glucose and xylose and, therefore, to higher ethanol yield (Lee et al., 2021). Similar results were shown for cow manure (Yan et al., 2018). However, the hydrolysis conditions such as acid concentration, temperature, and hydrolysis time play a crucial role in the production of bioethanol (Carrillo-Nieves et al., 2019), as shown for feedlot cattle manure (Vancov et al., 2015). For instance, rice straw and wheat straw are some of the most commonly used feedstocks for bioethanol (Rezania et al., 2020). They are reported to generate a bioethanol yield in the range of 1.38–83.10 g/L (Fonseca et al., 2018; Molaverdi et al., 2019) and 0.44–65 g/L (Prasad et al., 2018; Yuan et al., 2018), respectively. The yield was shown to depend on the hydrolysis method (Rezania et al., 2020).

The combination of different pretreatment methods has been reported as a way to overcome the limitations of individual pretreatment methods and improve process efficiency (Shirkavand et al., 2016). Based on the results shown in **Figure 2**, it was concluded that a combination of the acidic treatment with the enzymatic treatments with cellulose and protease was the preferred pretreatment pathway to obtain a material suitable for bioethanol production. The degradation of polysaccharides in the frass through acid hydrolysis and cellulase activity was critical for the effective release of fermentable sugar monomers. The combination of acid treatment with enzymatic treatment significantly enhanced the hydrolysis of polysaccharides compared with enzymatic treatment alone. Similar results have been observed with other feedstocks, such as rice straw and corn stover, highlighting the synergistic effect of these treatments in breaking down complex biomass (Rezania et al., 2020). It has been reported that severe chemical pretreatment methods that make cellulose more available for biofuel production can be improved when they follow a biological pretreatment (Shirkavand et al., 2016). The thermal treatment alone was not efficient in disrupting the lignocellulosic structure in the frass, while the activity of proteases was more efficient in protein hydrolysis, as expected. The frass hydrolysates also exhibited sugars like glucose, disaccharides, xylose/fructose, and arabinose, as shown in *section 3.3.1*.

3.2. Yeast identification and growth

Table 2 presents the identified yeast strains. All strains demonstrated reliable identification, except for *Saccharomyces* sp. isolated from municipal solid waste, which was identified with a high probability at the genus level. However, the presence of *Saccharomyces sp.* in the municipal solid waste was understandable. One of the strains that showed a highly probable identification and reliability was *Candida glabrata* from the frass itself. All identified strains have been considered appropriate for ethanol production, including *Candida glabrata* (Gherbi et al., 2023), *Candida colliculosa* (Saygili et al., 2023), and *Candida kefyr* (Tesfaw, 2023). The growth of the strains that are shown in **Table 2** using the substrates that are mentioned in *section 2.3* are presented in **Figures S1–S10** in the supplementary material.

Table 2.
Identified yeast strains that have been isolated from various sources and were tested for their growth using hydrolysates from insect frass.

Strain Number	Source	Identified Strain	Reliability
AX58	Municipal solid waste	*Saccharomyces* sp.	88.2%
AX79	Cricket frass	*Candida glabrata*	99.9%
A93	Coconut milk	-	<75%
A97	Sugar beet juice	*Candida colliculosa*	96.1%
A662	*Saccharomyces cerevisae* (DSM 70468, Heerlen, Netherlands)	*S. cerevisiae*	95%
A663	*S. cerevisae* (DSM 2155, Heerlen, Netherlands)	*S. cerevisiae*	99.9%
A687	Fresh tomato pomace	*Candida kefyr*	96.6%

According to **Figure S1**, no strain was able to grow on frass after only thermal treatment, while the addition of sugar beet molasses to this substrate facilitated the growth of strains A687, A93, A663, and A662 (**Fig. S2**). The combination of the thermal treatment with the hydrolysis with cellulases was efficient in generating a hydrolysate that was appropriate for all strains, with and without the addition of sugar beet molasses (**Figs. S3** and **S4**). In both cases, the strain AX79 (*Candida glabrata* isolated from the cricket frass) showed the fastest growth, which reached a plateau after only 5 h. However, the Saccharomyces strains exhibited slower growth when sugar beet molasses was added, with the growth plateau reached after nearly 10 hours. Despite the slower rate, the OD was higher, indicating increased biomass production (**Fig. S4**).

The same results were observed for the frass hydrolysate obtained through thermal, acidic, and enzymatic treatment with cellulases (**Figs. S5** and **S6**), as well as for the hydrolysate obtained through thermal, alkaline, and enzymatic treatment with cellulases (**Figs. S7** and **S8**). However, the growth of all yeast strains, except for *Candida glabrata* isolated from cricket frass, was slower compared to that observed in samples subjected to no chemical treatment or acidic treatment. Hydrolysates treated only with enzymatic proteases and cellulases were less effective as substrates, as the yeasts exhibited reduced growth rates (**Fig. S9**). Adding sugar beet molasses to these substrates improved yeast growth significantly (**Fig. S10**). Among the tested yeasts, *Candida glabrata* isolated from cricket frass displayed the fastest (reaching a plateau at approximately 5 h) and highest growth, followed by all Saccharomyces strains.

The selection of the appropriate strain for bioethanol production was based both on the reliability of its identification and on its growth performance on the different substrates. *Candida glabrata* isolated from the cricket frass showed a very reliable identification and a fast and high exponential growth on almost every substrate. The addition of molasses did not affect its growth, while it facilitated and improved the growth of all the *Saccharomyces* strains. However, the identification of strains found in municipal solid waste was not reliable enough. The results for *Candida glabrata* isolated from cricket frass were promising. However, this strain poses a potential health hazard, as it has been associated with infections (Steffen et al., 2023). Therefore, the strain used for bioethanol production was *S. cerevisiae* from DSMZ (Braunschweig, Germany).

3.3. Fermentation of frass

3.3.1. Sugar identification and reduction

The reduction in total sugars during the fermentation is shown in **Figure 3**. It was observed that after 23 h, the consumption of sugars from all substrates was almost complete and had reached a plateau. A further sugar consumption was observed only for the plain sugar beet molasses at higher concentrations. The individual sugars that were measured in the substrates are presented in **Figure 4**. The main identified sugar monomers were glucose, disaccharides, xylose, and arabinose. According to **Figure 4a**, glucose was observed mainly in substrates containing the frass hydrolysate, with the sugar beet molasses showing a low glucose content. Glucose was consumed completely during the fermentation, with its content reaching 0 g/L at t=29 h of the fermentation. Regarding the disaccharides, their content was found particularly high in the substrates containing sugar beet molasses, with the plain frass hydrolysate containing only 2.30 g/L of disaccharides at t=0 h of the fermentation. The consumption of disaccharides followed the trend of the total sugars, and at the end of the fermentation, over 80% of disaccharides were consumed for all substrates.

The consumption of xylose and fructose is shown in **Figure 4c**. An amount of xylose and fructose was measured in the plain frass hydrolysate that was equal to 5.78 g/L at t=0 h of the fermentation and by t=36 h was consumed completely. The xylose/fructose content of all substrates containing sugar beet molasses showed a small increase during the first 12 h of the fermentation. A similar result was seen also for the glucose content during the fermentation with plain sugar beet molasses (**Fig 4a**). Sugar beet molasses contains a high amount of the disaccharide sucrose (Gruska et al., 2022), which is composed of glucose and fructose (de Andrade Silva et al., 2024). This increase was attributed to a partial hydrolysis of the disaccharides during the fermentation and the release of glucose and fructose, which were then consumed by the yeast.

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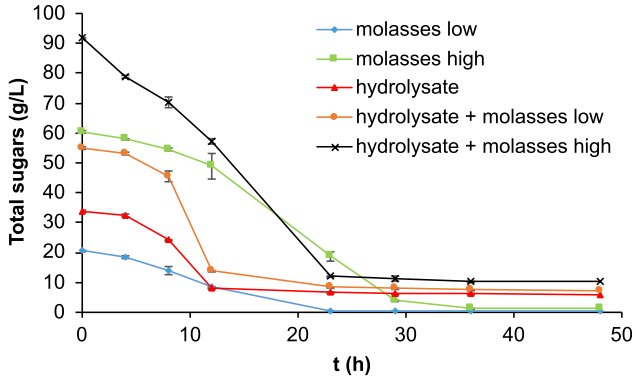

Fig. 3. Consumption of total sugars (g/L) from each substrate during the fermentation. Error bars correspond to the standard error among means obtained from replicates of the same measurement.

Arabinose was detected in the frass hydrolysates and, consequently, in the substrates containing these hydrolysates (**Fig. 4**). However, the yeast did not consume arabinose. The primary sugar monomers in the frass hydrolysates were glucose, at a concentration of 23.55 g/L, and xylose/fructose, at 5.78 g/L, prior to their consumption during hydrolysis. Glucose, a hexose, is a product of cellulose hydrolysis, while xylose and fructose, both pentoses, are products of hemicellulose hydrolysis (Rezania et al., 2020). These findings indicate that cricket frass is richer in cellulose than hemicellulose.

3.3.2. Bioethanol production

The bioethanol yield during the 48-h fermentation is presented in **Figure 5**. The most efficient substrate was the frass hydrolysate combined with the added sugar beet molasses at a high concentration, which led to a yield of 30.57 g/L. Substrates with a higher total sugar content led to a higher bioethanol yield. At t=23 h of fermentation, the plateau of bioethanol yield was reached for every sample. At the end of the fermentation, the bioethanol yield showed significant differences among substrates ($p<0.05$). The frass hydrolysate without the addition of sugar beet molasses was appropriate for bioethanol production, generating a final bioethanol yield of 10.53 g/L. The observed yield was within the range reported for a wide variety of substrates that have been fermented with *S. cerevisiae*, such as seaweed biomass, rice straw, and coconut meal (Verma and Kumar, 2021). **Table 3** discusses the bioethanol production and yield from various unconventional biomasses. The yield reported by the present study was comparable to the one reported for manure from conventional livestock. Therefore, the production of bioethanol using cricket frass as feedstock was seen as appropriate, especially considering the increasing interest in edible insects as livestock from both the academic and the industrial sectors (Megido et al., 2024). Additionally, combining acid hydrolysis with enzymatic treatment has been shown to enhance bioethanol production from manure derived from cattle, pigs, and chickens (Bona et al., 2018).

Regarding the evaluation of process efficiency (**Table 4**), significant differences in bioethanol yield over consumed sugars were observed among the substrates ($p > 0.05$).

It was observed that the bioethanol yield over consumed sugars ($Y_{p/s}$) was significantly higher ($p < 0.05$) when frass hydrolysate was combined with sugar beet molasses. Additionally, the frass hydrolysate alone exhibited a significantly higher ($p < 0.05$) $Y_{p/s}$ compared to substrates

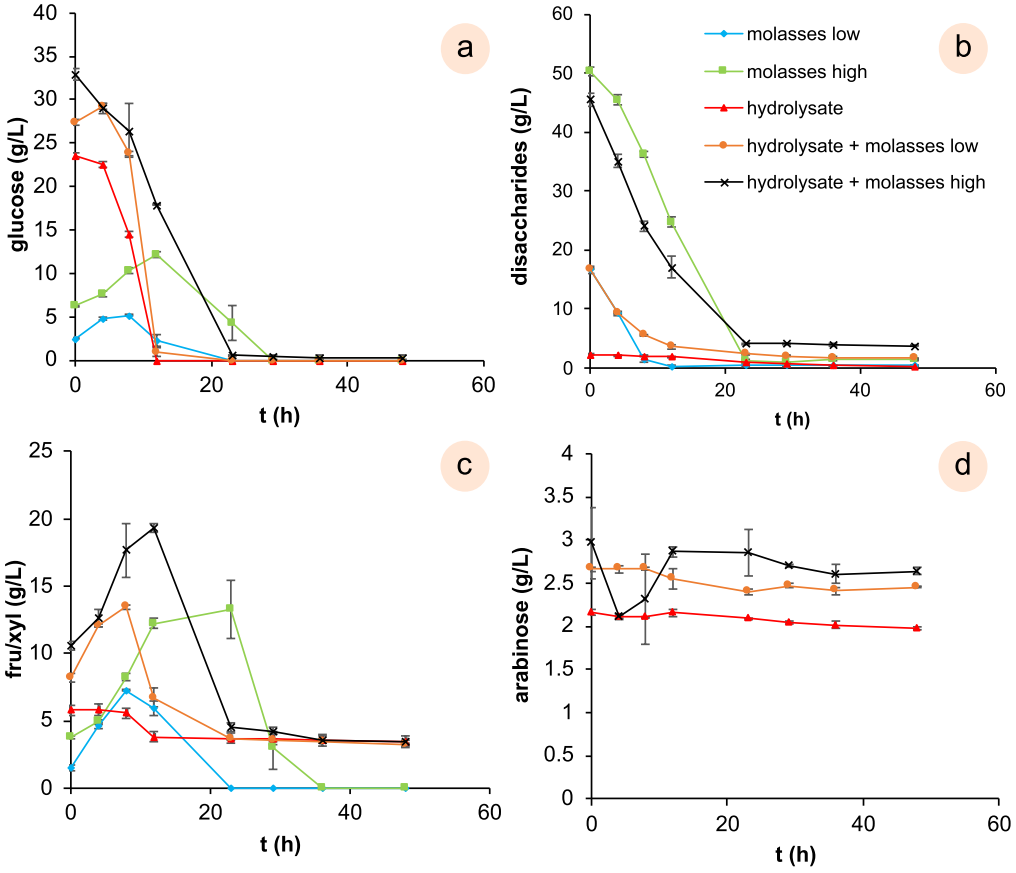

Fig. 4. Consumption of individual sugars (g/L) from each substrate during the fermentation: (a) glucose, (b) disaccharides, (c) xylose, (d) arabinose. Error bars correspond to the standard deviation among means obtained from replicates of the same measurement.

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Table 3.
Examples of studies that focus on bioethanol production from manure from conventional livestock and unconventional biomasses.

Type of biomass	Pretreatment	Maximum Bioethanol Production (g/L)	Reference
Cow manure	Acid hydrolysis with 2.5% H2SO4 at 121 °C for 30 min followed by enzymatic hydrolysis with cellulases 20 FPU/g DM for 72 h	7.30	Vancov et al. (2015)
Cow manure	Fed-batch hydrolysis with 20 FPU/g cellulose for12 h at 50 °C and simultaneous saccharification and fermentation	25.65	Yan et al. (2018)
Horse manure	Acid hydrolysis with 2% sulfuric acid at 121 °C for 2 h	7.60	Lee et al. (2021)
Poultry manure	Acid hydrolysis with 0.8 M sulfuric acid at 130 °C for 30 min	140.00	Woldesenbet et al. (2013)
Macroalgal biomass (*Sargassum* sp.)	Acid hydrolysis with 3% sulfuric acid at 121 °C for 30 min followed by 1 h of shaking at 30 °C and then by enzymatic hydrolysis with cellulases (53 FPU/g dry substrate) and β-glucosidase (30 U/g dry substrate) at 50 °C and 150 rpm for 4 h	28.70	Saravanan et al. (2018)
Coconut meal	Alkaline hydrolysis with 50% NaOH at 121 °C for 40 min followed by enzymatic hydrolysis with cellulases at 50 °C and 150 rpm for 72 h	8.50	Sangkharak et al. (2020)
Supermarket food waste from Japan	Enzymatic treatment with glucoamylases (20,000 U/g) at 180 mg protein/kg wet waste and at 50 °C, 200 rpm for 6 h	98.17	Ma et al. (2016)
Household food waste that was collected in Greece	Enzymatic treatment at 60 °C and 200 rpm for 8 h with cellulases produced from the thermophilic fungus *Myceliophthora thermophila*.	16.26	Matsakas and Christakopoulos (2015)
House cricket frass	Thermal/Acidic/Cellulases/Proteases	10.53	**Present Study**
House cricket frass combined with sugar beet molasses at 60 g/L	Thermal/Acidic/Cellulases/Proteases	30.57	**Present Study**

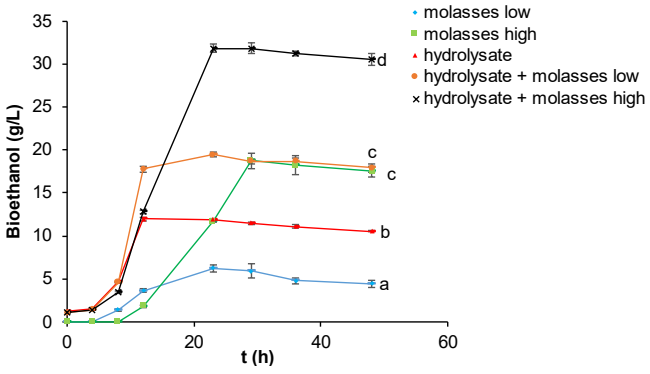

Fig. 5. Generation of bioethanol (g/L) from each substrate during the fermentation. Error bars correspond to the standard deviation among means obtained from replicates of the same measurement. Dashed lines represent the fitting of experimental data to the modified Gompertz model. Superscript letters (a,b,c, and d) indicate significant differences ($p<0.05$) among the mean values for the bioethanol production at the end of the fermentation obtained from replicates of the same measurement.

Table 4.
Indexes of the efficiency of the bioethanol production process.

Substrate	$Y_{p/s}$ (g/g)*	K	E (MJ/kg$_{ethanol}$)
Molasses low	0.22 ± 0.02^{a}	97.61 ± 0.08^{a}	6.77 ± 0.52^{a}
Molasses high	0.30 ± 0.01^{b}	97.60 ± 0.09^{a}	2.17 ± 0.06^{b}
Hydrolysate	0.34 ± 0.00^{c}	82.97 ± 0.17^{b}	3.30 ± 0.01^{c}
Hydrolysate + molasses low	0.35 ± 0.01^{c,d}	86.64 ± 0.16^{c}	2.12 ± 0.04^{b}
Hydrolysate + molasses high	0.36 ± 0.01^{d}	88.97 ± 0.12^{d}	1.37 ± 0.03^{d}

* Results are presented as mean ±SD. Superscript letters (a, b, c, and d) indicate significant differences ($p<0.05$) among the mean values obtained from replicates of the same measurement.

containing only plain sugar beet molasses. It was also observed that the energy requirement to distill the produced bioethanol was significantly different ($p<0.05$) among samples. The energy requirement became lower as the yield increased, reaching the minimum value ($p<0.05$) regarding the bioethanol generated from the frass hydrolysate combined with the added sugar beet molasses at high concentrations.

The modified Gompertz model was selected for its ability to account for the lag phase and its widespread use in describing bioethanol production from various sources, such as sugar beet raw juice and sugarcane leaf waste (Moodley and Kana, 2019). Numerous models exist to describe the fermentation profile of glucose-xylose mixtures for bioethanol production, with each tailored to specific processes, parameters, and conditions, such as batch or continuous processes, substrate composition, microorganism growth, and the effects of substrate and product inhibition. For example, high concentrations of both substrate and product can inhibit fermentation (Nosrati-Ghods et al., 2020). The modified Gompertz model was deemed suitable for estimating parameters like the maximum bioethanol production rate and potential maximum product generation, providing insights into process efficiency for each substrate (**Table 5**). The model was applied to data from all three process replicates to evaluate reproducibility before being used on mean values. It showed a good fit to the experimental data, with performance metrics ranging as follows: $0.5 < R^2 < 0.99$, $0.53 < RMSE < 1.13$, and $0.45 < \chi^2 < 2.03$ for all data sets across replicates.

Table 5.
Parameters of the modified Gompertz model that were estimated for the mean values of the bioethanol production from each substrate.

Substrate	P_m	$r_{p,m}$	t_L	R^2	RMSE	χ^2
Molasses low	5.31	0.68	6.11	0.95	0.53	0.45
Molasses high	18.78	1.09	10.00	0.98	1.13	2.03
Hydrolysate	11.40	10.19	7.54	0.97	0.79	0.99
Hydrolysate + molasses low	18.70	5.73	7.21	0.99	0.72	0.83
Hydrolysate + molasses high	31.76	3.15	7.72	0.99	1.00	1.61

The frass hydrolysate showed the highest. $r_{p,m}$, which underlines its potential as a fermentation substrate and was significantly higher ($p<0.05$) considering its variation among replicates of the same experiment. Regarding the P_m, the plain frass and the plain molasses at low concentrations show significantly lower values, with the combination of the

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two substrates leading to a higher P_m value ($p<0.05$). All samples showed comparable lag phases (h), which correspond to the experimental data since the start of the exponential growth of *S. cerevisiae* in the present study was observed at approximately 7 h (**Figs. S3–S10**). The reliability of the application of the modified Gompertz model on the data is further confirmed by the low RMSE and χ^2, apart from the high R^2 value. For instance, the application of models to describe the utilization of sweet sorghum juice for the production of bioethanol led to much higher RMSE values, especially for equations describing substrate consumption (Salakkam et al., 2023).

3.4. Life cycle assessment

The LCA comparing the environmental impacts of different ethanol production methods is presented in **Figure 6**. The results highlight the complex environmental impacts of ethanol production processes, particularly focusing on the use of hydrolysate and molasses. It reveals that these combinations significantly influence human health through factors like ozone layer depletion and human toxicity while also posing risks to ecosystems, particularly freshwater environments, due to increased ecotoxicity. Although metal consumption was negligible across all scenarios, variations in fossil fuel consumption highlighted differences in the energy sources used during production processes. The findings underscore the need for improved raw materials and production methods to mitigate ecological risks and promote the use of renewable energy, paving the way for more sustainable ethanol production practices. In every possible case, the production of fuel ethanol that is described in the present study revealed a much lower impact than the conventional fuel ethanol production that is taking place on the industrial level (**Fig. S11**).

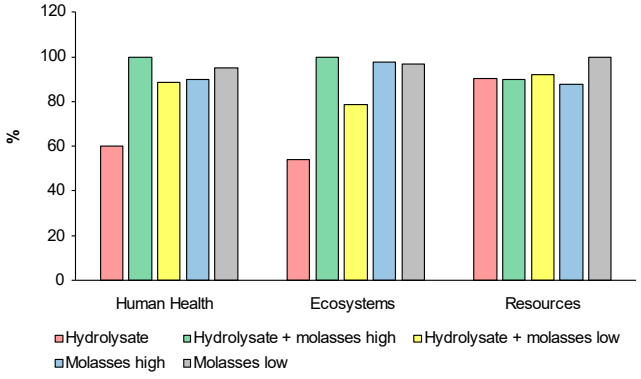

Fig. 6. Damage assessment results.

Single Score Impact Results are shown in **Figure 7**. The highest and the lowest impact values are calculated as 0.0074 and 0.0043 Pts at hydrolysate + molasses high and hydrolysate, respectively. The analysis of hydrolysate and molasses production reveals significant environmental engineering concerns, particularly regarding human health, ecosystem quality, and resource depletion. The processes involved contribute to climate change, ozone depletion, and particulate matter formation, while the addition of molasses generally exacerbates negative health impacts due to increased emissions and byproducts. Certain combinations of hydrolysate and molasses lead to heightened terrestrial acidification and freshwater eutrophication, posing risks to various ecosystems. While metal depletion is not a direct consequence, fossil resource consumption varies among production methods, indicating a need for a careful selection of combinations to mitigate these environmental impacts. Overall, the findings underscore the importance of evaluating production processes and implementing strategies to reduce adverse effects on health and ecosystems. The addition of hydrolysate as a co-substrate exhibited a varying effect on the impact value depending on the molasses concentration. Low molasses concentrations reduced the impact value, while higher concentrations increased it. Conversely, ethanol production declined with increasing molasses levels. However, this trend reversed with the addition of hydrolysate, demonstrating a positive correlation between hydrolysate addition and ethanol production. These differences in impact value can be attributed to the varying influence of the co-substrate on sugar utilization and ethanol formation during fermentation.

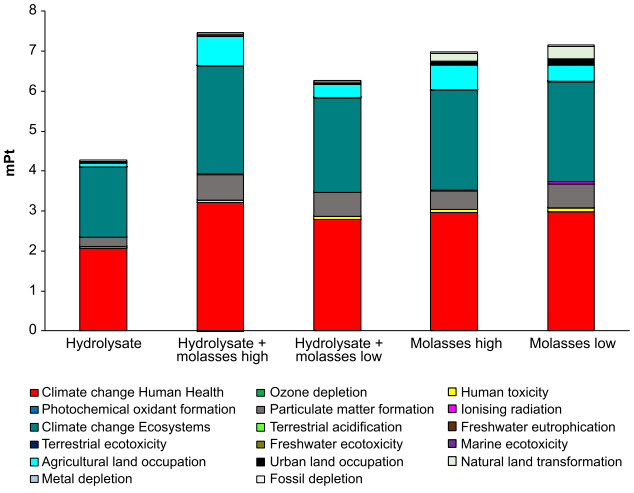

Fig. 7. Single score impact results for different substrates used for the bioethanol production.

3.5. Techno-economic analysis

Figure 8 illustrates the expenses for five distinct categories of ethanol, with laboratory-produced ethanol costing between 2.42 and 3.22 Euro/L. In comparison, the highest ethanol cost reported in the literature is 1.1 Euro/L. This threefold increase in unit cost highlights the high expenses associated with the laboratory production process, primarily due to the use of lab-scale infrastructure and the limited scale of the process. The conversion efficiency plays a vital role, as it dictates the yield of ethanol per unit of feedstock, with different production methods exhibiting varying efficiencies. The capital investment required to establish an ethanol production facility is another crucial factor heavily influenced by the choice of feedstock and technology. Furthermore, ongoing operating costs, which include energy consumption, labor, and maintenance, contribute to overall production expenses. Lastly, the potential value of byproducts generated during ethanol production, such as animal feed or chemicals, can provide additional revenue streams. Sugar beet molasses, a byproduct of sugar production, serves as a low-cost feedstock with high conversion efficiency, albeit requiring additional processing. Sugarcane ethanol benefits from high yields but is geographically limited to tropical and subtropical regions, which restricts its global applicability. Lastly, biowaste ethanol, derived from food scraps and agricultural residues, offers a sustainable alternative with generally low feedstock costs, though its conversion efficiency can be inconsistent based on the waste's type and quality. Overall, the choice of feedstock for bioethanol production involves a trade-off between cost and efficiency (Arshad et al., 2021).

4. Limitations of the study

This study is the first to explore the production of bioethanol using biomass generated during the farming of edible insects as a feedstock. House crickets, an insect species widely produced for food in various regions, generate cricket frass as a byproduct, posing a challenge for producers. This study demonstrates that cricket frass can indeed be used for bioethanol production. However, as a case study, it has limitations, particularly in addressing all aspects of the process. A variety of physical, chemical, and biological pretreatment methods exist for degrading the lignocellulosic structure of biomass to produce fermentable sugars (Rezania

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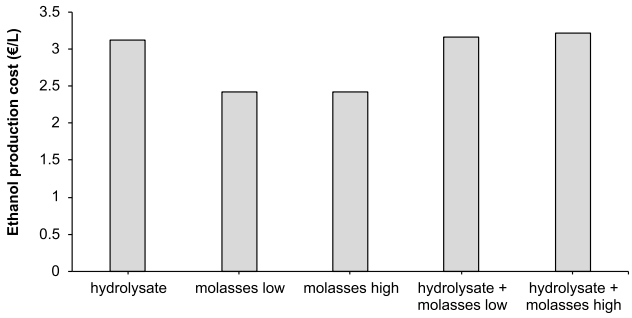

Fig. 8. Ethanol production cost.

et al., 2020), but this study evaluates only a limited number of these methods.

Additionally, the pretreatment methods and processing conditions used in this study have not been optimized. A broader range of enzymes, microorganisms, and chemical hydrolysis methods is necessary to enhance the conversion of frass to bioethanol. Predictive and optimization tools, such as artificial neural networks and response surface methodology, are commonly employed in bioethanol production (Coşgun et al., 2023) and could be used to design a tailored process with optimized conditions. Moreover, the study did not explore the variety of processing pathways available for bioethanol production. Inhibitors formed during pretreatment methods, which can hinder bioethanol production (Sjulander and Kikas, 2020), were not investigated. Additionally, downstream processes were not explored, and the study does not provide insights into the environmental sustainability of the production process. The variability in the composition of insect frass, influenced by abiotic rearing conditions and life stages (Psarianos et al., 2024), presents another challenge. Variations in the protein and polysaccharide content of frass could significantly impact the process. Furthermore, different edible insect species produce frass with distinct physicochemical properties. As such, the process described in this study is not standardized for all insect-rearing systems, limiting its broader applicability.

A major limitation of this study lies in its practical application. According to the LCA results, using frass as a feedstock is more sustainable than using sugar beet molasses or a combination of both. Specifically, frass as the sole feedstock was shown to have positive impacts on both human health and ecosystems compared to the other feedstocks. However, it also resulted in the lowest yield and highest cost, as indicated by the techno-economic analysis. A more holistic approach, including a detailed analysis, could identify potential solutions to bridge the gap between process efficiency, cost reduction, and sustainability. Furthermore, the study was conducted on a laboratory scale, where the infrastructure is not exclusively dedicated to this process. To assess its industrial applicability, the process needs to be scaled up and evaluated under real-world production conditions.

5. Practical implications and industrial relevance

Since frass is a non-edible feedstock, the bioethanol produced from it is classified as second-generation (2G) bioethanol. This is an advantage since frass does not compete with food sources. There is an increasing demand for biofuels and bioethanol, in particular, since fuel ethanol is one of the most commonly used additives to gasoline (Aghaei et al., 2022). This demand comes from the need to reduce fossil fuels, which are responsible for the majority of the global greenhouse gas emissions and the emitted CO_2 (Bashir et al., 2024). The replacement of fossil fuels with biofuels is one of the strategies that have been suggested to tackle the use of fossil fuels. Of course, further strategies have been suggested, such as the intervention of fossil fuel prices (Khatibi et al., 2024). The demand for biofuels and the already high use of fuel ethanol make the process in the current study highly relevant. House crickets are an insect species that is being farmed for food purposes (Pilco-Romero et al., 2023). The amount of frass that is produced globally from insect farming is estimated to be thousands of tonnes per year and 40 times larger than the produced animal biomass (Poveda, 2021; Rossi et al., 2024). The large scale of insect farming and the increasing interest in edible insects will create a demand for the utilization of frass. The large-scale nature of insect farming underscores the potential of the process presented in this study for broader application and industrial scalability.

A key consideration in this study is the environmental and socio-economic sustainability of the process. Fuel ethanol is expected to have a low market price, with reported costs ranging from 0.22 to 1.11 Euro/L. However, market fluctuations and high production costs can make 2G bioethanol more expensive (Correia et al., 2024). According to the techno-economic analysis performed in this study, incorporating frass as a feedstock increased production costs. Despite this, the pressing demand for a transition to renewable energy sources is undeniable (Gheewala, 2023). The process described in this study was found to be more sustainable than conventional methods. Utilizing frass for ethanol production demonstrated lower impacts on human health and ecosystems compared to other feedstocks, aligning with the United Nations' Sustainable Development Goals (SDGs) for good health and well-being, affordable and clean energy, climate action, and the protection of life below water and on land. With the European Union's recent initiatives to transition toward a more sustainable economy, the adoption of 2G biofuels like those explored in this study is particularly timely and relevant.

Similarly, an LCA performed on bioethanol production from cattle manure revealed that the drying step had the highest environmental impact. The study also demonstrated that producing bioethanol from manure positively affected climate change mitigation and fossil resource depletion by eliminating residual raw materials and disposal treatment (de Azevedo et al., 2017). In the case of cricket frass, the drying step was unnecessary due to its low moisture content, highlighting the sustainability of the process proposed in this study compared to similar biomasses. Furthermore, since ethanol yield was shown to improve when frass was combined with sugar beet molasses, it can be suggested that combining frass with other non-edible feedstocks, such as manure, could lead to a more efficient and sustainable bioethanol production process.

6. Conclusions and future directions

The present study shows that frass from house crickets is appropriate for the production of bioethanol. Different methods for hydrolysis of frass, such as acidic, alkaline, cellulases, and proteases, are appropriate to generate free sugars and FAN and facilitate the growth of various yeast strains. A combined pretreatment of acidic hydrolysis with enzymatic treatment with cellulases and proteases was successfully applied for bioethanol production. The addition of sugar beet molasses can enhance the bioethanol yield. Cricket frass shows major utilization potential for the production of biofuel. Future directions should focus on optimizing the bioethanol production process by refining pretreatment methods and exploring a wider range of enzymes, as well as investigating frass from other insect species. Alternative processes, such as simultaneous saccharification and fermentation, should also be tested to enhance efficiency. Additionally, the presence of inhibitors in the process must be thoroughly investigated, and if inhibitors are identified, appropriate mitigation strategies should be developed to address their impact. The use of frass as a feedstock was found to be more sustainable than its combination with sugar beet molasses but came with higher production costs. To address these challenges, a more holistic approach is necessary, and the process should be tested on a larger scale to identify solutions that balance high efficiency, sustainability, and cost-effectiveness. One potential solution is combining frass with other biomasses that have been demonstrated to be sustainable feedstocks for ethanol production, which could enhance process efficiency while maintaining environmental benefits.

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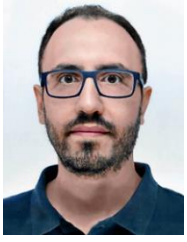

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Supplementary Material

1. Identification of cellulose and hemicellulose in insect frass

The dry weight of the frass was measured gravimetrically after drying the sample overnight at 105 °C overnight. The protein content of frass was measured by applying the 6.25 conversion factor to the nitrogen content that was estimated with the Kjeldahl method (AOAC, 2000 - method 992.15). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were estimated gravimetrically after digestion at 100°C with neutral and acid detergent, respectively, followed by drying for 3 h at 105 °C. Further digestion with 70% sulfuric acid for 3 h was used to estimate the acid detergent lignin (ADL). The difference between NDF and ADF is used to estimate hemicellulose content, while the difference between ADF and ADL (Acid Detergent Lignin) is used to estimate cellulose content (Peguero et al., 2023).

Table S1.
Chemical characterization of frass from house crickets.

Dry matter (%)	Crude protein (g/100g FM)	Cellulose (g/100g DM)	Hemicellulose (g/100g DM)	Lignin (g/100g DM)	Ash (g/100g DM)
85.42	37.01	12.31	9.41	3.50	1.64

Figures S1 - S10 show the growth of all yeast strains using as substrate the insect frass after all hydrolysis processes, with and without the addition of molasses.

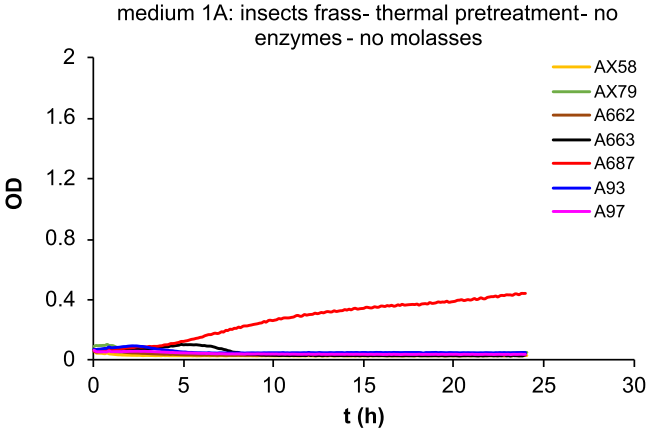

Fig. S1. Growth of yeast strains with frass hydrolysates after thermal treatment and without the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

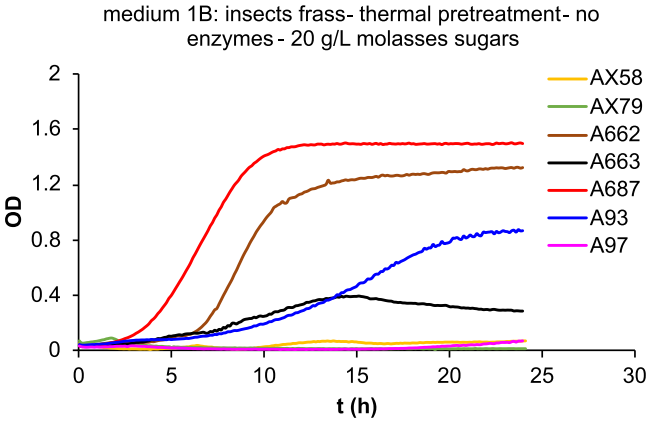

Fig. S2. Growth of yeast strains with frass hydrolysates after thermal treatment and with the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

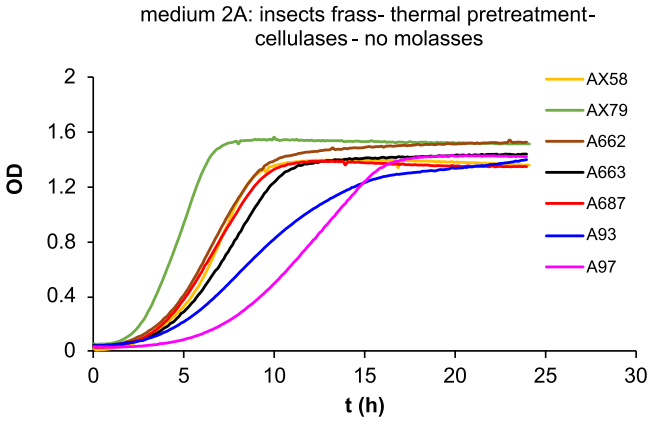

Fig. S3. Growth of yeast strains with frass hydrolysates after thermal and enzymatic treatment with cellulases and without the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

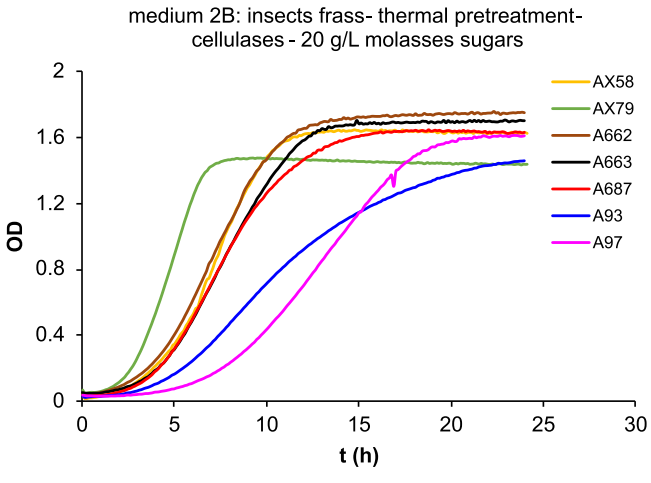

Fig. S4. Growth of yeast strains with frass hydrolysates after thermal and enzymatic treatment with cellulases and with the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

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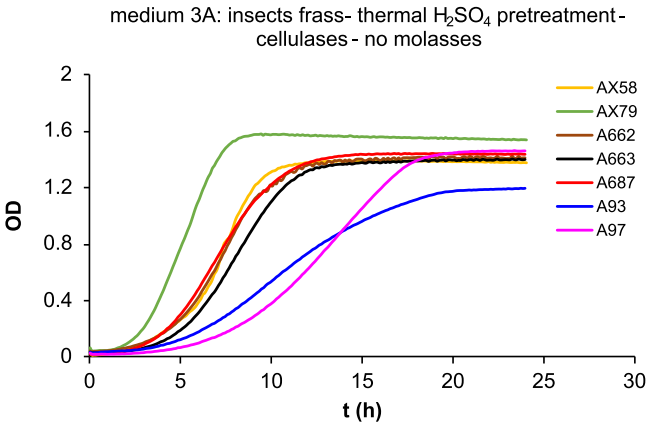

Fig. S5. Growth of yeast strains with frass hydrolysates after thermal, acidic, and enzymatic treatment with cellulases and without the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

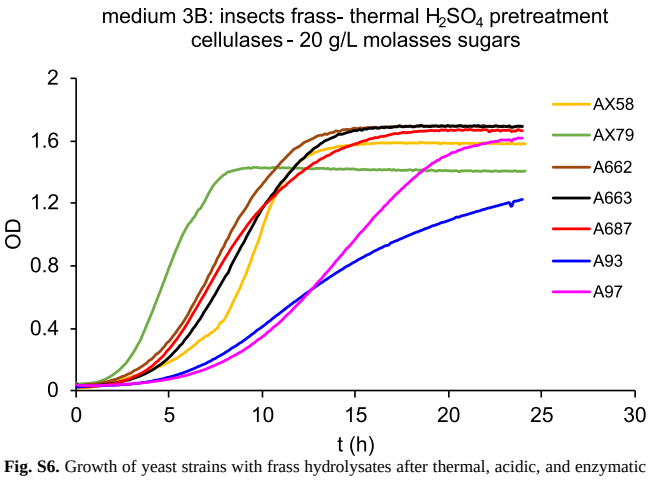

Fig. S6. Growth of yeast strains with frass hydrolysates after thermal, acidic, and enzymatic treatment with cellulases and with the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

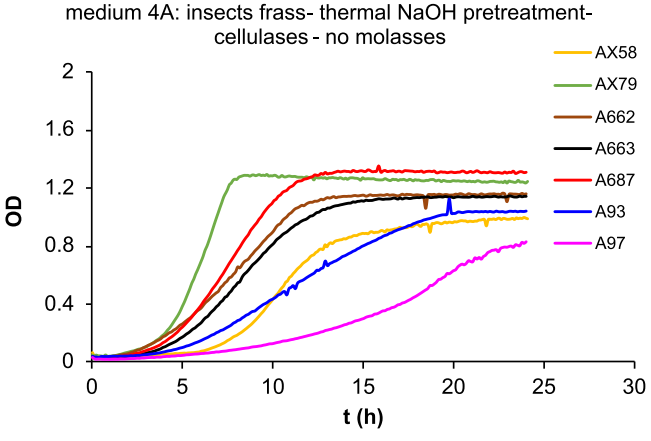

Fig. S7. Growth of yeast strains with frass hydrolysates after thermal, alkaline, and enzymatic treatment with cellulases and without the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

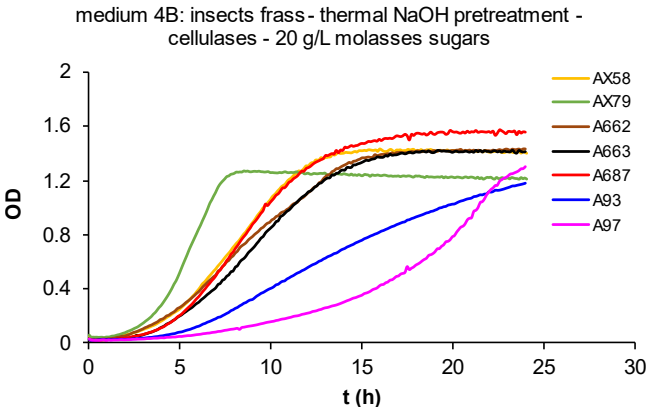

Fig. S8. Growth of yeast strains with frass hydrolysates after thermal, alkaline, and enzymatic treatment with cellulases and with the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

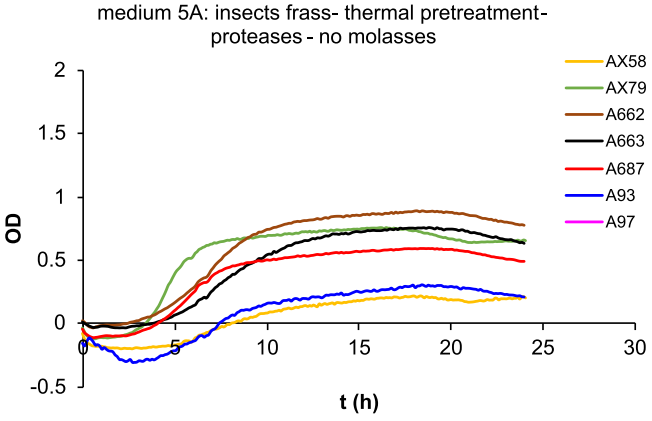

Fig. S9. Growth of yeast strains with frass hydrolysates after thermal and enzymatic treatment with cellulases and proteases, without the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

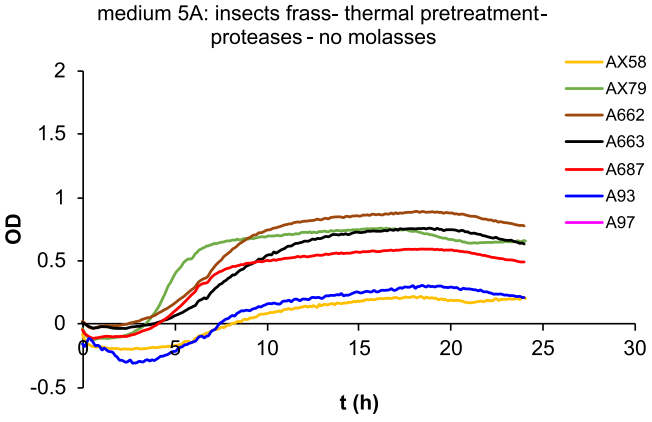

Fig. S10. Growth of yeast strains with frass hydrolysates after thermal and enzymatic treatment with cellulases and proteases, with the addition of sugar beet molasses. The data shown correspond to the means obtained from replicates of the same measurement.

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2. Life cycle assessment (LCA)

Table S2.
Background datasets adopted from SimaPro 7.2 for representing the material and energy parameters considered during the life cycle assessment.

Field	Adopted datasets
Electricity	Electricity, medium voltage, at grid [for Europe] | Unit process
Heat	Heat, at cogeneration with biogas engine, allocation exergy [for Europe] | Unit process
Heat	Heat, natural gas, at industrial furnace > 100 kW [for Europe] | Unit process
Water	Tap water, at user [for Europe] | Unit process
Water	Water, cooling, unspecified natural origin [for Europe] | Unit process
H_2SO_4	Sulfuric acid, liquid, [for Europe] | Unit process
Enzyme	Enzymes as an Organics [for Europe] | Unit process
Ethanol	Ethanol fermentation plant [for Europe] | Unit process
Molasses	Molasses from sugar beet, at sugar refinery [for Europe] | Unit process

Table S3.
Inventory data for different ethanol products*.

Processes	Input/Output	Units	Hydrolysate	Molasses low	Molasses high	Hydrolysate + Molasses low	Hydrolysate + Molasses high
Substrate Preparation							
Material inputs	Frass	g	50.82	0	0	50.82	50.82
	Water	mL	432.21	500	500	432.21	432.21
	Enzymes (cellulase)	g	4.3197	0	0	4.3197	4.3197
	Enzymes (Protease)	g	0.8639	0	0	0.8639	0.8539
	50% sulfuric acid solution	g	13.1058	0	0	13.1068	13.1058
	Sugars beet molasses	g	0	10	30	8.5442	25.9325
Autoclaving (for hydrolysate)	Electricity (121°C for 15 min)	kWh	0.0875	-	-	0.0875	0.0875
Mixing (for hydrolysate)	Electricity (at 200rpm with a blade impeller for 48 h)	kWh	0.0144	-	-	0.0144	0.0144
Centrifuge (for hydrolysate)	Electricity (at 4800 rpm for 15 min)	kWh	0.0075	-	-	0.0075	0.0075
Fermentation							
Autoclaving (for fermentation substrates)	Electricity (100°C for 15 min)	kWh	0.0625	0.0625	0.0625	0.0525	0.0625
Ethanol Production	Inoculum	g	3.20	3.20	3.2	3.20	3.2
	Sugar (total fermentable/input)	g	0.8242	2.4149	1.3513	2.2038	3.6788
	Sugar (output)	g	0.0197	0.0579	0.2301	0.2943	0.4057
	Ethanol (input)	g	0	0	0.0454	0.0424	0.0408
	Ethanol (output)	g	0.1777	0.7000	0.4211	0.7176	1.2227
	Electricity	Wh	0.0083	0.0051	0.0182	0.0255	0.0503
Molasses substrate production	Heat (during fermentation process)	MJ	-	0.0777	0.2254	0.0777	0.2254

** Input parameters of conventional ethanol production taken from SimaPro 7.2. The data in this table are the process and material/energy data used during production.*

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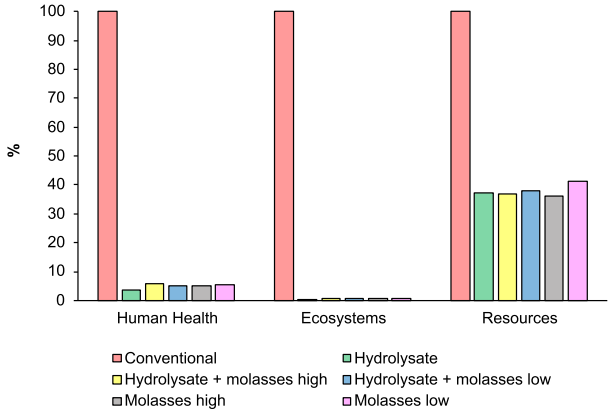

Fig. S11. Damage assessment of bioethanol production in comparison to the conventional fuel ethanol as estimated with average values from Germany and the European Union.

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[1] Peguero, D.A., Gold, M., Endara, A., Niu, M., Zurbrügg, C., Mathys, A., 2023. Evaluation of ammonia pretreatment of four fibrous biowastes and its effect on black soldier fly larvae rearing performance. Waste Manage. 160, 123-134.

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