NEWFEED

Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes

Grant Agreement number: 2013, Call 2020 Section 1 Farming IA

Orange peel valorisation strategy: including the peels composition, the optimized process and the setting up of pilot plant

Deliverable number 2.2

Work Package 2	Optimization of the Valorisation and Feeding strategies
Task 2.2	Case study 2: Orange peel-based ingredients for dairy sheep
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Foreword

The work described in this report was developed under the project NEWFEED: Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes (Grant Agreement number: 2013/ Call 2020 Section 1 Farming IA). If you wish any other information related to this report or the NEWFEED project please visit the project web-site (www.newfeed-prima.eu) or contact:

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Executive Summary

This is Deliverable D2.2 of the NEWFEED project “Turn food industry by-products into secondary feedstuffs via circular-economy schemes”, which documents the progress of Task 2.2 “Case study 2: orange peel-based ingredients for dairy sheep”. NTUA is the responsible partner for this task, SEVT and HAO-Demeter are other involved partners. The objectives of this deliverable were to define and optimize an innovative valorisation strategy to turn orange juice industry by-products into high value secondary feedstuff for animal feed. A sourcing of orange peels in a safe manner and the set up the pilot plant for the scaling up process in WP 3 are also included.

In view of orange peels sourcing, Hellenic Fruit Juices (www.hfj.gr) has agreed to stand as the orange juice industry that provides the raw material for all experimentation.

Optimisation of drying of the unprocessed orange peel waste took place and the preliminary animal trials with unprocessed orange peels were successful implemented.

The 1st integrated valorisation strategy (Strategy A), that was examined, included the production of advanced bioethanol and animal feed for ruminants. The concept was to take advantage of the sugar content of the hydrolysed substrate towards bioethanol while the hydrolysed, fermented residue enriched in protein could stand as an interesting feedstuff. Nevertheless, according to the experimental results, the fermentation process was inhibited, resulting in low ethanol yields (6-15%). Therefore, the prospect of recovering a biofuel and simultaneously producing advanced animal feed was abandoned and an alternative strategy was examined.

In the context of strategy B, the enzymatic hydrolysis of orange peels was studied, from which a liquid fraction rich in sugars and a hydrolyzed solid residue were obtained. The liquid fraction is used for yeast cultivation with the ultimate goal of producing single cell protein. The latter is mixed with the hydrolyzed solid residue to produce advanced animal feed. Finally, the formulated feedstock shall be dried in order to stabilize the product in terms of shelf life and feed safety. The saccharification process and the aerobic fermentation were optimised. According to the optimised conditions, the final animal feedstuff should be formulated by mixing the solid residue of orange peels after the saccharification process under the optimum conditions (50°C, 24h, 7.5% solids loading, Pectinex 25μL/g TS, CellicCTec3 25 μL/g TS) with the harvested yeast cultivated aerobically on orange peels hydrolysate (30°, 24h, orange peels hydrolysate as sugar source, nutrients addition,

pH =5, pH and DO control). The final feedstuff was a mixture of excess yeast and hydrolysed solid (14% yeast) with 23,11% higher in vitro organic matter digestibility and doubled protein content.

A pre-existing pilot plant installed in the premises of NTUA was used for the successful validation of the processes in pilot scale.

All the objectives of this deliverable as defined in the DoA have been fully achieved.

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1. Introduction

The overall objective of the project is to develop alternative feed ingredients using a circular economy approach through the conversion of food by-products into high-value secondary animal feed.

The purpose of this Deliverable is to define the orange peel valorisation strategy: including the orange peels composition, the optimized process, and the setting up of pilot plant. This process is included in the 2nd case study developed in the project:

- ✓ The 2nd case study will evaluate the use of orange peels as a second-generation ingredient for dairy sheep feed (Task 2.2). This case study is led by NTUA and will be validated in a continuous operating pilot plant, with real feedstock and under optimised conditions.

The analysis of the data obtained will allow the definition of the optimized process for the scaling up in the WP3 to ensure the successful valorisation of these by-products as alternative ingredients for animal feed.

Orange peel waste is a byproduct generated in large quantities during the juice extraction process in the citrus industry. Due to its high content of bioactive compounds and organic matter, orange peel waste has the potential to be used in various industrial applications, thus reducing the environmental impact associated with its disposal. One of the most promising applications of orange peel waste is in the production of animal feed, where it can be used as a source of dietary fiber, pectin, and antioxidants. In addition, orange peel waste can be used as a source of natural pigments and flavors in the food industry, particularly in the production of confectionery, bakery products, and beverages. Another potential application of orange peel waste is in the production of biofuels and biochemicals. The high concentration of carbohydrates in orange peel waste makes it a suitable substrate to produce ethanol, which can be used as a fuel or a feedstock for the production of other chemicals. Furthermore, orange peel waste can be used as a source of essential oils, which can be used in the fragrance and cosmetics industries. Other industrial applications of orange peel waste include the production of biodegradable polymers, wastewater treatment, and the remediation of contaminated soils. However, the utilization of orange peel waste in industrial applications is still limited by several factors, such as the high variability in composition and the lack of standardization in processing methods. Thus, orange peel waste is a promising source of value-added products that can be used in various industrial applications.

Orange peels are used in animal feed since they contain a range of nutrients that can be beneficial to animals, including fiber, carbohydrates, vitamins, and minerals. Additionally, the peel contains essential oils that can have antimicrobial and antioxidant properties, which can help improve animal health and welfare. Orange peel can be used as a partial replacement for traditional feed sources, such as corn or soybean meal, in ruminant and non-ruminant diets (Andrianou et al., 2023; Tahir et al., 2023). Nevertheless, the full-scale case studies/success stories that have been applied mainly involve drying as a treatment step. Thus, there is no alteration or upgrade in the composition of the raw material, apart from the removal of moisture. The only example of upgrade of orange peels as animal feed ingredient is the production of silage from orange peels where few physico-chemical processes occur. Within NEWFEED project, the 2nd case study aspires to produce an improved animal feed ingredient with increased protein content and higher digestibility thus meeting the big challenges in the animal feed industry.

2. Methodology

2.1 Chemical analysis

2.1.1 Determination of total solids and moisture in biomass

Introduction

Biomass samples can contain large and varying amounts of moisture, which can change quickly when exposed to air. To be meaningful, the results of chemical analyses of biomass are typically reported on a dry weight basis.

Parts of this procedure are similar to ASTM E1756-01 and T412 om-02.

The following procedure describes the methods used to determine the amount of solids or moisture present in a solid or slurry biomass sample. It also covers the determination of dissolved solids in a liquor sample.

Apparatus

- Convection drying oven, with temperature control of 105 ± 3°C
- Analytical balance, accurate to 0.1 mg
- Desiccator containing dessicant
- Convection drying oven, with temperature control of 105 ± 3°C

Materials

- Aluminum pans, made to fit infrared moisture analyzer if necessary
- Glass fiber pads for liquor samples
- 0.2 µm pore size filters, either large syringe filters with syringes or 50 mm filter units, for liquor samples only

Procedure

Solid samples usually require 0.5 to 2 grams, slurry samples require 2-5 grams, and liquor samples require 10 mL, per duplicate. Liquor samples should be filtered through a 0.2 µm pore size filter prior to analysis.

Pre-dry aluminum weighing dishes by placing them in a 105 ± 3ºC drying oven for a minimum of four hours. Cool the dishes in a desiccator. Using gloves or tweezers to handle the dishes, weigh a pre-dried dish to the nearest 0.1 mg. Record this weight.

Thoroughly mix the sample and then weigh out an appropriate amount to the nearest 0.1 mg, into the weighing dish. Liquor samples should be passed through a 0.2 µm filter prior to analysis. Record the weight of the sample plus weighing dish. Analyze each sample in duplicate, at minimum.

Place the sample into a convection oven at 105 ± 3ºC for a minimum of four hours. Remove the sample from the oven and allow it to cool to room temperature in a desiccator. Weigh the dish containing the oven-dried sample to the nearest 0.1mg and record this weight.

Place the sample back into a convection oven at 105 ± 3ºC and dry to constant weight. Constant weight is defined as ± 0.1% change in the weight percent solids upon one hour of re-heating the sample. Overnight drying is usually required for very wet or liquid samples (Sluiter et al., 2008).

Calculations

Calculate the percent total solids for a liquor sample, on a 105ºC dry weight basis as follows:

$$\%\ \boldsymbol{Total\ Solids} = \frac{Weight_{dry\ pan\ plus\ dry\ sample} - Weight_{dry\ pan}}{Weight_{sample\ as\ received}} \cdot 100 \qquad \textit{Equation 1}$$

The percent moisture can also be calculated:

$$\%\ \boldsymbol{Moisture} = 100 - \frac{Weight_{dry\ pan\ plus\ dry\ sample} - Weight_{dry\ pan}}{Weight_{sample\ as\ received}} \cdot 100 \qquad \textit{Equation 2}$$

2.1.2 Determination of volatile mass and ash in biomass

Introduction

The amount of inorganic material in biomass, either structural or extractable, should be measured as part of the total composition. Structural ash is inorganic material that is bound in the physical structure of the biomass, while extractable ash is inorganic material that can be removed by washing or extracting the material.

This procedure is substantially similar to ASTM Standard Method Number E1755-01 “Standard Method for the Determination of Ash in Biomass”.

This test method covers the determination of ash, expressed as the percentage of residue remaining after dry oxidation at 550 to 600ºC. All results are reported relative to the 105ºC oven dry weight of the sample.

Apparatus

- Muffle furnace, equipped with a thermostat, set to 575 + 25 °C
- Analytical balance, accurate to 0.1 mg.
- Desiccator containing desiccant
- Ashing crucibles, 50 mL, porcelain, silica, or platinum
- Porcelain markers, high temperature, or equivalent crucible marking method
- Ashing burner, ignition source, tongs, and clay triangle with stand
- Convection drying oven, with temperature control of 105 ± 3°C, optional

Procedure

Using a porcelain marker, mark an appropriate number of crucibles with identifiers, and place them in the muffle furnace at 575 +25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator. If using a furnace set to 575 + 25 °C, cool for a specific period of time, one hour is recommended. Record the cool time. Weigh the crucibles to the nearest 0.1 mg and record this weight.

Place the sample back into the muffle furnace at 575 ± 25°C and dry to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

Weigh 0.5 to 2.0 g, to the nearest 0.1 mg, of a test specimen into the tared crucible. Record the sample weight. If the sample being analyzed is a 105°C dried test specimen, the sample should be stored in a desiccator until use.

Ash the samples using a muffle furnace set to 575 + 25 °C for 24 + 6 hours. When handling the crucible, protect the sample from drafts to avoid mechanical loss of sample.

Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.

Place the sample back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the

crucible. When allowing samples to cool in a desiccator, it is necessary to maintain the initial cool time (Sluiter, Hames, et al., 2005).

Calculations

Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content.

$$ODW = \frac{Weight_{air\ dry\ sample} \cdot \%\ Total\ solids}{100} \qquad \textit{Equation 3}$$

where:

ODW_{sample} = weight of sample in milligrams

Calculate and record the percentage ash on an ODW basis.

$$\%\ Ash = \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{ODW_{sample}} \cdot 100 \qquad \textit{Equation 4}$$

$$\%\ Volatile\ Solids = 100 - \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{ODW_{sample}} \cdot 100 \qquad \textit{Equation 5}$$

2.1.3 Determination of extractives in biomass

Introduction

It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material, among others. Inorganic material in the water soluble material may come from both the biomass and any soluble material that it is associated with the biomass, such as soil or fertilizer. Some biomass may require both extraction steps, while other biomass may only require exhaustive ethanol extraction.

This method is similar to ASTM Standard Test Method E 1690 “Determination of Ethanol Extractives in Biomass” for extraction procedures for isolation and characterization of extractives.

This procedure covers the determination of soluble non-structural materials in a biomass sample. The results are reported, on a dry weight basis, as a weight percentage of the biomass. Extractives percentages are measured and used to convert compositions from an extractives-free basis to and as-received basis.

Apparatus

- Analytical balance, accurate to 1 mg or 0.1 mg
- Medium to large capacity oven set to 105 + 5 °C for glassware drying
- Vacuum oven set to 40 + 2 °C or drying oven set to 45 + 2 °C
- Apparatus for extraction, either Soxhlet or automatic
- Heating mantles, suitable for 500 mL boiling flasks
- Rotary evaporator with trap and water bath set to 40 + 5 °C

Reagents and materials

Reagents

- Water, HPLC grade
- Ethyl alcohol, 190 proof, USP grade

Materials

- Materials necessary for extraction
- Boiling flasks, round bottom, 500 mL capacity, 24/40 joint, equal to the number of extractions desired
- Teflon boiling chips or stir bars (stir bars may only be used with heating mantles equipped with stirring capacity)
- Cellulose filter paper, medium porosity, of appropriate size
- Buchner funnels, for paper diameter 70 mm or larger
- 200 mL volumetric flasks,
- Desiccator(s) containing desiccant, of a volume large enough to accommodate appropriate glassware

Procedure

Prepare the sample for extraction.

The moisture content of a biomass sample can change rapidly when exposed to air. Weigh samples for total solids determination at the same time as the samples for the extractives determination to avoid errors due to changes in humidity.

Prepare the apparatus for extraction.

Dry boiling flasks and other relevant glassware in a 105 + 5 °C drying oven for a minimum of 12 hours. Remove the glassware and allow it to come to room temperature in a desiccator. Add boiling stones (or stir bars if using heating mantles with stirring capacity) to the flasks, label clearly, and record the oven dry weight (ODW) to the nearest 0.1 mg.

Add 2-10 g of sample to a tared extraction thimble. Record the weight to the nearest 0.1 mg. The amount of sample necessary will depend on the bulk density of the biomass. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube. If the biomass height does exceed the siphon height, incomplete extraction will occur. Label the top edge of the thimble with a pencil.

Assemble the Soxhlet apparatus. Add a 250 mL bump trap between the receiving flask and the Soxhlet tube to control foaming if necessary. Insert the thimble into the Soxhlet tube.

Analyze the sample for water extractives

Add 190 + 5 mL of HPLC grade water to the tared receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 4-5 siphon cycles per hour.

Reflux for 6-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate. In some biomass, the reflux time is usually around eight hours, and any remaining water soluble material will be extracted during the ethanol extraction.

When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature.

If a successive ethanol extraction is to be performed, leave the thimble in the Soxhlet extractor, removing as much residual water from the Soxhlet tube as possible. If an ethanol extraction is not necessary, remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh HPLC grade water. Allow the solids to dry using vacuum filtration or air dry (Sluiter, Ruiz, et al., 2005).

Calculations

Calculate the amount of extractives in the sample, on a percent dry weight basis.

$$\%\ \boldsymbol{Extractives} = \frac{\boldsymbol{Weight}_{flask\ plus\ extractives} - \boldsymbol{Weight}_{flask}}{\boldsymbol{ODW}_{sample}} \cdot \mathbf{100} \qquad \textit{Equation 6}$$

2.1.4 Determination of nitrogen in biomass

Introduction

The Kjeldahl method is used to determine the nitrogen content in organic and inorganic samples. For longer than 100 years the Kjeldahl method has been used for the determination of nitrogen in a wide range of samples. The determination of Kjeldahl nitrogen is made in foods and drinks, meat, feeds, cereals and forages for the calculation of the protein content. Also the Kjeldahl method is used for the nitrogen determination in wastewaters, soils and other samples. It is an official method and it is described in different normatives such as AOAC, USEPA, ISO, DIN, Pharmacopeias and different European Directives. The Kjeldahl procedure involves three major steps: digestion, distillation and titration.

Apparatus

- Analytical balance, accurate to 1 mg or 0.1 mg
- Medium to large capacity oven set to 105 + 5 °C for glassware drying
- Distillation apparatus (Gerhard Vapodest 30s): 500- to 900-ml Kjeldahl digestion flask connected to distillation trap by rubber stopper; distillation trap connected to condenser with low-S tubing (outlet of condenser tube should be <4 mm in diameter)
- Digestion apparatus (Gerhard Kjeldatherm KB / KBL)

Reagents and materials

Reagents

- Salicylic acid/ Concentrated sulfuric acid solution
- Catalyst mixture (K_2SO_4 - $CuSO_4 \cdot 5H_2O$ - TiO_2)
- Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5\ H_2O$)
- Boric acid solution

- Concentrated sulfuric acid (H_2SO_4; 95% to 98%, nitrogen-free)
- 0.5 N hydrochloric or sulfuric acid standard solution
- Methyl red indicator solution
- Concentrated NaOH solution
- 0.1 N NaOH standard solution

Materials

- 500- to 900-ml Kjeldahl digestion flasks
- 8 to 14 mesh alumina boiling stones (Thomas Scientific)
- 500-mL or equivalent size titration beaker
- 25-mL burette

Procedure

Digestion process

In a digested tube, the weighed amount of solid sample is quantitatively transferred, and then 20 mL of the salicylic acid solution in sulfuric acid are added. The tube is shaken until the acid is thoroughly mixed with the solid sample. The mixture is left for several hours (or overnight). Then 2.5 g of sodium thiosulfate are added through a dry funnel to the bottom of the tube and the mixture is heated to 170±10˚C in the special Gerhard Kjeldatherm KB / KBL digestion apparatus for 30 minutes (until foaming stops). Then, the tube is cooled and 5.5 g of catalyst mixture are added.

The tube is placed back into the digestion apparaus where it is gently heated to boiling conditions for 2 hours at 400±10 ˚C. The tube is allowed to cool (10 min/300±10 ˚C, 10 min/200 ±10 ˚C).

NOTE: The temperature of the solution should not exceed 400˚C. The mixture is boiled gently for up to 5 hours, so that the sulfuric acid liquefies about 1/3 of the way up the neck of the tube. In most cases 2 h of boiling is sufficient.

At the end of the digestion, the tube is allowed to cool and transferred to the Vapodest distillation apparatus.

At least one blank sample (20 mL of salicylic acid/sulfuric acid solution, 2.5 g of sodium thiosulfate and 5.5 g of catalyst mixture) is prepared at the same time and follows the same digestion, distillation and titration process.

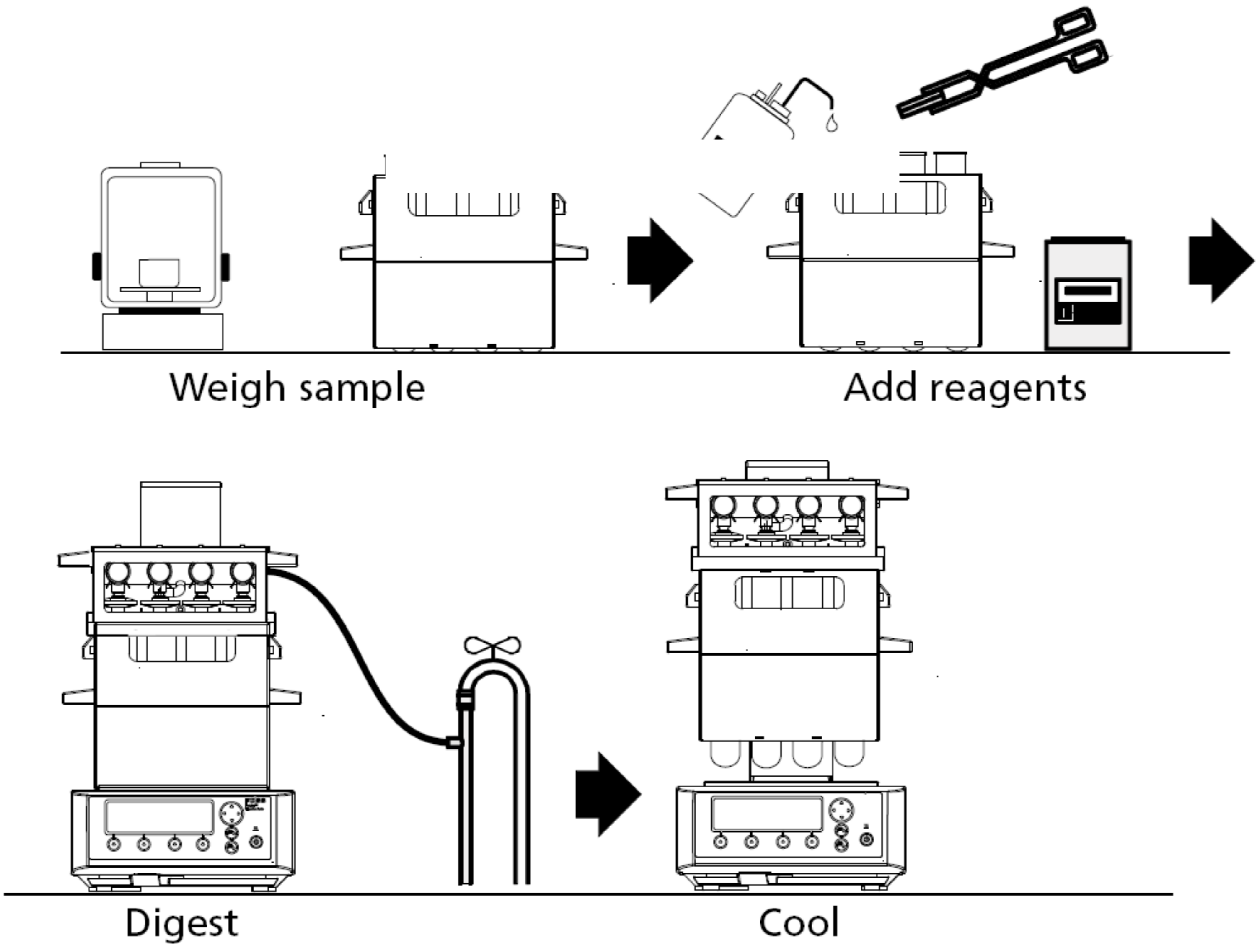


Figure 1. Digestion process

Distillation process

By opening the distillation apparatus, the tap water connected to the instrument should be also opened, so that there is flow in the system and the vapors formed are cooled down. When starting the still, it takes a quarter of an hour to warm up. There are four programs in the device:

00 TEST: Initial cleaning of the device

01 NITROGEN: Nitrogen determination process. 100 mL of 40% w/w sodium hydroxide solution and 100 mL of deionized water are automatically added. Distillation immediately starts capturing ammonia, which is released in the alkaline environment, from 25 mL of 2% w/v boric acid solution also containing an indicator.

02 CLEAN: Performed after each sample analysis for cleaning due to contamination

03 KATH: Performed at the end of all analyzes for the final cleaning of the device

The steps of the procedure are the following:

An initial cleaning of the device is carried out with the program 00 TEST.

The tube with the digested sample is attached to the distillation apparatus.

25 mL of boric acid and 3-4 drops of indicator are added to a 250 mL conical flask and the flask is placed under the condenser of the distillation apparatus.

The distillation program 01 NITROGEN is carried out.

The collected distillate in the conical flask is transferred to the titration unit.

The distillation program 02 CLEAN is carried out.

At the end of all distillations, the 03 KATH distillation program is carried out.

The collected distillate in the conical flask is titrated with the sulfuric acid to the end point with a color change from green to violet.

2.1.5 Determination of proteins in biomass

Introduction

Biomass used as a feedstock for conversion to fuels and chemicals may contain protein and other nitrogen containing materials. These constituents are measured as part of a comprehensive biomass analysis. Protein in biomass is difficult to measure directly. In many cases the nitrogen content of the biomass sample is measured by combustion or Kjeldahl methods and the protein content is estimated using an appropriate Nitrogen Factor (NF). There are published methods that recommend using an NF of 6.25 for all types of biomass except wheat grains where an NF of 5.70 is recommended (Hames et al., 2004).

2.1.6 Determination of structural carbohydrates and lignin in biomass

Introduction

Carbohydrates and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis. Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural

carbohydrates can be removed using extraction or washing steps. Lignin is a complex phenolic polymer.

Parts of this procedure are substantially similar to ASTM E1758-01 “Standard Method for the Determination of Carbohydrates by HPLC.”

This procedure is suitable for samples that do not contain extractives. Thus, extractives should be removed prior to this procedure. It uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into acid insoluble material and acid soluble material. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolyzed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. Protein may also partition into the liquid fraction.

Interferences

This procedure has been optimized for the particle size range specified in LAP “Preparation of Samples for Biomass Compositional Analysis”. Deviation to a smaller particle size may result in a low bias in carbohydrate content (and consequent high lignin bias) due to excessive carbohydrate degradation. Deviation to a larger particle size may also result in a low bias in carbohydrate content (and consequent high lignin bias) due to incomplete hydrolysis of polymeric sugars to monomeric sugars.

Samples containing extractives are not suitable for this procedure. Extractives will partition irreproducibly, resulting in a high lignin bias. Furthermore, samples with an ash content above 10 wt % may not be suitable for this procedure, as the sample may contain soil or other minerals that will interfere with appropriate acid concentrations and may catalyze side reactions. Samples with a moisture content above 10 wt % may also not be suitable for this procedure, as the excess moisture will interfere with appropriate acid concentrations. Samples should be dried (air-dried or oven dried at less than 40°C) prior to this procedure.

Samples containing protein will bias the acid insoluble lignin high unless the protein is accounted for in the gravimetric determination of acid insoluble material. An independent nitrogen analysis is required to estimate the protein content of the residue. The protein estimate is then subtracted from the acid insoluble residue measurement.

Apparatus

- Analytical balance, accurate to 0.1 mg.
- Convection drying oven, with temperature control of 105 ± 3°C.
- Muffle furnace, equipped with a thermostat, set to 575 ± 25 °C or equipped with optional ramping program.
- Water bath, set at 30 ± 3 °C.
- Autoclave, suitable for autoclaving liquids, set to 121 ± 3 °C.
- Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles.
- Desiccator containing desiccant.
- HPLC system equipped with refractive index detector and suitable columns.
- UV-Visible spectrophotometer, diode array or single wavelength, with high purity quartz cuvettes of pathlength 1 cm.
- Automatic burette, optional.

Reagents and Materials

Reagents

- Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C)
- Calcium carbonate, ACS reagent grade.
- Water, purified, 0.2 µm filtered.
- 7.1.4 High purity standards: D-cellobiose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose.
- 7.1.5 Second set of high purity standards, as listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS).

Materials

- Pressure tubes, minimum 90 mL capacity, glass, with screw on Teflon caps and o-ring seals.
- Teflon stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes.

- Filtering crucibles, 25 mL, porcelain, medium porosity.
- Bottles, wide mouth, 50 mL.
- Filtration flasks, 250 mL.
- Erlenmeyer flasks, 50 mL.
- Adjustable pipettors, covering ranges of 0.02 to 5.00 mL and 84.00 mL.
- pH paper, range 4–9.
- Disposable syringes, 3 mL, fitted with 0.2 µm syringe filters.

Procedure

Prepare the sample for analysis and hydrolyze.

Place an appropriate number of filtering crucibles in the muffle furnace at 575 ± 25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator and cool for a specific period of time, one hour is recommended. Weigh the crucibles to the nearest 0.1 mg and record this weight. It is important to keep the crucibles in a specified order, if they are not marked with identifiers.

Place the crucible back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

Weigh 300.0 ± 10.0 mg of the sample into a tared pressure tube. Record the weight to the nearest 0.1 mg. Each sample should be analyzed in duplicate, at minimum.

Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

Place the pressure tube in a water bath set at 30 ± 3 °C and incubate the sample for 60 ± 5 minutes. Using the stir rod, stir the sample every 5 to 10 minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.

Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 ± 0.04 mL deionized water using an automatic burette. Dilution can also be done by adding 84.00 ± 0.04 g of purified water using a balance accurate to 0.01

g. Screw the Teflon caps on securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

The volume of the 4% solution will be 86.73 mL.

Prepare a set of sugar recovery standards that will be taken through the remaining hydrolysis and used to correct for losses due to destruction of sugars during dilute acid hydrolysis.

Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C, usually the liquids setting. After completion of the autoclave cycle, allow the hydrolyzates to slowly cool to near room temperature before removing the caps.

Analyze the sample for acid insoluble lignin as follows.

Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.

Transfer an aliquot, approximately 50 mL, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. Acid soluble lignin determination must be done within 6 hours of hydrolysis.

Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible. Rinse the solids with a minimum of 50 mL fresh deionized water. Hot deionized water may be used in place of room temperature water to decrease the filtration time.

Dry the crucible and acid insoluble residue at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.

Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.

Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.

Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight.

Analyze the sample for acid soluble lignin as follows.

On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.

Using the hydrolysis liquor aliquot obtained, measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer. Dilute the sample as necessary to bring the absorbance into the range of 0.7–1.0, recording the dilution. Deionized water or 4% sulfuric acid may be used to dilute the sample, but the same solvent should be used as a blank. Record the absorbance to three decimal places. Reproducibility should be ± 0.05

Calculate the amount of acid soluble lignin present using Equation 9.

Analyze the sample for structural carbohydrates

Prepare a series of calibration standards containing the compounds that are to be quantified. Use a four point calibration. A fresh set of standards is not required for every analysis.

Using the hydrolysis liquor obtained, transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.

Use calcium carbonate to neutralize each sample to pH 5–6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5–6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.

Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 µm filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate

HPLC conditions:

Injection volume: 10–50 µL, dependent on concentration and detector limits

Mobile phase: HPLC grade water, 0.2 µm filtered and degassed

Flow rate: 0.6 mL/minute

Column temperature: 80–85 °C

Detector temperature: as close to column temperature as possible

Detector: refractive index

Run time: 35 minutes (Sluiter et al., 2012).

Calculations

Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis.

$$\%\ \boldsymbol{AIR} = \frac{\boldsymbol{Weight_{crucible\ plus\ AIR} - Weight_{crucible}}}{\boldsymbol{ODW_{sample}}} \cdot \mathbf{100} \qquad \textit{Equation 7}$$

$$\%\ \boldsymbol{AIL} = \frac{(\boldsymbol{Weight_{crucible\ plus\ AIR} - Weight_{crucible}}) - (\boldsymbol{Weight_{crucible\ plus\ ash} - Weight_{crucible}}) - \boldsymbol{Weight_{protein}}}{\boldsymbol{ODW_{sample}}} \cdot \mathbf{100} \qquad \textit{Equation 8}$$

where:

$Weight_{protein}$ = Amount of protein present in the acid insoluble residue. This measurement is only necessary for biomass containing high amounts of protein.

Calculate the amount of acid soluble lignin (ASL) on an extractives free basis.

$$\%\ \boldsymbol{ASL} = \frac{\boldsymbol{UV_{abs} \cdot Volume_{filtrate} \cdot Dilution}}{\boldsymbol{\varepsilon \cdot ODW_{sample} \cdot Pathlength}} \cdot \mathbf{100} \qquad \textit{Equation 9}$$

where:

UVabs = average UV-Vis absorbance for the sample at appropriate wavelength

$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 86.73 mL

$$\boldsymbol{Dilution} = \frac{\boldsymbol{Volume_{sample} + Volume_{diluting\ solvent}}}{\boldsymbol{Volume_{sample}}} \qquad \textit{Equation 10}$$

ε = Absorptivity of biomass at specific wavelength

ODW_{sample} = weight of sample in milligrams

Pathlength = pathlength of UV-Vis cell in cm.

Calculate the total amount of lignin on an extractives free basis.

$$\%\ \boldsymbol{Lignin_{ext\ free}} = \%\ \boldsymbol{AIL} + \%\ \boldsymbol{ASL} \qquad \textit{Equation 11}$$

Calculate the total lignin value to an as received basis, if necessary:

$$\%\boldsymbol{Lignin_{as\ received}} = \left(\%\ \boldsymbol{Lignin_{ext\ free}}\right) \cdot \frac{(\mathbf{100} - \%\boldsymbol{Extractives})}{\mathbf{100}} \qquad \textit{Equation 12}$$

where:

% Extractives = percent extractives in the prepared biomass sample.

Create a calibration curve for each analyte to be quantified using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.

Calculate and record the amount of each calibration verification standard recovered following HPLC analysis.

$$\%\ \boldsymbol{CVS\ recovery} = \frac{\boldsymbol{conc.detected\ by\ HPLC,mg/mL}}{\boldsymbol{known\ conc.of\ standard,mg/mL}} \cdot \mathbf{100} \qquad \textit{Equation 13}$$

For the sugar recovery standards, calculate the amount of each component sugar recovered after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis. Average any replicate (%R_{sugar}) values obtained for each individual sugar and report %$R_{avg,\ sugar}$.

$$\%\ \boldsymbol{R_{sugar}} = \frac{\boldsymbol{conc.detected\ by\ HPLC,mg/mL}}{\boldsymbol{known\ conc.of\ sugar\ before\ hydrolysis,mg/mL}} \cdot \mathbf{100} \qquad \textit{Equation 14}$$

Use the percent hydrolyzed sugar recovery values calculated in the previous step to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis.

$$\boldsymbol{C_x} = \frac{\boldsymbol{C_{HPLC} \cdot dilution\ factor}}{\%\ \boldsymbol{R_{ave.sugar}}/\mathbf{100}} \qquad \textit{Equation 15}$$

where:

C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL

% $R_{ave.\ sugar}$ = average recovery of a specific SRS component

C_x = $C_{cor.\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis.

Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or 162/180) for C-6 sugars (glucose, galactose, and mannose).

$$\boldsymbol{C_{anhydro} = C_{corr} \cdot Anhydro\ correction} \qquad \text{Equation 16}$$

2.1.7 Determination of starch in biomass

Introduction

Starch, a non-crystalline glucose polymer, is often found in biomass feedstock that contains grain. This is a procedure that is based on the Megazyme Total Starch Assay (amyloglucosidase/α-amylase method). Extraction of the biomass is recommended prior to the starch assay to remove any nonstructural free glucose. Failure to remove free glucose will artificially elevate the starch content of the biomass sample. If this procedure is performed in conjunction with carbohydrate (cellulose

and hemicelluloses) determination, the contribution of glucose from starch will be included in the total glucose value.

Apparatus

- Grinding mill: centrifugal, with 12-tooth rotor and 0.5 mm sieve, or similar device.
- Bench centrifuge: capable of holding 101 x 65 mm polypropylene tubes, with rating of approx. 3250 rcf (~ 4000 rpm)
- Microfuge centrifuge: capable of 13000 rpm.
- Spectrophotometer: capable of operating at 510 nm, (10 mm path length).
- Analytical balance: 0.1 mg readability, accuracy and precision.
- Thermostatted water bath: set at 50°C.
- Boiling water bath: with tube rack.
- Magnetic stirrer
- Magnetic stirring bars
- Vortex mixer
- Pipettors: capable of delivering 100 µL or 1.0 mL
- Dispensers to dispense 4 mL and 10 mL of 100 mM sodium acetate buffer (pH 5.0)
- Disposable polypropylene tube: 13 mL, 101 x 16.5 mm
- Disposable 2.0 mL polypropylene microfuge tubes
- Glass test tubes
- Digestion tubes

Reagents

- Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM): Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L and store the buffer at 4°C. Stable for > 6 months at 4°C.

- Sodium acetate buffer (200 mM, pH 4.5) plus calcium chloride (5 mM): Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 60 mL is required). Adjust the volume to 1 L. Stable for > 6 months at 4°C.
- Sodium acetate buffer (600 mM, pH 3.8) plus calcium chloride (5 mM): Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 1600 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Add 1.48 g of calcium chloride dihydrate and dissolve. Adjust the volume to 2 L with distilled water. Stable for > 12 months at room temperature.
- Sodium hydroxide solution (1.7 M): Add 68 g NaOH to 900 mL of deionised water and dissolve by stirring. Adjust the volume to 1 L. Store in a sealed container. Stable for > 2 years at room temperature.
- Ethanol (~ 50% and ~ 80% v/v): Add 500 mL of either ethanol (95% v/v) to 500 mL of distilled water. ~80% v/v: Add 800 mL of either ethanol (95% v/v) to 200 mL of distilled water. Store in a 1 L Duran bottle. Stable for > 4 years at room temperature.

Procedure

Mill sample to pass a 0.5 mm screen.

Accurately weigh ~ 100 mg of test sample, in duplicate (one as a sample blank) into Corning culture tubes (16 x 120 mm). Record the exact weight. Tap the tube so that sample drops to the bottom of the tube.

To both of the tubes, add 10 mL of sodium acetate buffer (100 mM, pH 5) plus calcium chloride (5 mM). Stir the tubes vigorously on a vortex mixer for 5 sec.

To one of the tubes (sample tube), add 0.1 mL of undiluted thermostable α-amylase with a 5 mL tip. To the second tube (sample blank) add 0.1 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM).

Vortex the tubes for 3 sec, cap the tubes loosely and immediately transfer them to a boiling water bath and start the timer. After approx. 2 min, tighten the caps and mix the tube contents vigorously on a vortex mixer. After further 5 and 10 min, vortex the tube contents again for 5 sec and return the tubes to the boiling water bath. After 15 min (from addition of α-amylase), remove tubes from

the boiling water bath and mix the contents vigorously for 5 sec on a vortex mixer. Place the tubes in a water bath at 50°C and allow them to equilibrate to temperature over 5 min.

To one of the tubes (the sample tube), add 0.1 mL of undiluted AMG (3300 U/mL) and vortex for 3 sec. To the second tube (the sample blank) add 0.1 mL of sodium acetate buffer (100 mM pH 5.0) plus calcium chloride (5 mM). Incubate the tubes at 50°C for 30 min with no further mixing.

Remove the tubes from the water bath and allow them to cool to room temperature over 10 min. Invert the tubes a few times to ensure condensed water on the inside of the lid is mixed with liquid in the tube.

Transfer 2.0 mL of each solution (sample and sample blank) to microfuge tubes and centrifuge the tubes at 13000 rpm for 5 min. Accurately transfer a 1.0 mL aliquot of the supernatants to 12 x 120 mm tubes containing 4 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) and mix the contents.

Accurately transfer duplicate 0.1 mL aliquots of each sample to the bottoms of 16 x 120 mm glass test tubes. Also transfer a single 0.1 mL aliquot of sample blanks to a 16 x 120 mm glass test tube.

Add 3.0 mL of GOPOD reagent and incubate the solutions at 50°C for 20 min and measure absorbance against the reagent blank at 510 nm.

Concurrently incubate:

Glucose controls: 0.1 mL of glucose standard solution (1.0 mg/mL) plus 3.0 mL of GOPOD reagent, in quadruplicate.

Reagent Blank: 0.1 mL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) with 3.0 mL of GOPOD reagent in duplicate (Neogen, 2022).

Calculations

Calculations for solid samples:

$$\boldsymbol{Starch}, \% = \boldsymbol{\Delta A} \cdot \boldsymbol{F} \cdot \frac{\boldsymbol{EV}}{\boldsymbol{0.1}} \cdot \boldsymbol{D} \cdot \frac{\boldsymbol{1}}{\boldsymbol{1000}} \cdot \frac{\boldsymbol{100}}{\boldsymbol{W}} \cdot \frac{\boldsymbol{162}}{\boldsymbol{180}} = \boldsymbol{\Delta A} \cdot \boldsymbol{F} \cdot \boldsymbol{EV} \cdot \frac{\boldsymbol{D}}{\boldsymbol{W}} \cdot \boldsymbol{0.90} \quad \textit{Equation 17}$$

where:

ΔA = absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank (only where a sample blank is determined).

F = factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose).

EV = sample extraction volume [10.2 mL for procedure].

0.1 = volume of sample analysed.

D = further dilution of sample solution (either undiluted, or diluted 5-fold or 11-fold)

1/1000 = conversion from mg to mg.

100/W = conversion to 100 mg sample; W = sample weight in mg.

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

$$\boldsymbol{Starch}\ \%\ \boldsymbol{w/w}\ (\boldsymbol{dry\ wt.basis}) = \boldsymbol{Starch}\ \%\ \boldsymbol{w/w}\ (\boldsymbol{as\ is}) \cdot \frac{\mathbf{100}}{\mathbf{100} - \boldsymbol{moisture\ content}\ (\%\ \boldsymbol{w/w})}$$

Equation 18

2.1.8 Determination of glucose in liquid samples

Introduction

The method of glucose determination is based on the conversion of glucose into a red product, which is the result of the successive action of the enzymes oxidase and glucose peroxidase. Initially, glucose is converted to gluconic acid by oxidase with simultaneous production of hydrogen peroxide. Subsequently, hydrogen peroxide in the presence of aminophenazone and a phenolic derivative by the action of peroxidase is converted to a red product which has a maximum absorption of 510 nm. The 2 reactions mentioned are presented below (GOD = oxidase, POD = peroxidase):

$$\text{Glucose} \xrightarrow{\text{GOD}} \text{Gluconic acid} + \ H_2O_2$$

$$H_2O_2 + \text{Aminophenazone} + \text{Phenolic derivative} \xrightarrow{\text{POD}} \text{Product (of red colour)}$$

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of glucose.

Reagents and Materials

Reagents

A commercial kit by BIOSIS S.A. (https://www.biosis.com.gr/) is used that includes a sugar reagent R1 and a standard glucose solution R4 (100mg/dL).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

To determine the free glucose in the samples, the samples are initially filtered and then the filtrates are properly diluted. Then, 2 mL of working solution R1 and 0.1 mL of filtrate from each diluted solution are added to test tubes. To prepare the blank, 2 mL of working solution and 0.1 mL of deionized water are added to a test tube. The test tubes are then placed for incubation in a water bath at 37 °C for 15 minutes. At the end of the incubation period, each test tube is stirred in the Vortex-Genie 2 device and then its absorbance is measured on a Spectroquant Pharo 300 Merck photometer, at a wavelength of 510 nm. The photometer is zeroed out with deionized water before starting the measurement of the samples.

The presence of glucose in the sample is evident by the coloring of the solution from colorless to light pink when the sample is added. If the sample is not coloured, this would mean that either the sample does not contain glucose or that the dilution is very high.

Calculations

Free glucose is calculated by the following equation:

$$\boldsymbol{Free\ glucose}\ (\%\,{}^{W}/_{W}) = \frac{0{,}71 * \boldsymbol{ABS_{mean} * D * (Vtot) * 10^{-3}}}{\boldsymbol{initial\ solid}} * \mathbf{100} \qquad \textit{Equation 19}$$

where:

ABSmean = the average absorption

D = the dilution (D = 1 when the solution remains undiluted, D = 10 when the dilution is 1:10)

Vtot = the total volume of the filtrate

initial solid = the initial mass of the sample.

2.1.9 Determination of total reducing sugars in liquid samples

Introduction

Carbohydrates are classified into 3 categories, which are analyzed below:

1. **Monosaccharides:** These are the carbohydrates that can not be hydrolyzed further in order to give a simpler poly-hydroxy unit aldehyde or ketone. Monosaccharides can be classified according to the number of carbon atoms and the active group of their molecule in trioses (with 3 carbon atoms), tetroses (with 4 carbon atoms), pentoses (with 5 carbon atoms), hexoses (with 6 carbon atoms) etc. Also, if the monosaccharide has an aldehyde group in the molecule, it belongs to the group of aldoses and if it has a ketone group it belongs to the group of ketoses. There are about 20 monosaccharides in nature and most commonly contain 5 (pentoses) or 6 (hexoses) carbon atoms: glucose, fructose, ribose, arabinose, xylose etc.

2. **Oligosaccharides:** These are the carbohydrates that during their hydrolysis can yield 2 to 10 units of monosaccharides. Depending on the number of monosaccharides yielding by hydrolysis, they are divided into disaccharides, trisaccharides, tetra-saccharides, etc. Typical examples of disaccharides are sucrose which when hydrolyzed gives an equimolar mixture of glucose and fructose and maltose which when hydrolyzed gives 2 molecules of glucose.

3. **Polysaccharides:** These are the carbohydrates that during their hydrolysis produce large number of monosaccharides. Examples of polysaccharides are starch, cellulose, hemicellulose, glycogen etc.

In addition, carbohydrates can be categorized as reducing or non-reducing. Reducing carbohydrates are those that can function as reducing agents due to the presence of free aldehyde or ketone group in their molecule. This property is used by the DNS method to quantify sugars in a solution. Reduction of 5- dinitrosalicylic acid leads to the formation of a 3-amino-5-nitro-salicylic acid product which shows an absorption maximum at 540 nm.

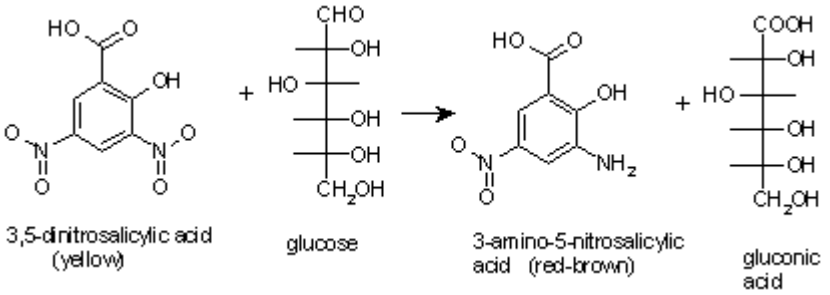


Figure 2. Reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitro-salicylic acid in the presence of glucose.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of total reducing sugars (TRS).

Reagents and materials

Reagents

- 3,5- dinitrosalicylic acid solution.
- NaOH solution.

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

Total reducing sugars are determined by the 3,5-dinitrosalicylic acid method. Reduction of 3,5-dinitrosalicylic acid (yellow) leads to the formation of a 3-amino-5-nitro-salicylic acid product (orange-yellow) which shows an absorption maximum at 540 nm. 250µL of each sample (diluted or not) is added to test tubes along with 250µL DNS. The test tubes are boiled for 15 min to incubate and destroy any microorganisms that have grown and consumed a certain amount of sugar.

Immediately after heating, the test tubes are removed from the water bath and then 2 mL of water are added. Finally, the absorptions at a wavelength of 540nm are recorded from which, the concentrations of reducing sugars are calculated.

Calculations

The measured absorptions are introduced into the proper calibration curves. More specifically:

*TRS (mg/L) = 1.6058 * ABSmean * D* *Equation 20*

where:

ABSmean: the average absorption

D: The dilution performed in the solution (D = 1 when the solution is not diluted, D = 5 when the dilution is 1: 5 and D = 10 when the dilution is 1:10).

2.1.10 Determination of ethanol in liquid samples

Introduction

Ethanol is ubiquitous in its natural occurrence, and thus its quantitative determination is not only important in the manufacture of intoxicating wines, beers and spirits, but also for low-alcohol and non-alcoholic beverages, fruit juices and a range of other foodstuffs, including chocolates, sweets, jam, honey, vinegar and dairy products. A large range of non-foods also contain significant quantities of ethanol, such as cosmetics and pharmaceuticals.

Alcohol dehydrogenase (ADH) catalyses the oxidation of ethanol to acetaldehyde coupled with the reduction of nicotinamide-adenine dinucleotide (NAD^+).

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

The amount of NADH formed in this reaction pathway is stoichiometric with the amount of ethanol. It is the NADH which is measured by the increase in absorbance at 340 nm.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of ethanol.

Reagents and materials

Reagents

A commercial kit by Megazyme (https://www.megazyme.com/) is used that includes the following reagents:

- Reagent 1 (120 mL) Contains sodium azide (0.02% w/v) as a preservative.
- Reagent 2 (30 mL) Contains sodium azide (0.02% w/v) as a preservative.
- Ethanol Standard (5 mL, 5 mg/mL).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

The following procedure is used to determine ethanol:

Place 2 mL of Reagent 1 in a glass vial and add 0.1 mL of the sample.

In another glass vial prepare the blank sample. 2 mL of Reagent 1 ethanol are placed in a glass vial and 0.1 mL of deionized water is added.

After 3 minutes, add 0.5 mL of Reagent 2 to the glass vials and leave to stand.

After 7 minutes the samples are photometered at 340nm, after stirring in the Vortex Genie 2 stirrer and resetting the photometer with deionized water.

Calculations

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{ethanol}$.

The value of $\Delta A_{ethanol}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of ethanol can be calculated as follows:

$$c = \frac{V \cdot MW}{\varepsilon \cdot d \cdot v} \cdot \Delta A \quad (\frac{g}{L}) \qquad \textit{Equation 21}$$

where:

V = final volume (mL)

MW = molecular weight of ethanol (g/mol)

ε = extinction coefficient of NADH at 340 nm = 6300 (l x mol^{-1} x cm^{-1})

d = light path (cm)

v = sample volume (mL)

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

2.1.11 Determination of total organic carbon

Introduction

Total Organic Carbon (TOC) is a measure of the total amount of carbon in organic compounds in pure water and aqueous systems. TOC has become an important parameter used to monitor overall levels of organic compounds present. This has happened despite the lack of any direct quantitative correlation between total organic carbon and the total concentration of organic compounds present and reflects the importance of having an easy-to-measure, general indicator of the approximate level of organic contamination. It also reflects the appeal of a parameter which has a name which sounds more fundamental than it is. In many cases, the TOC is used as an on-going monitor of change or lack of change in organic content.

Apparatus

A SHIMADZU TOC-VCSH TOC analyser is used in order to quantify the concentrations of total, organic and inorganic carbon in liquid samples. The carbon content of solid samples is quantified by applying the Solid Sample Module (SSM5000A).

Materials

- Volumetric flasks (50 mL and 100 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Porcelain capsules.
- Whatman filter papers.

Procedure

The operation principle of the method is based on the calculation of the amount of carbon dioxide produced resulting from the oxidation of carbonaceous compounds present in the test solutions. The samples enter the combustion tube of the apparatus and the total carbon contained in the sample is oxidized at 720^{o}C to form carbon dioxide. A carrier gas is then utilized which carries the various combustion products along with the carbon dioxide. In the case of this instrument (SHIMADZU TOC-VCSH), ultra-clean air was used as the carrier gas. With the help of ultra-clean air, the combustion products are transferred to a dehumidifier in order to remove the moisture while at the same time all the gases produced during the initial combustion are cooled. The carrier gas then passes through a halogen trap and ends up in the NDIR (Non Dispersive Infra Red gas analyzer) cell, where carbon dioxide is detected. It is worth noting that in the way described above the total carbon present in the samples, organic and inorganic, is measured. Inorganic carbon is in the form of carbon dioxide and is not bound to organic compounds. Therefore, when the measurement of inorganic carbon (IC) is required, then the instrument automatically removes the IC from the total carbon (TC) and thus calculates the organic carbon that was initially present in the sample.

The experimental TOC determination process of solid samples starts by weighing a 40 mg sample, which is ground to a size of 1 mm and dried at 80°C. These samples are placed in porcelain capsules. The capsules are then placed in the special oven of the instrument where the sample are heated to 900^{o}C. At this temperature the organic carbon is burned and its exhaust gases are carried to the TOCVCSH central unit. The mass of organic carbon (mg) of CO_2 produced during combustion is calculated in this unit.

Calculations

The instrument provides the concentrations of TC, TOC or TIC of the liquid samples and just the dilutions made should be taken into account.

The (%) percentage of organic carbon in the solid samples is given by the following formula:

$$TOC\ (\%) = \frac{\frac{m_1}{M_1} + \frac{m_2}{M_2}}{2} \cdot 100 \qquad \textit{Equation 22}$$

where:

m_1: the measured mass of organic carbon of the first sample (mg)

m_2: the measured mass of organic carbon of the second sample (mg)

M_1: the initial mass of the first sample (mg)

M_2: the initial mass of the second sample (mg).

2.1.12 Determination of volatile fatty acids

Introduction

Volatile fatty acids (VFA) are the important intermediates indicating the stability and performance of fermentation process but they are also the product of delignification process. In anaerobic digestion, VFA accumulation reflects a kinetic uncoupling between acid formers and consumers and is typical of the stress situation caused by hydraulic or organic overloading, sudden temperature variations, or the presence of toxic or inhibitory compounds, among other factors. Thus, the monitoring of their concentration is of crucial importance.

In an acidic medium lower fatty acids (VFA) react with a diole to form fatty acid esters, which are subsequently converted into hydroxamic acids with hydroxylamine. These in turn react with iron(III) ions to form red complexes that are determined photometrically.

Apparatus

A UV/Vis photometer Spectroquant Pharo 300 Merck is used for the quantification of volatile fatty acids.

Reagents and materials

Reagents

A commercial kit (Spectroquant Volatile Organic Acids Test 1018909) by Merck (https://www.merckmillipore.com/) is used that includes Reagents OA-1 to OA-5.

Materials

- Volumetric flasks (50 mL and 100 mL).
- Disposable plastic cuvettes (1 cm light path, 3.0 mL).
- Micro-pipettors (200 µL and 1000 µL).
- Positive displacement pipettor.
- Analytical balance.
- Vortex mixer.
- Whatman filter papers.

Procedure

The samples must be analyzed immediately after sampling. Otherwise they should be stored at 4 °C for less than 24 hours.

The pH must be within the range 2 - 12.

Turbid samples must be filtered.

Pipette 0.75 mL Reagent OA-1 into a clean round cell.

Add 0.50 mL Reagent OA-2 with pipette.

Add 0.50 mL of pretreated or diluted sample with pipette, close the cell tightly, and mix.

Heat the cell at 100 °C in the preheated thermoreactor for 15 min, then cool to room temperature under running water.

Add 1.0 mL Reagent OA-3 with pipette.

Add 1.0 mL Reagent OA-4 with pipette, close the cell tightly, and mix.

Add 1.0 mL Reagent OA-5 with pipette, close the cell tightly, and mix.

A transient turbidity or precipitate may form.

Leave to stand for 1 min (reaction time), then measure the sample in the photometer.

Calculations

The instrument provides the concentration of volatile fatty acids as mg/L of acetic acid and just the dilutions made should be taken into account.

2.1.13 Determination of in-vitro in vitro organic matter digestibility

Procedure

4 in vitro series were set up for the determination of in-vitro digestibility of the samples. In each series, ruminal fluid was collected from one multiparous Latxa ewe slaughtered for this production purpose. Before slaughtering, ewes were fed a basal diet (80% meadow hay and 20% compound feed) for 3 weeks and had free access to fresh water and feed. Ruminal fluid was collected before the morning feeding and filtered through four layers of cheesecloth into a volumetric flask. Then, it was diluted in culture medium in a 1:4 ratio (ruminal fluid and phosphate-bicarbonate buffer, respectively) under anaerobic conditions according to Menke et al. (Menke et al., 1979).

Approximately 500 mg of orange peel samples were weighed into 125 mL serum bottles. Each sample was incubated in 50 mL of culture medium in triplicate, the bottles were crimp sealed and incubated at a constant temperature (39°C) in the incubator for 24 hours. Gas production was released at 2, 4, 6 and 22 hours post-inoculation to avoid the pressure in the bottle headspace exceeding 48 kPa, as suggested by Theodorou et al. (Theodorou et al., 1994). After 24 hours of incubation, the bottles were put in the fridge for 20 min to stop fermentation for subsequent sampling of short chain fatty acids (SCFA) and in vitro organic matter digestibility (IVOMD) determination.

In vitro organic matter digestibility, in the short term in vitro trial was calculated as described by Pell and Schofield (A.N. & P., 1993), where 45 mL of a neutral detergent solution was added to each bottle and warmed at 105 °C for 1 h; then, the bottles were cooled, filtered through glass filter crucibles (Porosity 2) and washed with distilled water, ethanol and acetone. The remaining sample was dried at 100 °C overnight and then burned in a muffle furnace at 500 °C to obtain true IVOMD values.

The analysis of the SCFA (acetic, propionic, butyric, isobutyric, valeric and isovaleric) of rumen samples was performed by gas chromatography using a flame ionization detector.

2.1.14 Determination of fiber and lignin contents (Van Soest analysis)

Introduction

The Van Soest analysis aims to identify fractions (polymer classes) of cell wall components, which are as free as possible from other feed components (e.g. proteins) by removing the cellular content with appropriate solutions.

This method is applicable to grains, feeds, forages, and all fiber-bearing materials. The Neutral Detergent Fiber (NDF), at first, determines the residue remaining after digesting in a detergent solution. These fiber residues are predominantly hemicelluloses, cellulose and lignin. Then, a follow-up procedure aims to determine the Acid Detergent Fiber (ADF), which is the fiber cellulose and lignin residue (cellulose and lignin) remaining after processing with acid detergent. Last, the Acid Detergent Lignin (ADL) can then be quantified using sulfuric acid extraction and ashing to determine the residue lignin.

Apparatus

- Electronic analytical balance with the least accuracy of 0.1mg.
- Desiccator.
- Autoclave, able to achieve 100°C at 10-25 psi.
- Furnace at 550°C.
- Oven at 105°C.

Reagents and materials

Reagents

- Distilled De-Ionized water (DDI water)
- Purified acetone
- NDS:

In 1 L distilled H_2O,

- Add 30.0 g Sodium dodecyl sulfate, USP ($NaC_{12}H_{25}SO_4$),
- Add 18.61g of EDTA disodium salt, dehydrated,
- Add 6.81 g Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$),
- Add 4.56 g Sodium phosphate dibasic, anhydrous (Na_2HPO_4) or 5.72 g $Na_2HPO_4 \cdot H_2O$,
- Add 10.0 ml Triethylene glycol ($C_6H_{14}O_4$),

- Check pH ranges from 6.9 to 7.1. Agitate and heat to aid the solution.

- 1N H_2SO_4:
  - Prepare in a fume hood.
  - Add approximately 500 mL of DDI water in a 1L volumetric flask followed by 28 mL concentrated H_2SO_4.
  - Allow the solution to cool in a water bath before bringing it to volume with Distilled De-Ionized (DDI) water.
- ADS:
  - Add 20 g of Cetyl-trimethylammonium bromide (CTAB) to 1 L of 1N H_2SO_4. Agitate and heat to aid the solution process.
- 72% Sulfuric Acid (H_2SO_4)
- Na_2SO_3.

Materials

- Fiber filter bags.
- Heat Sealer.
- 50 mL, 500 mL, and 1 L beakers.
- Chemical resistant marker.
- 250 mL Borosilicate glass bottles (BORO bottles).
- Crucibles, large enough to hold 40-50 mL liquid.
- Forceps.

Procedure

Weigh and record the weight of each empty filter bag (W1). It is not necessary to pre-dry filter bags. Any moisture will be accounted for by the blank bag correction.

Weigh 0.45-0.55 g of prepared sample (W2) directly in the filter bag. Avoid placing the sample on the upper 4mm of the bag. Using a heat sealer, completely seal the upper edge of the filter bag within 4 mm of the top.

Weigh one blank bag and include it in the run to determine blank bag correction (**C**).

Next, the samples are placed into BORO bottles of 250 mL volume with 100 mL of NDS and 0.5 g/50mL NDS of Na_2SO_3. The process of heating (NDF extraction) takes place in an autoclave (ISOLAB Laborgerate) for 75 minutes at 100°C.

After the samples are cooled to ambient temperature, rinse with hot DDI water to remove NDS (About 8-10 washouts for 5 minutes). During the incubation, stir and pressurize the samples with the help of forceps. If pH levels are above 6, stop the rinsing process.

Then the bags are placed in a 250 mL beaker with enough acetone to soak the bags for 5 - 10 minutes. Remove the samples and wait till the evaporation of acetone to place them into the oven at 105°C for 2-4 hours. *CAUTION: Do not place bags in the oven until the acetone has completely evaporated. Extended drying times or too high temperatures can compromise the bag's filtration media.*

Remove the filter bags from the oven and immediately place them directly into a collapsible desiccant pouch and flatten them to remove any air. Cool to ambient temperature and weigh the filter bags (**W3**). Calculate blank bag correction using weight loss of a blank bag upon NDF procedure (**C1**). At this point, the NDF content can be calculated.

Afterwards, place the fiber bags in BORO bottles of 250 mL volume and repeat the previous steps, while using the ADS Solution instead of the NDS Solution. In addition, the processing time is 60 minutes instead of 75. In the last step weigh the filter bags (W4) and calculate blank bag correction using the weight loss of a blank bag upon ADF procedure (C2), to determine the ADF content.

After the ADF extraction, the dry bags/samples are placed into 100 mL beakers along with a sufficient quantity (approximately 20 – 30 mL) of 72% H_2SO_4 to cover the bags.

CAUTION: Bags must be completely dry and at ambient temperature before adding concentrate acid to prevent heat generation by the acid and water reaction and affect the results.

Place weight inside the beaker to submerge the bags. Agitate bags at the beginning and at 30-minute intervals for 3 hours, by pushing and lifting the weight up and down approximately 30 times.

After 3 hours, pour off H_2SO_4 and rinse with warm water to remove all acid. Repeat rinses until the pH is neutral.

Rinse with approximately 250 ml of pure acetone for 3 minutes to remove water and let it evaporate. *CAUTION: Do not place bags in the oven until acetone is completely evaporated.*

Place bags in the oven at 105°C for 3 – 4 hours.

Remove bags from the oven and cool in a desiccator. Flatten it to remove air and weigh the pouch (**W5**). Calculate blank bag correction using weight loss of a blank bag upon sulfuric acid extraction (**C3**).

Then, add bags to a dry crucible and weigh them before (**W6**) placing them in the furnace at 550°C for 3 hours. Cool the crucibles to ambient temperature post and weigh (**W7**). Calculate blank bag ash correction using weight loss of a blank bag upon ignition (**C4**). Finally, calculate the ADL content.

Calculations

The Neutral Detergent Fiber (NDF), the Acid Detergent Fiber (ADF) and the Acid Detergent Lignin (ADL) contents can be calculated as follows:

$$\%\ \boldsymbol{NDF} = \frac{(W3-(W1*C1)}{W2} * 100 \qquad \textit{Equation 23}$$

$$\%\ \boldsymbol{ADF} = \frac{(W4-(W1*C2)}{W2} * 100 \qquad \textit{Equation 24}$$

$$\%\ \boldsymbol{ADL} = \frac{(W5-(W1*C3)) - (W6-W7)-(W1*C4)}{W2} * 100 \qquad \textit{Equation 25}$$

where,

W1: Empty fiber filter bag

W2: Initial dry sample weight prior processing

W3: Dried sample weight after NDF extraction process

W4: Dried sample weight after ADF extraction process

W5: Dried sample weight after sulfuric acid extraction process

W6: Dried sample weight prior ashing process in crucible

W7: Sample weight after ashing process

C1: NDF extraction blank bag correction

C2: ADF extraction blank bag correction

C3: Sulfuric acid extraction blank bag correction

C4: Ash blank bag correction.

Neutral detergent insoluble Nitrogen (NDIN) and acid detergent insoluble Nitrogen (ADIN) were determined by analyzing NDF and ADF residues for Kjeldahl N (Licitra et al., 1996). Neutral detergent insoluble CP (NDICP) and acid detergent insoluble CP (ADICP) were estimated by multiplying the NDIN and ADIN by the coefficient of 6.25.

2.2 Experimental Protocol

2.2.1 Drying

The feedstocks (unprocessed or processed) are dehydrated and simultaneously milled by a rotary drum waste dryer. The main operational parameters are drying temperature and duration. The feedstocks with 84.47±0.82% initial moisture is dehydrated at 100-140 °C for 9-15 h aiming to avoid all possible microbial development but also to avoid alterations in the feedstock composition. The simultaneous milling results in a homogeneous coarse powder feedstock. After the dehydration process, the moisture of the dried feedstock is measured.

Operational Parameters: Drying temperature and time

Monitoring Parameters: Moisture

Performance: Moisture reduction.

2.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis of biomass is conducted in 100-mL Duran laboratory borosilicate glass bottles with sealing screw caps. The initial pH of the mixture is measured and when it is corrected when necessary by dilute alkaline solution ($CaCO_3$) in order to set the pH at the optimum range (pH 5-6) of each enzymatic formulation. Cellulose and pectin hydrolysis is performed at 50 °C and 150 rpm for 24 h in a rotary shaker incubator (Constant Temperature Shaking Incubator FS-70B) by use of either CelliCTec2, CellicCTec3, NS22177 or/and Pectinex Ultra. The first two enzymes are commercial Novozymes products and are a blend of cellulases, β-glucosidases and hemicellulases. Their hydrolysis action is proven to work on a range of pretreated or not lignocellulosic materials. NS22177 is a non-commercial Novozymes formulation that aims to attack cellulose from food biomass, while Pectinex Ultra is a commercial enzymatic formulation that targets the hydrolysis of pectin.

For all enzymatic formulations, their activity is estimated based on the standard methods.

All experiments are replicated twice, and the average values are evaluated.

The saccharification yield, SG, is adopted as a measure of enzymatic hydrolysis efficiency according to the following equation:

$$S_G = \frac{Glucose\ production}{Maximum\ theoretical\ glucose\ production\ from\ the\ total\ conversion\ of\ carbohydrates\ targeted} \cdot 100\% \quad \textit{Equation 26}$$

In addition the sugar yield (Y_S) is expressed as the mass of glucose per 100 g of total solids (in the feedstock used).

Operational Parameters: Hydrolysis temperature and time, type and dosage of enzymes, solids loading, wet or dry feedstock

Monitoring Parameters: Glucose concentration, solid content, carbohydrates content

Performance: Saccharification yield, Sugars yield, carbohydrates degradation.

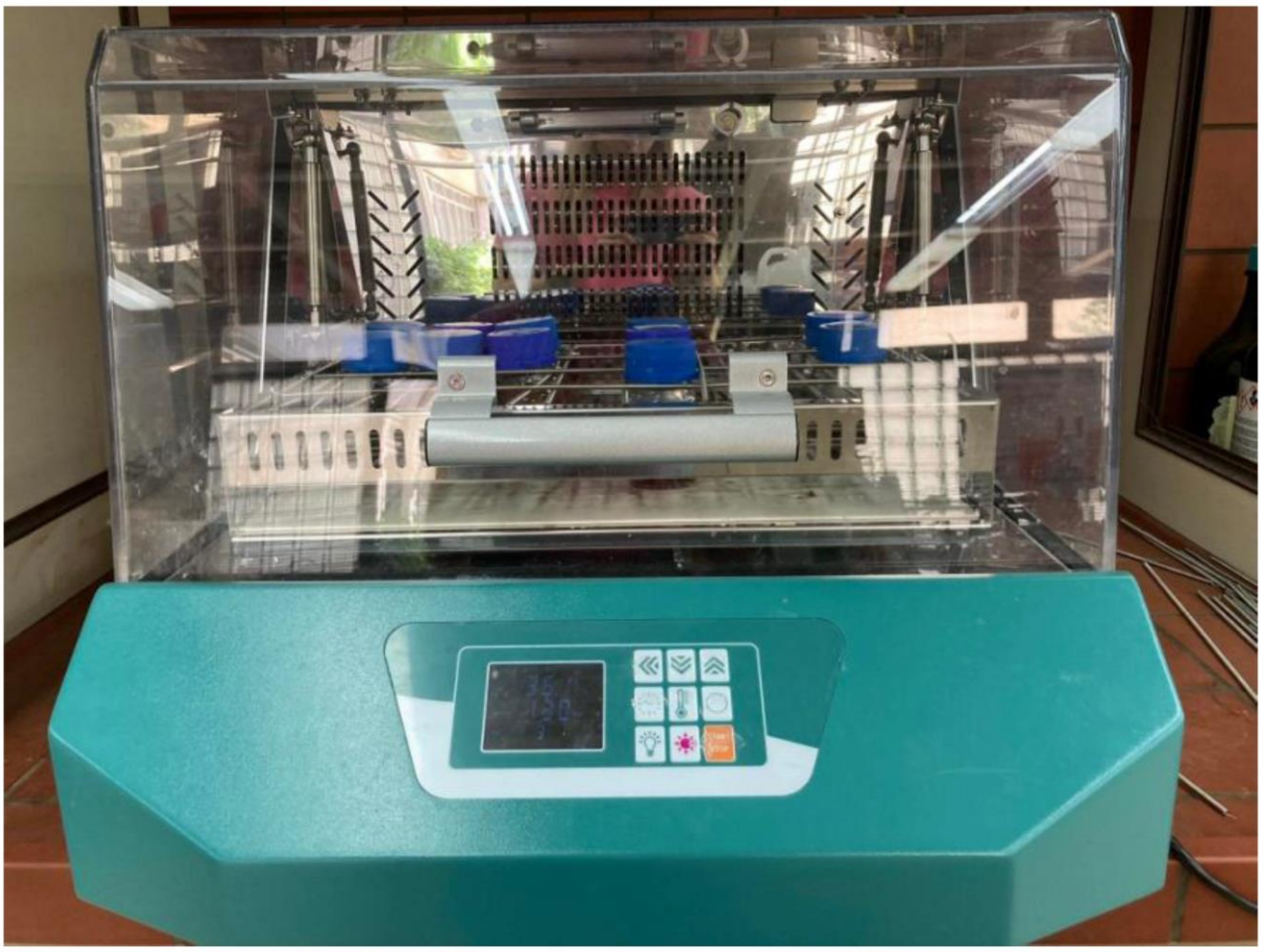


Figure 3. Constant Temperature Shaking Incubator FS-70B

2.2.3 Ethanolic fermentation

After applying enzymatic hydrolysis, the released glucose is biologically converted to ethanol through fermentation process by adding 2% Saccharomyces cerevisiae (in the form of instant dry baker's yeast) into the same 100-250 mL Duran laboratory borosilicate glass bottles with sealing screw cap, at 30 °C for 24 h in the same incubator shaker with agitation speed 150 rpm.

All experiments are replicated twice, and the average values are evaluated.

The ethanol yield, Y_{eth}, is adopted as a measure of ethanol production according to the following equation:

$$S_G = \frac{Glucose\ production}{Maximum\ theoretical\ glucose\ production\ from\ the\ total\ conversion\ of\ carbohydrates\ targeted} \cdot 100\% \quad Equation\ 26$$

$$Y_{eth} = \frac{Ethanol\ production}{Maximum\ theoretical\ ethanol\ production\ from\ the\ total\ conversion\ of\ carbohydrates\ targeted} \cdot 100\% \quad Equation\ 27$$

Fermentation efficiency, Y_{ferm} can also be estimated according to the following equation:

$$Y_{ferm} = \frac{Ethanol\ production}{Maximum\ theoretical\ ethanol\ production\ from\ the\ total\ conversion\ of\ glucose\ produced} \cdot 100\% \quad Equation\ 28$$

Operational Parameters: Fermentation time, dosage of yeast

Monitoring Parameters: Remaining glucose concentration, Ethanol concentration, solid content, carbohydrates content

Performance: Ethanol yield, fermentation efficiency, carbohydrates degradation.

2.2.4 Aerobic Fermentation

In 500 mL autoclavable bottles, 200 mL of liquid rich in glucose (either synthetic or from the residue of the enzymatic hydrolysis of orange peels) are added. The pH is corrected (different pHs to find the optimum) with $CaCO_3$ and the nutrient solution presented in Table 1 is added.

Table 1. Nutrient solution composition concentrations

Nutrients	KH_2PO_4	$(NH_4)_2SO_4$	$MgSO_4$
Concentration (g/L)	5	2	0.4

1 g/L Saccharomyces cerevisiae yeast is then added to all bottles. Finally, air pumps are used for continuous aeration of each sample and the samples are placed in a water bath at 30°C. After 24

hours the samples are centrifuged and filtered. The solid residue is placed in pre-weighed capsules and transferred to an oven for 24 hours at 50°C, while the concentration of ethanol and residual glucose is measured in the liquid phase. Experiments are also carried out on a larger scale using a 4L reactor where 1L of liquid orange fraction is added.

The aerobic fermentation yield, Y_{aer}, is adopted as a measure of biomass production according to the following equation:

$$Y_{aer} = \frac{Biomass\ production}{Theoretical\ biomass\ production} \cdot 100\% \quad Equation\ 29$$

Where the theoretical aerobic biomass production is considered equal to 0.5g/g substrate (Vieira et al., 2013).

Biomass yield, Y_{obs} is also estimated as:

$$Y_{obs} = \frac{Biomass\ production}{Glucose\ utilised} \cdot 100\% \quad Equation\ 30$$

3. Feedstock supply and logistics in a safe manner and characterization of orange peels

SEVT has ensured the feedstock supply and the respective logistics. Special care is taken in order to ensure the safe transportation of the feedstock to the NTUA premises. Hellenic Fruit Juices (www.hfj.gr) has agreed to stand as the orange juice industry that provides the raw material for all experimentation. At first, all confidentiality issues were discussed and resolved and an non-disclosure agreement (NDA) was signed by the parties involved in October 2021. Then, the first sample was received in October 2021, the second large batch of 700kg in December 2021, and in May 2022 the third batch was delivered in NTUA. It is worth noticing that despite the difficulties in the trasportation and delivery of the feedstock, Hellenic Fruit Juices always supports these procedures and the samples are successfully delivered in NTUA premises.

Upon feedstock delivery, the characterization of orange peels took place according to the analytical procedures described in Section 2.1. The mean values of the main physicochemical and nutritional characteristics that were analysed are presented in Table 2.

Table 2. Physicochemical and nutritional characteristics of orange peel waste

	26/10/21 (1)	**13/12/21 (2)**	**10/5/2021 (3)**	**Average**
TS (%)	14.63	16.22	15.75	15.53 ± 0.82
Moisture (%)	85.36	83.78	84.25	84.46 ± 0.81
Ether extract (%)	6.35	1.55	4.51	4.14 ± 2.42
Water Soluble Solids (%)	27.67	32.45	17.51	25.88 ± 7.63
VS (%)	95.12	95.99	95.92	95.68 ± 0.48
Ash (%)	4.87	4.01	4.08	4.32 ± 0.48
Cellulose (%)	20.30	15.47	15.74	17.17 ± 2.71
Hemicellulose (%)	38.18	33.52	39.79	37.16 ± 3.26
Acid Soluble Lignin (%)	1.56	1.71	1.81	1.69 ± 0.13
Acid Insoluble Residue (%)	14.51	10.34	22.37	15.74 ± 6.11
TN (%)	1.34	1.07	1.07	1.16 ± 0.16
Crude Protein (%)	8.35	6.69	6.66	7.23 ± 0.97

Neutral detergent fibre (NDF) (%)	55.61	37.72	35.15	42.83	±	11.15
Acid detergent fibre (ADF) (%)	35.61	29.19	25.09	29.96	±	5.30
Lignin Acid Detergent (ADL) (%)	11.46	5.15	4.68	7.10	±	3.79
Neutral detergent insoluble nitrogen (NDIN) (%)	0.44	0.36	0.35	0.38	±	0.05
Neutral detergent insoluble crude protein (NDICP) (%)	2.77	2.23	2.18	2.39	±	0.33
Acid detergent insoluble nitrogen (ADIN)	0.11	0.08	0.07	0.09	±	0.02
Acid detergent insoluble crude protein (ADICP) (%)	0.67	0.52	0.44	0.54	±	0.12

Cellulose content presents relatively small deviations from the values reported in literature and is within the reported range (13-37% by weight). Regarding lignin, the values found in literature are very close to the experimental values and are within the literature range (2-15% by weight). The same applies for ash. A significant discrepancy with the literature is observed in the value of hemicellulose, where there is a relative repeatability among the values of the 3 feedstocks that were characterized. This fact could be attributed to different production processes followed in each factory and in general in the different orange varieties.

As far as contaminants like pesticides, molds, bacteria are concerned, the application of good agricultural practices followed by industrial plant minimises the risk of their growth or presence. The recommended pesticide application practices are followed and the pre-harvest intervals specified on pesticide labels are adhere to ensure that residues are within safe limits. A washing process step within the juice production line is included for the removal of any surface pesticides residue. Additionally, the water used for washing or irrigation is of high quality and does not contain contaminants that could transfer to the orange peels. Since the extraction of essential oil is also performed, even any traces of contaminants absorbed by the wax of the orange peel would be removed. Furthermore, the juice industry ensures the safe transportation and Storage by using clean and food-grade containers for transporting orange peels to prevent contamination during transit and by storing the peels in a clean, cool, and dry environment to reduce the risk of mold growth and spoilage. The absence of potential contaminants is also ensured by conducting regular microbiological testing to monitor for the presence of harmful bacteria and molds in orange peels

and by testing for mycotoxin contamination, especially if there are signs of mold growth, as mycotoxins can be harmful. In general, regular monitoring for any physical signs of contamination, such as mold growth, off-odors, or spoilage during processing and storage is performed. Therefore, taking into consideration the industrial safety procedures and actions, along with the high temperature drying step in the downstream processing, it was considered that the possibility of contaminants presence is very low.

4. Optimization of the unprocessed orange peels drying process

The next step included the drying of the unprocessed orange peel waste. During drying of these samples, several issues came up due to the presence of free sugars and the hydrophilic nature of pectin. This obstacle was overcome with the addition of a small amount of oil that helped the drying process itself and the particle size distribution of the dried product.

In December 2021, around 50kg dried unprocessed orange peel waste were sent to ELGO-Dimitra (Figure 4) in order to supplement the feeding of ruminants and stand as the baseline of the animal trials. The composition of this substrate is presented in Table 3.

Figure 4. Dried unprocessed orange peels

Table 3. Composition of dried unprocessed orange peels

Parameter	Value
TS (%)	91.27
Moisture (%)	8.73
ASH (%)	4.81
VS (%)	95.19
Oil (%)	2.71
TN (%)	1.15
Crude Protein (%)	7.18
Cellulose (%)	20.58
Hemicellulose (%)	24.62
Acid Insoluble Residue (%)	12.98
Ether extract (%)	3.57
Neutral detergent fibre (NDF) (%)	38.32
Acid detergent fibre (ADF) (%)	29.66

Lignin Acid Detergent (ADL) (%)	5.24
Neutral detergent insoluble nitrogen (NDIN) (%)	0.41
Neutral detergent insoluble crude protein (NDICP) (%)	2.58
Acid detergent insoluble nitrogen (ADIN) (%)	0.09
Acid detergent insoluble crude protein (ADICP) (%)	0.56
In vitro organic matter digestibility (IVOMD) (%)	72.7

The preliminary animal trials with unprocessed orange peels were successful since the feedstock was incorporated well in the ruminants' daily feed. After 2 days of adaptation, the ruminants consumed the new ingredient easily (Figure 5).

Figure 5. Ruminants eating animal feed supplemented with dried orange peels

5. Optimization of the process treatment train in laboratory scale

The main goal of this project is to produce an improved feed ingredient for dairy sheep from orange peels from orange juice industries. In this context, it was decided that the applied process should aim to decrease the pectin and free sugar content and to increase the protein content of the orange peel waste while preserving or even increasing the digestibility of the feedstock in order to receive a nutrient-balanced animal feed.

Thus, the next step was the optimization of the process treatment train in laboratory scale that included an enzymatic hydrolysis and/or fermentation step resulting in a new secondary feedstuff of higher nutritional value. The processed feedstock shall be also dried in order to stabilize the product in terms of shelf life and feed safety. Factorial experimental design was adopted as methodological approach. Nutritional and in vitro digestibility value of the final product are set as optimisation parameters, while the controlling parameters are the main operational conditions such as loading, type and dosage of enzymes and yeasts.

5.1 1st integrated valorisation strategy (Strategy A)

In line with the targets described above, the 1st integrated valorisation strategy (Strategy A) that was examined is presented in the following Figure (Figure 6). It includes the production of advanced bioethanol and animal feed for ruminants. The concept was to take advantage of the sugar content of the hydrolysed substrate towards bioethanol while the hydrolysed, fermented residue enriched in protein could stand as an interesting feedstuff.

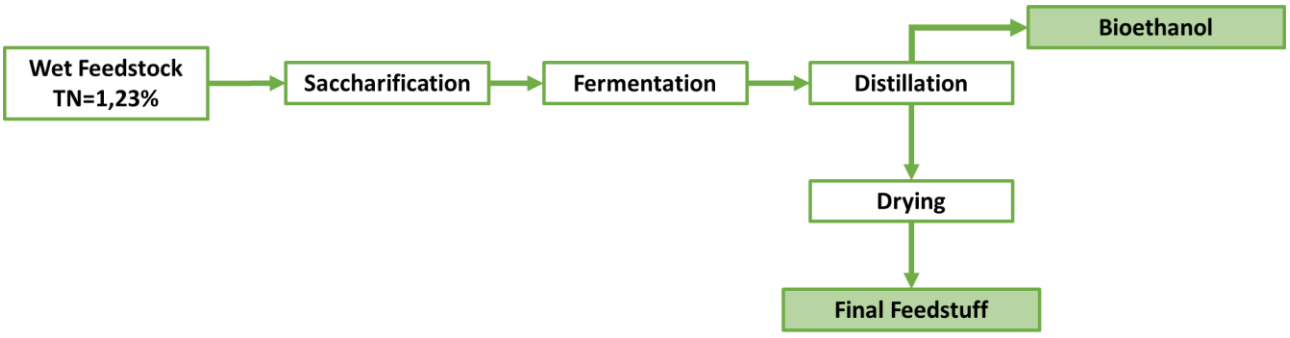


Figure 6. 1st integrated strategic approach (Strategy A)

Within this strategy, the first experimental trials included the enzymatic hydrolysis of cellulose with varying dosages of CellicCTec2 (0, 50, 150, 300 and 450μL/g cellulose) and subsequent fermentation with 2% Saccharomyces Cerevisiae (

Table 4). The effect of pH control was also studied. Enzymatic hydrolysis was performed at 50°C for 24h while fermentation at 30°C for 24h. Each experimental trial was perfomed twice. Figure 7 and Figure 8 present the time the time evolution of glucose and ethanol production during the experimental trials without and with pH control respectively.

Table 4. 1st experimental set studying the effect of enzyme dosage and pH control

	CellicCTec2 (µL/g cellulose) 50ºC, 24h	S. Cerevisiae (% d.b.) 30ºC, 24h
A1	0	2%
A2	50	2%
A3	150	2%
A4	300	2%
A5	450	2%

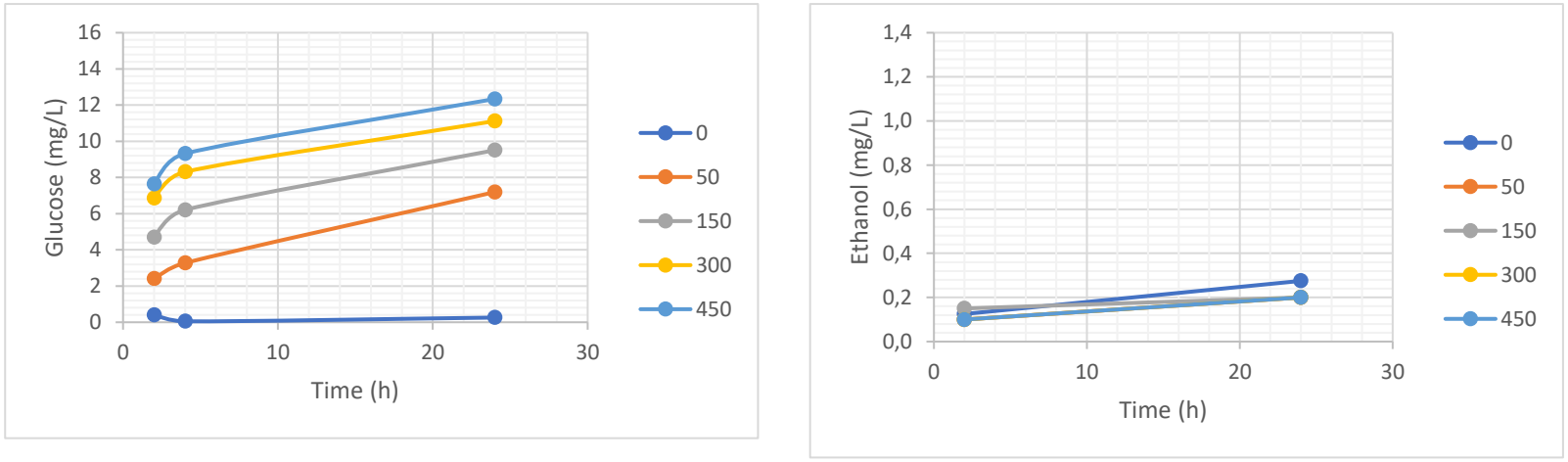


Figure 7. Glucose (a) and ethanol (b) production from orange peels during saccharification and fermentation without pH control

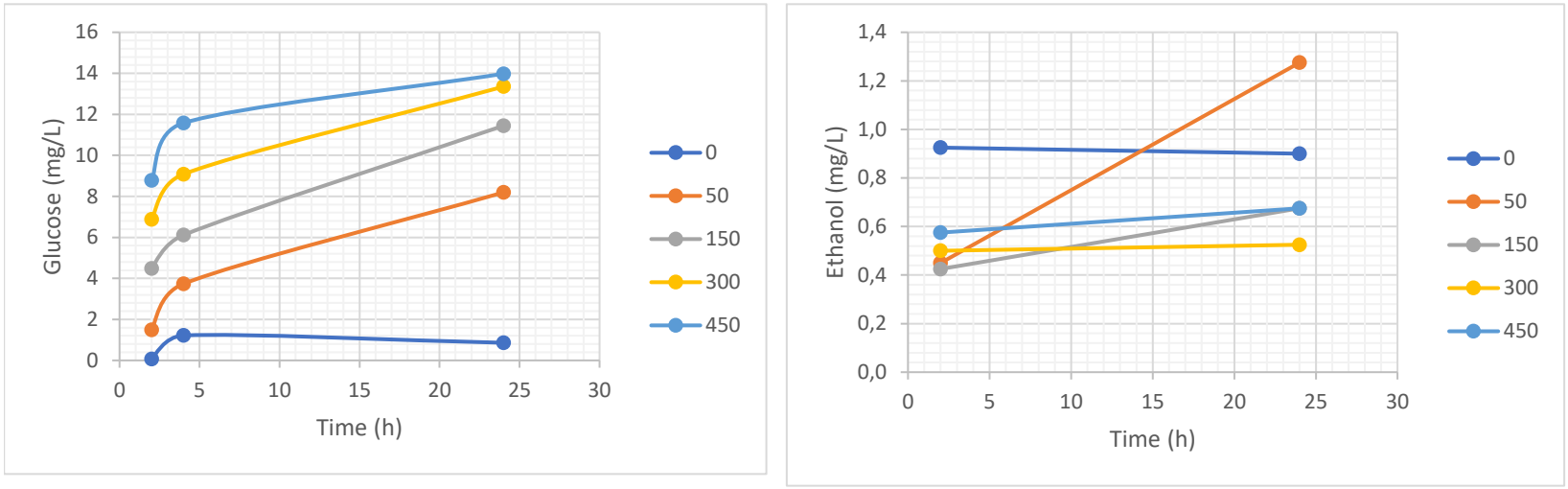


Figure 8. Glucose (a) and ethanol (b) production from orange peels during saccharification and fermentation with pH control

The saccharification and ethanol yields (SG and Yeth) are presented in Table 5 and Table 6 without and with pH control.

Table 5. Saccharification yield and ethanol yield of orange peels during the 1st experimental set without pH control (pH~3.8)

	Saccharification Yield S_G (%)			Ethanol Yield Y_{eth} (%)	
	2h	4h	24h	2h	24h
A1	2.44 ± 1.71	0.32 ± 0.04	1.59 ± 1.05	1.51 ± 0.3	3.32 ± 0.3
A2	14.815 ± 1.155	20.155 ± 1.975	44.255 ± 2.115	1.21 ± 0.2	2.41 ± 0.1
A3	28.955 ± 0.285	38.19 ± 2.21	58.49 ± 2.5	1.21 ± 0.1	2.41 ± 0.2
A4	42.23 ± 0.19	51.13 ± 0.72	68.395 ± 0.675	1.21 ± 0.2	2.41 ± 0.2
A5	46.98 ± 1.12	57.335 ± 0.095	75.9 ± 4.71	1.21 ± 0.0	2.41 ± 0.0

Table 6. Saccharification yield and ethanol yield of orange peels during the 1st experimental set with pH control (from 3.8 to 5.5)

	Saccharification Yield S_G (%)			Ethanol Yield Y_{eth} (%)	
	2h	4h	24h	2h	24h
A1'	0.415 ± 0.195	7.485 ± 5.295	5.29 ± 1.56	11.16 ± 6.33	10.86 ± 5.43
A2'	9.19 ± 0.53	22.99 ± 0.67	50.405 ± 1.925	5.43 ± 0	15.385 ± 0.905
A3'	27.61 ± 2.79	37.61 ± 0.77	70.415 ± 3.655	5.13 ± 0.3	8.145 ± 0.905
A4'	42.28 ± 1.3	55.89 ± 3.46	82.155 ± 0.575	6.035 ± 0.605	6.335 ± 0.905
A5'	54.015 ± 3.125	71.235 ± 4.765	86.005 ± 2.885	6.94 ± 0.3	8.445 ± 0.605

In all cases, for the experimental runs without pH control, a gradual increase in the saccharification yield was observed with the increase of the enzyme loading. A gradual increase in the saccharification yield was also observed over time. However, this was not the case for ethanol yield, since ethanol yields remained constant and at very low levels regardless of enzyme dosage. A slight increase in the ethanol yields was observed after 2 hours of fermentation. It is worth mentioning that the residual glucose concentrations remained high, indicating that the bioconversion of glucose to ethanol was inhibited.

The same pattern was observed for the experimental runs that pH was corrected using $CaCO_3$ from 3-3.5 to 5-5.5. It is evident that pH correction resulted in slightly higher yields. As before in all cases, with increasing enzyme concentration and over time a gradual increase in saccharification yield was observed. Regarding ethanol yield, the rates were relatively higher compared to the respective trials without pH corection. Nevertheless, ethanol yields remained at low levels and residual glucose concentrations remained high.

Conclusively, the enzyme loading as well as the pH control are factors that positively affect the saccharification performance. For this reason, it was decided in the following experimental tests to control the pH. On the other hand, the fermentation process was not favored accordingly indicating

the presence of inhibitory factors, resulting in low ethanol yields (6-15%). Therefore, the prospect of recovering a biofuel and simultaneously producing advanced animal feed does not seem viable. For this reason, an alternative strategy was examined.

5.2 2nd integrated valorisation strategy (Strategy B)

An alternative strategic approach was designed and is presented in Figure 9.

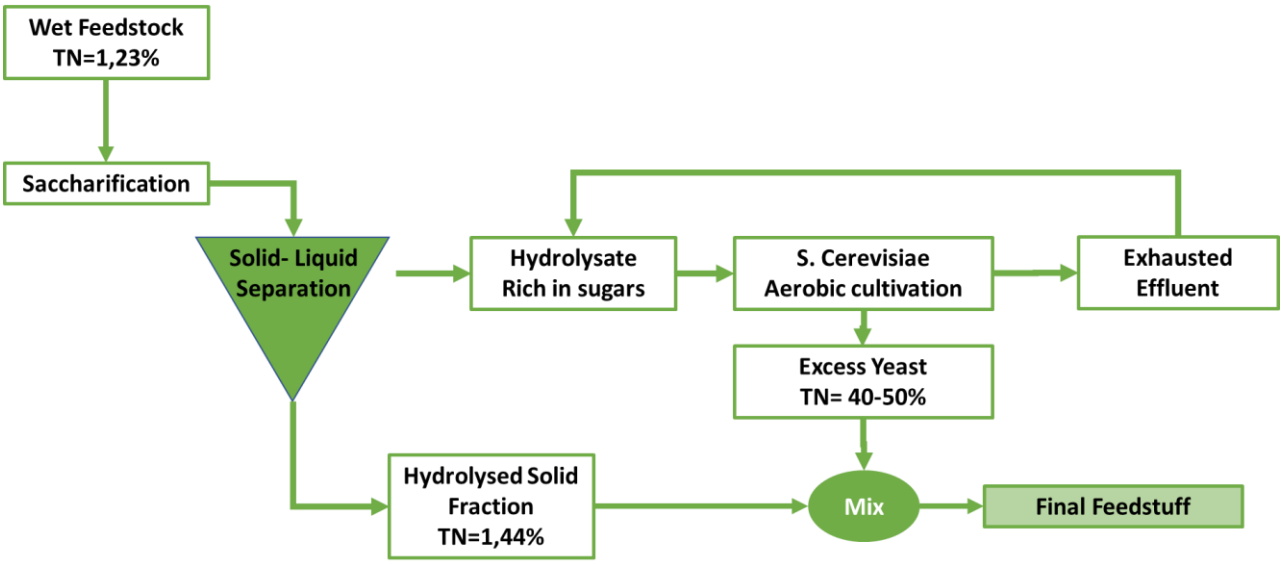


Figure 9. 2nd integrated valorisation strategy

In the context of this strategy, the enzymatic hydrolysis of orange peels was studied, from which a liquid fraction rich in sugars and a hydrolyzed solid residue are obtained. The liquid fraction is used for yeast cultivation with the ultimate goal of producing single cell protein. The latter is mixed with the hydrolyzed solid residue to produce advanced animal feed.

5.2.1 Optimisation of orange peels saccharification

Initially, various enzymatic formulations were tested to this direction to optimize the saccharification process (Table 7). The orange peels as received (wet feedstock) as well as the dried orange peels (dry feedstock) were both examined.

Table 7. Effect of enzymatic formulation and dosage on the saccharification of orange peels

No		Loading (%)	NS22177 (µL/g cel)	Pectinex (µL/g TS)	CellicCtec2 (µL/g cel)	CellicCtec3 (µL/g cel)	TSS degradation (%)	Glucose (g/L)	S_G (%)	TRS (g/L)	TN %
B.1.1	Wet	7.36	-	2.5	-	512	70.50	18.47	87.61	29.87	1.44
B.1.2	Wet	7.36	750		-	-	71.02	19.14	90.80	28.60	1.44
B.1.3	Wet	7.36	-	-	-	512	56.09	17.65	83.75	23.75	1.21
B.1.4	Wet	7.36	500	2.5	-	-	55.15	13.70	64.98	20.61	1.62
B.1.5	Wet	7.36	500	3.7	-	-	54.67	16.89	80.12	19.86	1.54
B.1.6	Wet	7.36	-	3.7	500	-	42.99	15.79	74.92	17.93	1.51
B.1.7	Wet	7.36	-	-	-	-	41.27	8.97	42.58	16.73	1.03
B.1.8	Wet	7.36	500	2.5	-	-	51.81	15.98	75.81	14.63	1.87
B.1.9	Wet	7.36	750	-	-	-	45.36	15.10	71.66	14.59	1.65
B.1.10	Wet	7.36	-	2.5	500	-	43.30	14.07	66.77	14.01	1.44
B.1.11	Wet	7.36	500	-	-	-	44.72	15.20	72.10	13.74	1.74
B.1.12	Wet	7.36	-	-	-	-	15.85	1.81	8.61	9.80	1.35
B.1.13	Wet	10.13	500				53.05	20.64	72.88	35.53	1.45
B.1.14	Dry	16.66	350	2.5	-	-	46.27	23.47	76.29	48.12	1.34
B.1.15	Dry	10.73	525	2.5	-	-	57.98	20.95	68.11	36.15	1.83
B.1.16	Dry	10.05	-	2.5	350	-	55.93	18.79	61.09	29.06	1.70
B.1.17	Dry	10.06	-	3.7	350	-	55.34	18.76	60.99	26.44	1.79
B.1.18	Dry	10.05	350	3.7	-	-	57.27	20.39	66.28	25.65	1.93

It is evident that quite high saccharification yields were observed (42.58-90.8%) for the wet feedstock. Initially the highest efficiency was achieved by using the cellulolytic enzyme NS22177 (750 (μL / g cel)) and then the enzyme CellicCtec3 in combination with pectinase (Pectinex). Pectinase positively affected the saccharification yield since in the test with CellicCtec3 without pectinase (test B.1.3) the saccharification yield was lower (83.75%). Regarding the nitrogen content in the residual solid, very small variations were observed between the untreated raw material (1.35% TN) and the other tests. Test B.1.8 seems to have the highest nitrogen content where 500 μL of NS22177 / g cel were added in combination with pectinase (48 Pectinex (μL/g).

Furthermore, the use of dried substate was studied in an effort to increase solid loading and subsequent sugar release. Similar observations occur in the case of the dry substrate. Satisfactory saccharification yields (60.99-76.29%) and low variations in nitrogen content were observed. Compared to the wet substrate, higher concentrations of glucose were observed (19 -23 g / L versus 13-19 g / L in the wet). However, this increase can not justify the implementation of a drying step due to the high energy requirements.

Therefore, this strategy adopts the use of fresh/wet substrate with the use of enzymes CellicCtec3 in combination with Pectinex. In order to to trace the optimum operational conditions, **a** 2^3 factorial experiment was designed.

The aim of this experimental procedure was to determine the influence of some basic process parameters on the saccharification efficiency SG (optimization parameter). A factorial experiment at lab-scale was designed in order to optimize the dosages of the enzymatic formulations (CellicCtec3, Pectinex) and the solid loading in the saccharification process of orange peels waste. The factorial design was applied as a useful technique to investigate the effect of the process variables (enzyme dosages both cellulolytic and pectinolytic, and solid loading) on the saccharification process output in terms of sugars yield (Y_s). The 3rd feedstock was used for these experimental runs. In general, by using a 2^n factorial design, "n" controlling parameters interrelate to an optimization parameter through an appropriate linear model. Their significance can also be estimated and assessed (Alder et al., 1975; Cochran & Cox, 1957). The levels of the controlling parameters are given in Table 8. The experimental area of the factorial design was pre-determined in the preliminary trials as presented before (Table 7).

Table 8. Levels of factorial experiment

Parameter	Low level	High Level	Center

	(-)	(+)	(0)
Pectinex (µL/ g TS)	25	75	50
CellicCTec3 (µL/g TS)	25	75	50
Loading (%)	2.5	7.5	5

In the 2^3 factorial design, 8 experiments were carried out in duplicate (Table 9). Four extra experiments in the centre of the design were also conducted for statistical purposes. From these data, a mathematical model was constructed, and its adequacy was checked by the Fisher criterion.

Table 9. Experimental runs of orange peels waste saccharification according to the factorial design (B4.1-B4.20)

Experiments	Pectinex (µL/ g TS)	CellicCTec3 (µL/g TS)	Loading (%)
B2.1	25	25	2.5
B2.2	25	25	2.5
B2.3	25	25	7.5
B2.4	25	25	7.5
B2.5	25	75	2.5
B2.6	25	75	2.5
B2.7	25	75	7.5
B2.8	25	75	7.5
B2.9	75	25	2.5
B2.10	75	25	2.5
B2.11	75	25	7.5
B2.12	75	25	7.5
B2.13	75	75	2.5
B2.14	75	75	2.5
B2.15	75	75	7.5
B2.16	75	75	7.5
B2.17	50	50	5
B2.18	50	50	5
B2.19	50	50	5
B2.20	50	50	5

At the beginning of each experimental trial, the pH was corrected to 5.5. Nevertheless, by the end of the saccharification process the measured pH was lower (4.06±0.28), implying the low buffering capacity of the mixture along with the possible production of short chain fatty acids. The hydrolysate in the end of the experimental trials was centrifuged and the liquid and solid phase were characterised.

The results of the factorial experiment in terms of glucose, total reduced sugars (TRS) and TOC concentrations are presented in the following table (Table 10).

Table 10. Liquid phase composition of the factorial experimental trials

Experiments	Glucose (g/L)	TRS (g/L)	TOC (g/L)
B2.1	3.810	1.696	8.172
B2.2	3.452	1.819	8.426
B2.3	13.150	8.821	25.640
B2.4	13.236	11.380	26.120
B2.5	4.383	2.084	9.076
B2.6	4.985	0.734	8.886
B2.7	13.302	19.629	28.550
B2.8	12.589	20.835	27.330
B2.9	3.593	4.540	9.660
B2.10	3.546	3.585	9.365
B2.11	13.007	21.568	29.117
B2.12	13.143	21.313	28.445
B2.13	5.056	7.658	10.330
B2.14	4.916	6.827	9.660
B2.15	13.462	26.934	27.520
B2.16	13.696	29.318	29.305
B2.17	9.369	16.301	18.810
B2.18	9.397	13.706	18.450
B2.19	9.455	15.008	17.590
B2.20	9.798	13.044	18.220

From this Table (Table 10), it is obvious that the released sugars and overall organic compounds are increased with the solid loading implying that effective solids hydrolysis took place. In all cases, glucose contributed by 19.19±2.00% to the total organic carbon concentration.

The characteristics of the solid fraction in terms of Total Kjeldahl Nitrogen (TKN), Water Soluble Solids (WS), Cellulose, Hemicellulose, Acid Insoluble Residue (AIR) and Acid Soluble Lignin (ASL) is presented in the following table (Table 11).

Table 11. Solid phase composition of the factorial experimental trials

Experiments	TKN (%)	WS (%)	Cellulose (%)	HemiCellulose (%)	AIR (%)	ASL (%)
Feedstock	1.07	17.51	15.74	39.79	22.37	1.81
B2.1	1.54	14.49	9.08	23.91	22.80	2.16

B2.2	1.68	13.62	8.77	28.52	24.43	2.13
B2.3	1.62	29.13	6.13	19.88	19.74	1.97
B2.4	1.37	29.55	6.71	21.07	21.65	1.97
B2.5	1.87	10.80	9.06	28.44	23.22	2.43
B2.6	1.96	12.41	8.68	28.22	23.38	2.19
B2.7	1.41	32.18	5.58	20.33	19.76	1.47
B2.8	1.39	24.05	5.94	26.16	19.45	1.75
B2.9	1.72	18.34	7.02	24.80	25.34	1.80
B2.10	1.75	16.75	5.93	23.27	26.03	1.91
B2.11	1.94	28.60	5.50	23.28	20.13	1.53
B2.12	1.26	32.67	5.73	26.82	21.29	1.65
B2.13	1.42	13.14	6.41	28.94	22.69	2.15
B2.14	1.37	10.98	6.84	33.32	22.30	2.29
B2.15	1.75	29.16	4.88	22.65	18.80	1.78
B2.16	1.77	31.69	5.46	23.13	19.49	1.57
B2.17	1.54	17.08	6.45	26.33	24.94	1.84
B2.18	1.47	20.52	6.90	29.76	19.88	1.93
B2.19	1.51	21.09	6.29	29.57	19.52	1.81
B2.20	1.49	20.25	5.50	29.32	21.11	1.94

Given the experimental results and measurements, the degradation efficiencies of total solids, cellulose and hemicellulose along with the sugars yield were calculated and presented in the following Table (Table 12).

Table 12. Degradation efficiencies and saccharification yields of the factorial experimental trials

Experiments	Pectinex (µL/g TS)	CellicCTec3 (µL/g TS)	Loading (%)	TS degradation (%)	Cellulose degradation (%)	Hemicellulose degradation (%)	Sugars yield Y_s (g glucose/100g TS)
B4.1 -B4.2	25	25	2.5	57.14 ± 0.01	75.07 ± 0.60	71.04 ± 3.60	12.44 ± 0.99
B4.3 -B4.4	25	25	7.5	41.99 ± 2.93	75.77 ± 0.32	69.42 ± 0.29	14.57 ± 0.08
B4.5 -B4.6	25	75	2.5	59.4 ± 1.04	76.53 ± 1.31	70.36 ± 0.92	16.55 ± 1.66
B4.7 -B4.8	25	75	7.5	47.13 ± 0.92	80.16 ± 0.54	68.38 ± 5.07	14.27 ± 0.62
B4.9 -B4.10	75	25	2.5	59.41 ± 0.70	82.86 ± 2.34	74.84 ± 1.56	12.20 ± 0.13
B4.11 -B4.12	75	25	7.5	50.42 ± 0.63	81.86 ± 0.74	67.97 ± 3.61	14.43 ± 0.12
B4.13 -B4.14	75	75	2.5	66.25 ± 1.09	79.69 ± 0.61	62.26 ± 3.15	17.73 ± 0.39
B4.15 -B4.16	75	75	7.5	52.93 ± 0.76	88.65 ± 0.54	80.09 ± 0.35	15.05 ± 0.20
B4.17 -B4.20	50	50	5	55.06 ± 1.23	81.62 ± 1.40	66.73 ± 1.56	16.34 ± 0.38

According to the results of the factorial experiment and by following a specific analytical procedure (Alder et al., 1975; Cochran & Cox, 1957), the following mathematical model was developed, interrelating the sugars yield with the controlling parameters of the system:

$$Y=14.46+0.95*X1-0.04*X2+0.02*X3-.1*X1*X2+0.34*X1*X3-0.21*X2*X3-0.59*X1*X2*X3$$

Equation 31

Pareto chart of the standardized effects (Figure 10) compares the relative magnitude and the statistical significance of both main and interaction effects.

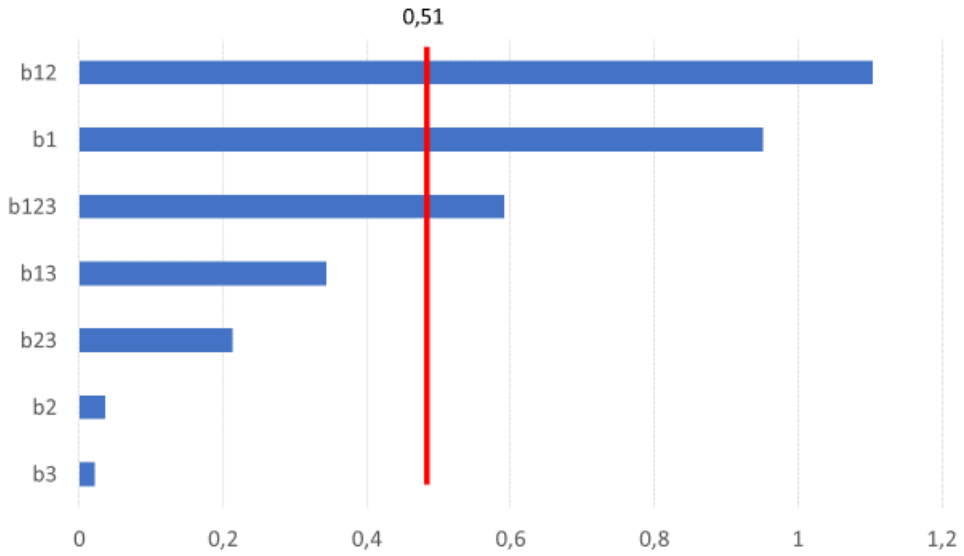


Figure 10. Pareto Chart of the Standardized Effects (response is Strength, α = 0.05)

According to these results, the dosage of Pectinex (X1) is statistically significant (α = 0.05), along with the interaction of both enzyme dosages and the interaction of all three controlling parameters. In addition, the largest effect is the interaction of enzymatic formulations (CelliccTec3 and Pectinex). The effect of the interaction of all parameters (b123) is the smallest because it extends the least.

The plus (+) in the above equation indicates that an increase in Pectinex dosage leads to a higher sugars yield and consequently to higher glucose concentrations. On the other hand, the minus (-) implies a negative impact as it is the case for interactions.

Taking into consideration only the statistical important parameters, the mathematical model is converted into the following equation:

$$Y=14.46+0.95*X1-1.1*X1*X2 -0.59*X1*X2*X3$$ *Equation 32*

The adequacy of the mathematical model derived from the factorial design was checked by the Fisher criterion and it proved to be adequate and could satisfactorily fit to the data within the examined range.

Converting the coded parameters to physical values the mathematical model is:

$$Y_s = 12.88+0.0316P-0.0064C-0.944L+0.00013PC+0.01888PL+0.01888CL-0.000378PLC \quad \text{Equation 33}$$

where Y_s saccharification yield expressed as g glucose per 100g TS.

P Pectinex dosage in µL/g TS

C CellicCTec3 in µL/g TS

L Loading (%)

In the experimental range studied, the highest sugars yield achieved was 17.73±0.39 % in the experimental point 75 µL Pectinex /g TS, 75 µL CellicCTec3 /g TS and 2.5% Loading. Nevertheless, the highest glucose concetrations 13.579± 0.166 g/L and 13.193±0.061 g/L were observed at B4.15-B4.16 and B4.3-B4.4 repsectively with high sugar yields over 15%. Conclusively, taking into consideration the experimental results and technoeconomic factors (e.g. enzymes cost, minimisation of fresh water needs), it was decided the pilot trials of saccharification process would be conducted at 50°C, 7.5% solids loading, Pectinex 25µL/g TS, CellicCTec3 25 µL/g TS.

5.2.2 Optimisation of aerobic fermentation

Regarding the aerobic fermentation of S.Cerevisiae, initially preliminary experiments with synthetic glucose solution and nutrients addition were conducted (Table 13). According to literature, pH, DO and Glucose/Yeast ratio are the crucial operational parameters.

For each experimental trial, the residual glucose concentration was measured. Ethanol concentration was also measured to evaluate the Grab effect and the solid residue in terms of suspended solids was determined.

Table 13. Aerobic fermentation with synthetic glucose solution and nutrients addition

No	Yeast (g/L)	Glucose initial (g/L)	pH corrected	pH final	Glucose final (g/L)	Ethanol (g/L)	Y_{aer} (%)	Y_{obs} (%)
B3.1	1	5	4.70	3.00	0.000	0	31.96	15.98

B3.2	1	5	6.03	5.70	0.000	0	20.90	10.45
B3.3	1	5	4.63	3.15	0.024	0.45	20.96	10.48
B3.4	1	5	4.65	4.27	0.004	0.22	26.64	13.32
B3.5	1	5	4.64	5.22	0.006	0	23.70	11.85

The cultivation of yeast with synthetic glucose resulted in narrow variations in biomass yields, Y_{obs} (10-16%). In the aerobic fermentation yields, Y_{aer},, a small deviation was observed in sample B3.1 (31.96%) compared to the rest of the samples which ranged between 20.9-26.64%.

As a next step, the liquid phase of the orange peels waste was used as substrate for the cultivation of yeast. The effect of working volume/scale, glucose to yeast ratio, the addition of nutrients, control and monitoring of DO and pH was assessed according to Table 14.

Table 14. Aerobic fermentation with the liquid phase of the orange peels waste

No	Nutrients Addition	Yeast (g/L)	Glucose (g/L)	Volume (L)	Glucose/ Yeast	Initial pH	Corrected pH	Final pH	Initial DO (mg/L)	Final DO (mg/L)	Final Glucose (g/L)	Final Ethanol (g/L)	Y_{obs} (%)
B4.1	No	10	0.96	1	0.0957	3.4	5.77	5.5	1.5-2	1.5-2	0.005	0.38	< 0
B4.2	Yes	1	0.98	1	0.98	3.62	5.87	3.6	-	-	n.d.	0.39	1.26
B4.3	No	1	1.06	1	1.06	3.44	5.97	3.9	-	-	n.d.	0.24	9.20
B4.4	Yes	1	0.92	0.2	0.916	3.5	5.92	6.2	-	0.29	0.007	0.21	15.56
B4.5	Yes	1	0.99	1	0.985	3.31	5.91	7.1	-	0.22	0.03	0.12	19.28
B4.6	No	1	1.75	0.2	1.75	3.35	5.81	5.86	-	-	0.009	0.06	20.69
B4.7	Yes	0.548	2.74	1	5	3.14	5.54	4	9	-	n.d.	0.292	21.58
B4.8	Yes	1	3.66	0.2	3.66	3.40	4.80	4.63	-	0.29	0.003	0.09	24.86
B4.9	No	1	0.96	1	0.9575	3.27	5.85	5.7	1.5-2	1.5-2	0.02	0.87	25.35
B4.10	Yes	1	2.23	0.2	2.23	3.42	4.80	4.9	-	-	0.02	0.70	28.09
B4.11	Yes	1	2.23	0.2	2.23	3.40	4.80	4.38	-	0.31	0.002	n.d.	33.16
B4.12	Yes	1	1.75	0.2	1.75	3.45	5.79	5.95	-	-	0.009	0.03	49.20
B4.13	Yes	1	0.95	0.2	0.95	3.49	5.85	5.9	-	-	0.03	n.d.	52.21
B4.14	Yes	1	0.94	1	0.9412	3.5	5.82	6	6.60	0.25	0.029	n.d.	74.17
B4.15	Yes	1	1.12	0.2	1.1227	3.6	5.88	5.7	6.98	0.23	0.033	n.d.	77.36

A wide range of biomass yields (1.26-77.36%) were observed for the aerobic fermentation with the liquid phase of the orange peels waste. Test B4.15 (77.36%) and B4.14 (74.17%) presented the highest yields. The low yield in tests B4.2 (1.26%) and B4.3 (9.20%) can be attributed to the fact that during the experiment some issues regarding the air diffussion were occured. Therefore, in these cases the anaerobic fermentation process was favored and the sugars were converted into bioethanol with concentrations of 0.39-0.24 g/L respectively.

Conclusively, it seems that the nutrients addition is necessary, and DO and pH control improve the efficiency while upscaling by a factor of 5 does not affect negatively the process.

As a next step, the hydrolysate from orange peels at the optimized conditions was tested as substrate for the aerobic cultivation of S. Cerevisiae in bench scale (1-2L working volume). According to the results described above the addition of nutrients and control of pH and DO were adopted . Furthermore higher glucose to yeast ratios were applied in order to minimise the water needs of dilution. Thus, the operational conditions and the corresponding results are presented in Table 15.

Table 15. Aerobic fermentation with thehydrolysate of the orange peels waste at the optimised saccharification conditions

No	Yeast (g/L)	Glucose (g/L)	Volume (L)	Glucose/ Yeast	Initial pH	Cor. pH	Final pH	Final DO (mg/L)	Final Glucose (g/L)	Final Ethanol (g/L)	Y_{obs} (%)
B5.1	1	16.5	1	16.5	3.4	5.00	4.3	0.31	0.860	3.01	45.27
B5.2	0.3	15.97	2	53.2	3.2	6.51	5.9	-	n.d.		49.99

High biomass yields were also observed by the use of orange peels hydrolysate. In view of these results, the optimum conditions that will be upscaled are use of orange peels hydrolysate as sugar source, nutrients addition, pH and DO control during aerobic fermentation.

5.2.3 Formulation of animal feedstuff

According to strategy B, the final animal feedstuff should be formulated by mixing the solid residue of orange peels after the saccharification process under the optimum conditions (50°C, 24h, 7.5% solids loading, Pectinex 25μL/g TS, CellicCTec3 25 μL/g TS) with the harvested yeast cultivated aerobically on orange peels hydrolysate (30°, 24h, orange peels hydrolysate as sugar source, nutrients addition, pH =5, pH and DO control).

According to the results of the experimental trials and the achieved yields, a mass balance of the applied treatment train is presented in Figure 11.

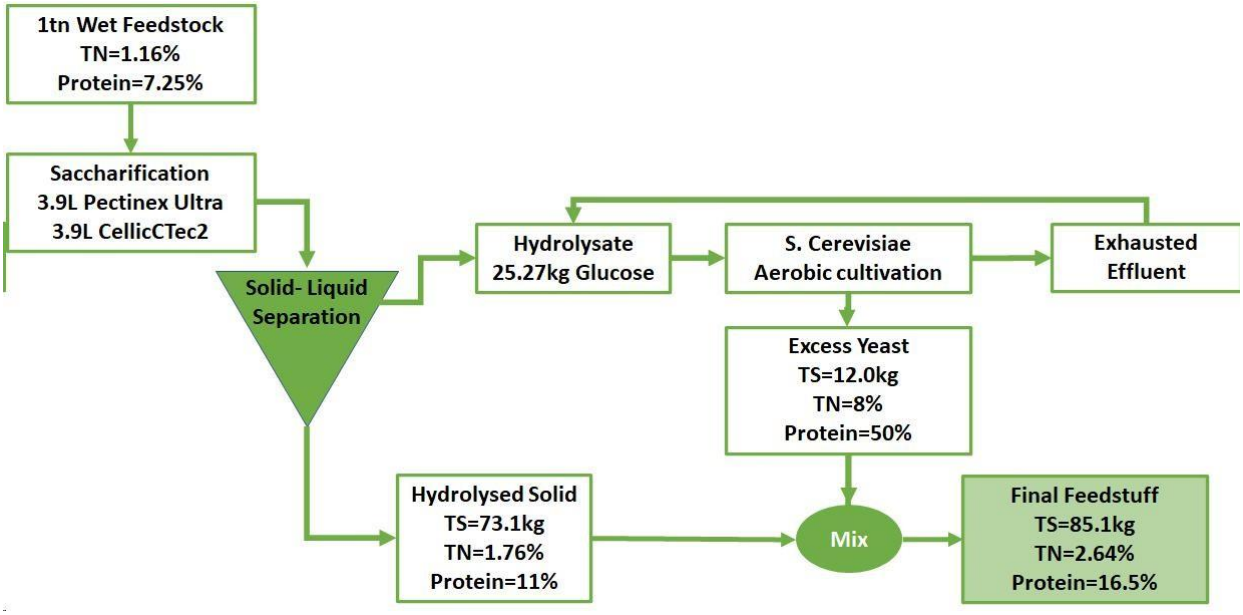


Figure 11. Flow chart of strategy B including mass balances

It is evident that the implementation of strategy B could lead to a final feedstuff with elevated protein content (16.5%). According to the presented mass balances and in line with a zero waste discharge concept, the mixing of excess yeast and hydrolysed solid (14% yeast) is final step for the formulation of the animal feedstuff prior to drying.

In this context, a sample of feedstuff prepared under the optimum conditions descripted above was formulated and characterized in physicochemical and nutritional terms.

Table 16. Composition of dried unprocessed orange peels and feedstuff prepared under the optimum conditions of strategy B

Parameter	**Dried unprocessed orange peels**	**Feedstuff prepared under the optimum conditions of strategy B**
TS (%)	91.27	94.78
Moisture (%)	8.73	5.22
ASH (%)	4.81	5.03
VS (%)	95.19	94.97
Oil (%)	2.71	2.25
TN(%)	1.15	2.36
Crude Protein (%)	7.18	14.75
Cellulose (%)	20.58	6.80
Hemicellulose (%)	24.62	17.94
Acid Insoluble Residue (%)	12.98	18.92
Ether extract (%)	3.57	2.96

Neutral detergent fibre (NDF) (%)	38.32	28.54
Acid detergent fibre (ADF) (%)	29.66	17.81
Lignin Acid Detergent (ADL) (%)	5.24	6.43
Neutral detergent insoluble nitrogen (NDIN) (%)	0.41	0.31
Neutral detergent insoluble crude protein (NDICP) (%)	2.58	1.96
Acid detergent insoluble nitrogen (ADIN) (%)	0.09	0.05
Acid detergent insoluble crude protein (ADICP) (%)	0.56	0.30
In vitro organic matter digestibility (IVOMD) (%)	72.7	89.5

It is worth noticing that the feedstuff prepared under the optimum conditions of strategy B presented higher (23.11%) in vitro organic matter digestibility, almost doubled protein content.

6. Start-up pilot operation

6.1 Location of the facility and other partners

The pilot plant that will materialize the case study 2 is located in the premises of NTUA, Athens, Greece. It is established in the Unit of Environmental Science and Technology, School of Chemical Engineering, National Technical University of Athens (Figure 12). The mail address is 9, Iroon Polytechniou str., Zografou, 15780, Greece and the coordinates are 37°58'36.9"N 23°47'06.0"E (https://maps.app.goo.gl/fwspxZCnPbXz8d5Y8).

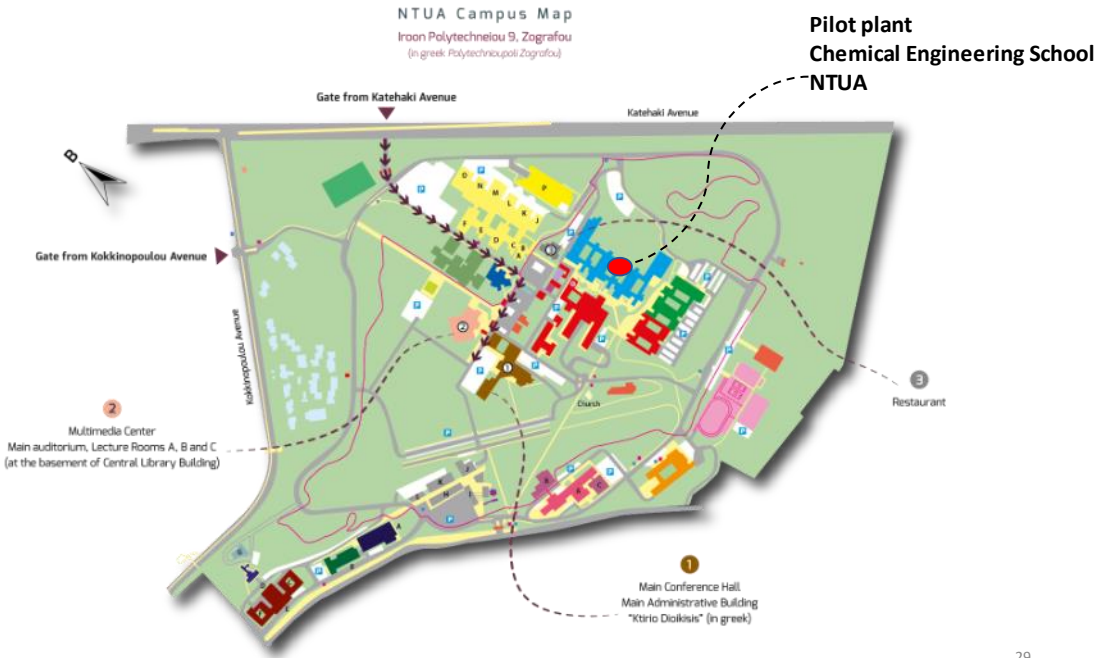


Figure 12. Pilot plant location in the NTUA campus

The pilot plant operates under the responsibility of National Technical University of Athens, NTUA. SEVT will provide orange peels from the collabolating orange juice industry; Hellenic Fruit Juices and ELGO-DIMITRA will determine the Feeding strategy based on the analysis of the obtained ingredients.

6.2 Pilot plant description

The pilot plant is a pre-existing installation developed in the framework of the LIFE WASTE2BIO project (LIFE11 ENV/GR/000949) which was upgraded within Horizon 2020 WaysTUP! Project (GA

no. 818308). This prototype plant will be used in order to meet the NEWFEED needs. The pilot plant includes the following:

a) 2 interconnected bioreactors (for enzymatic hydrolysis and fermentation)

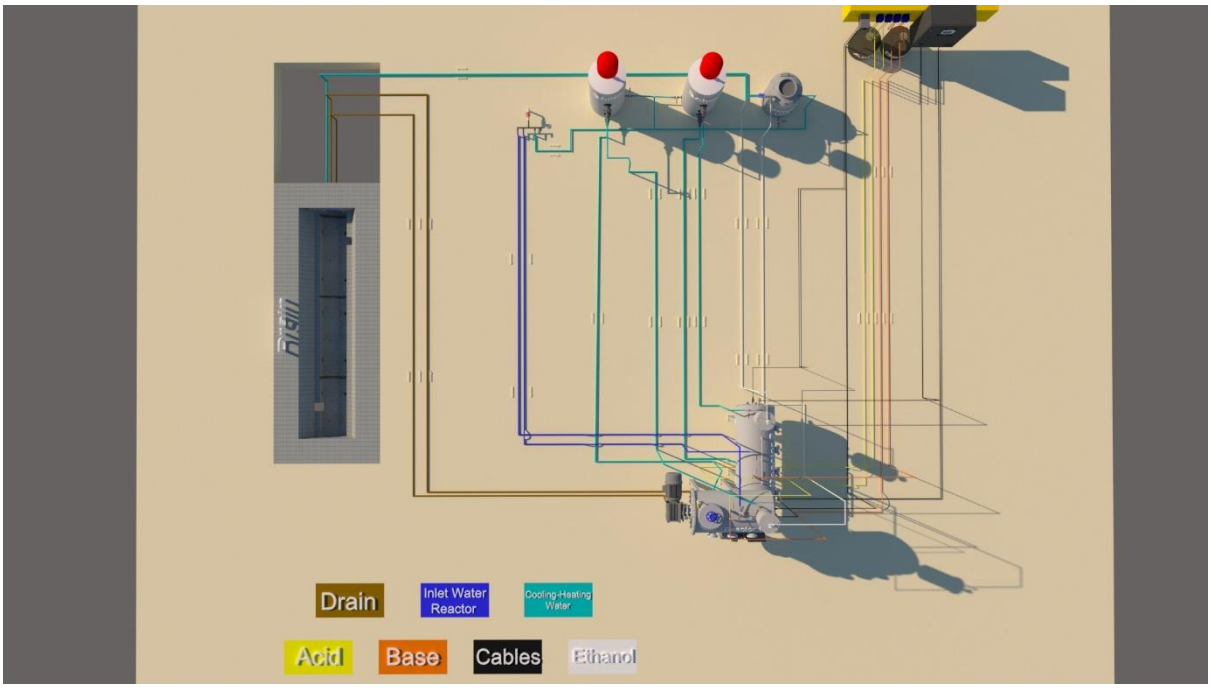
b) a distillation unit (solvents recovery)

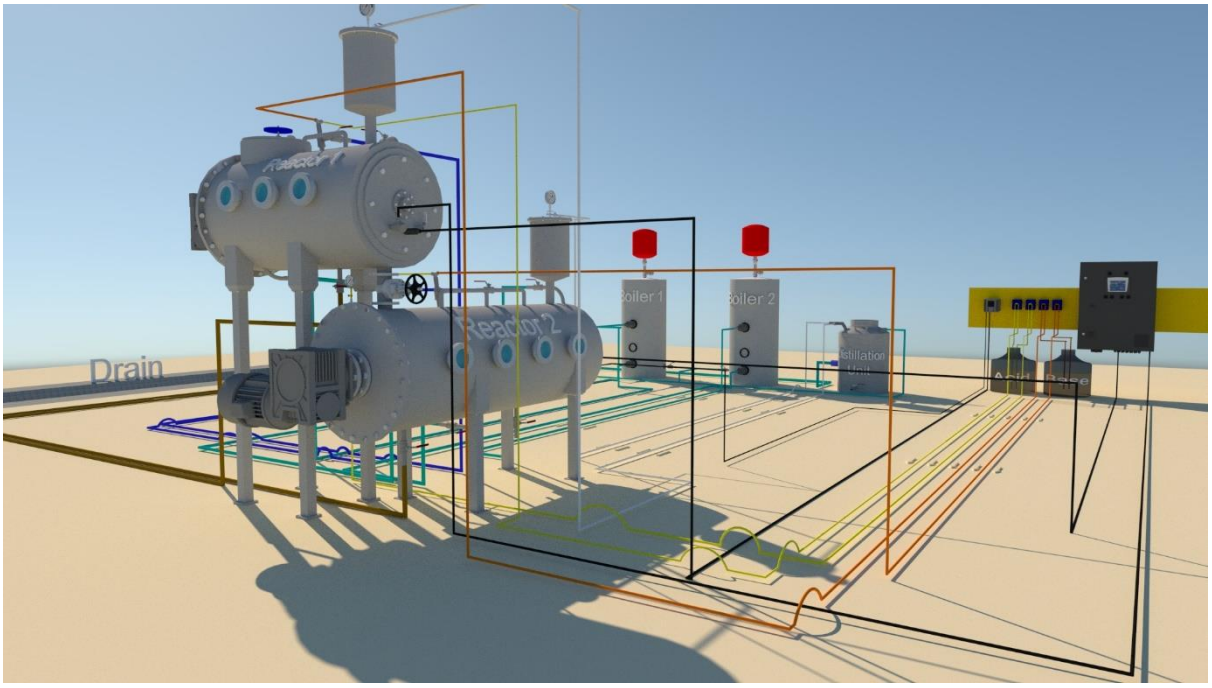
c) a GAIA GC-100 food waste dryer (dehydration).

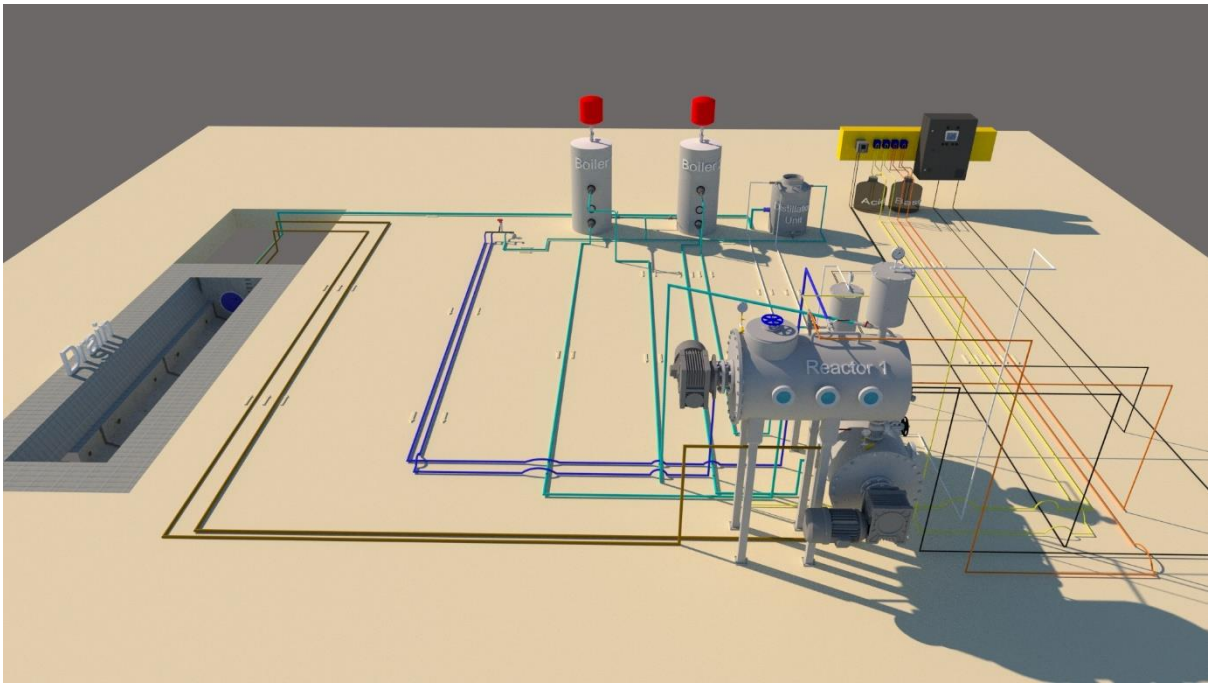
A concise description of each process unit as it will operate in the framework of NEWFEED project follows.

6.2.1 Bioconversion and Distillation Unit

The pilot plant is illustrated in Figure 13 presenting the main components along with the respective piping and wiring from different perspectives.




Drain
Boiler 1
Boiler 2


Boiler
Boiler
Reactor 1

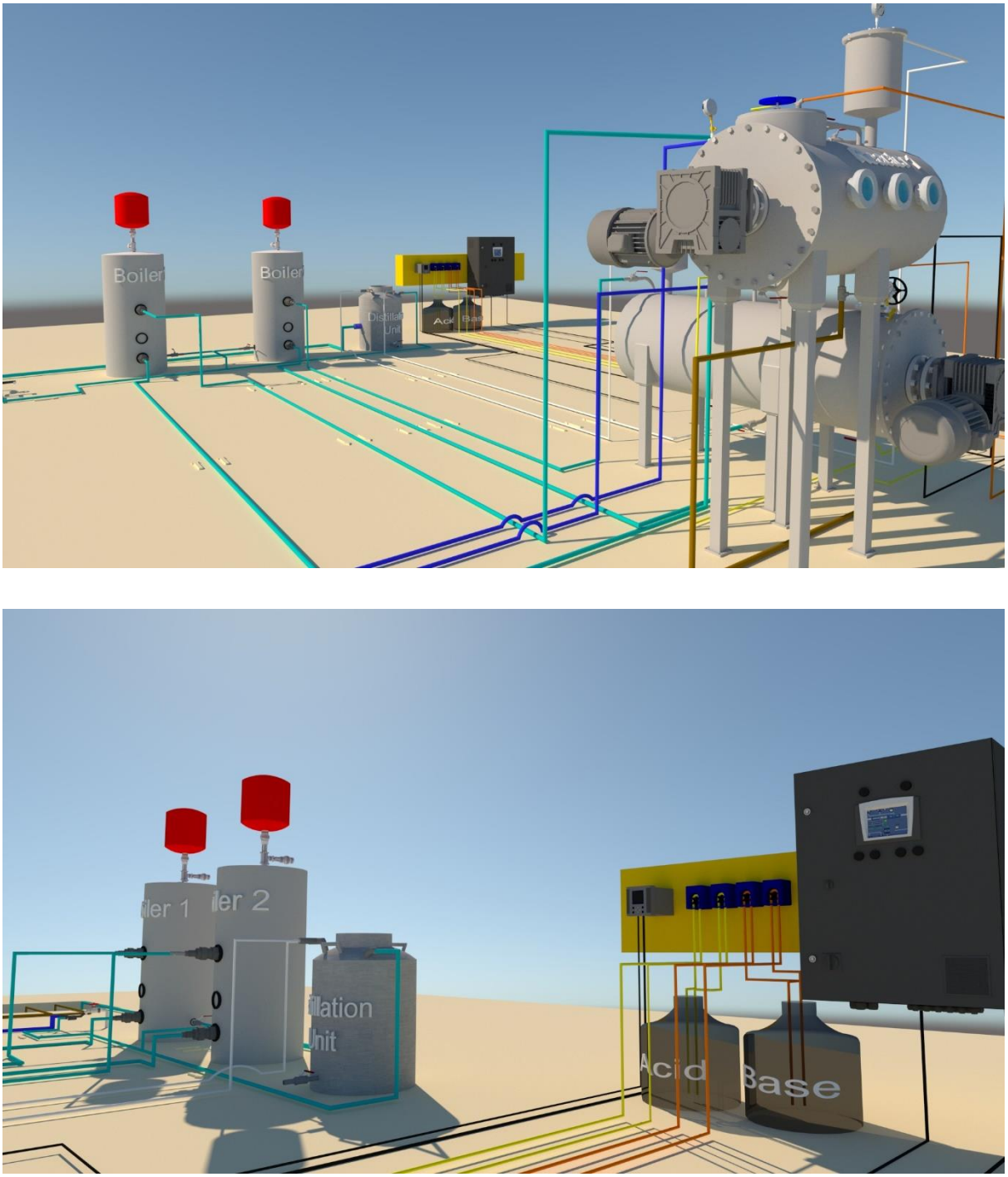


Figure 13. Photorealistic overview of bioconversion and distillation units of the pilot plant

The bioconversion unit consists of two interconnecting horizontal cylindrical bioreactors of 200 L, with a rotating shaft for the mixing of the material (Figure 14). The temperature is controlled by water jacket in a close loop. Each bioreactor has independent heating circuit that the reaction mixture temperature is set and controlled through the PLC. 4 digital temperature displays and control modulus are included in the central switchboard (Figure 15). The user has the ability to set both the reaction temperature and the recirculated water temperature. pH is monitored and

controlled, through a fully automated system that includes pH probes, display and controlling modulus, 4 peristaltic pumps for the addition of the necessary chemicals (Figure 16). The operation of the pilot plant is fully controlled through the upgraded PLC Siemens S7-1200 (Figure 17). The pilot operator may set all the operational parameters (T, pH, mixing time and direction, total duration) through the installed touch screen 7'' (Siemens). Additionally, stored programs may be used.

Figure 14. The 2 horizontal bioreactors of the pilot plant

Figure 15. Main components of the heating system of the bioreactors

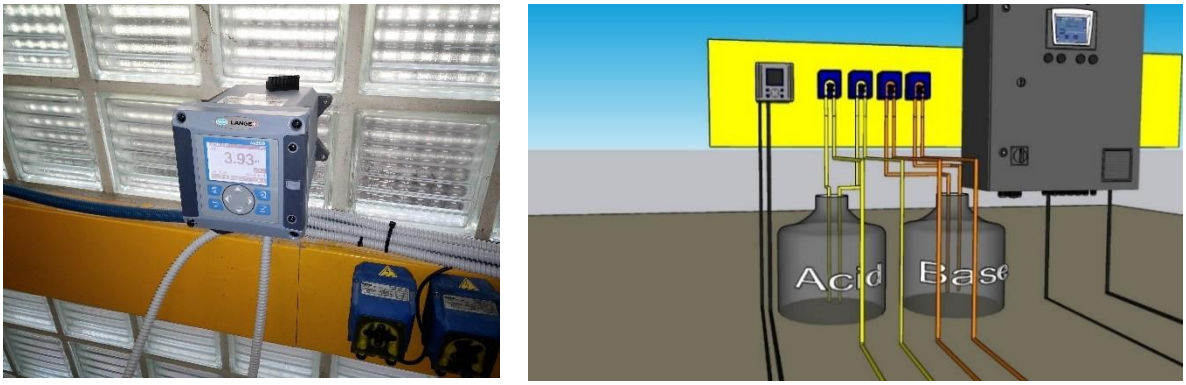


Figure 16. pH control system

Figure 17. Pictures of the PLC and the central switchboard of the pilot plant

A distillation unit is also included in the system that would be used for the ethanol recovery in case Strategy A was favourable.

6.2.2 Dehydration Unit

A commercial food waste dryer GAIA GC-100 will be used for the dehydration of the animal feedstuff (Figure 18).

Figure 18. Dehydration unit GAIA GC-100 of the pilot plant

6.3 Pilot capacity

The proposed technology has been tested and proven in a relevant environment that closely resembles its real-world use meeting the target of TRL 6, both for the valorisation technology and the innovative animal feed ingredient. The capacity of the demo scale bioconversion reactor is about 375 kg of raw material / day, and the capacity of the drier is 1 ton of raw material per day. This is a significant step, showing that the technology is ready for further development and integration into practical applications.

6.4 Pilot plant initial trial

After the verification of proper operation of all parts of the pilot plant, an initial pilot trial was performed.

Orange peels from Hellenic Fruit Juices were used as feedstock.

Enzymatic hydrolysis of wet orange peels was performed under the optimum conditions (50°C, 24h, 7.5% solids loading, Pectinex 25μL/g TS, CellicCTec3 25 μL/g TS). 37.8kg wet orange peels and 44L water were added in the bioreactor in order to achieve the desirerd solids loading (7.5%). The initial pH of the mixture was around 3.3 and was adjusted to 5.6. At the end of the hydrolysis experiment, the glucose concentration was calculated in the hydrolysate. The efficiency of enzymatic hydrolysis was assessed on the basis of the sugars yield, Y_s.

After 24h of enzymatic hydrolysis, the glucose concentration of the hydrolysate was equal to 15.25g/L, that is in accordance with the lab scale experimental results (13.19±0.06g/L). This implies that upscaling of saccharification did not affect negatively the saccharifiaction process.

A solid-liquid separation process was performed to the hydrolysate via sieving. 2.2kg wet solids were recovered that were mixed in a subsequent step with the produced yeast. The recovered liquid phase of hydrolysate was aerobically fermented in the bioreactor with 0.3 g/L Saccharomyces Cerevisiae (baker's yeast) at 30°C for 24 hours.

The initial pH of the hydrolysate was 3.2 and it was corrected to 5.84 with the respective chemicals ($CaCO_3$). The nutrients medium was added (Table 1) and the aeration was conducted by continuous and vigorous stirring. After 24h of aerobic fermentation, the final glucose concentration was very low (0.4g/L) and the biomass yield reached up to 42%. The yeast produced was recovered by sieving and it was added to the wet solids of the hydrolysate. This mixture was dried succesfully in GAIA dryer to produce the final feedstuff.

Conclusively, from the initial pilot trial, all the units of the pilot plant operated properly and the bioconversion of the orange peels to animal feedstuff was achieved. Thus, the treatment train of Strategy B was verified in pilot scale.

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