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# Editorial

We begin this first issue of 2022 with the illusion of opening a space for the dissemination of information related to the environment in all its dimensions. From the most scientific field to practical cases supported by applied research in which knowledge transfer has been achieved.

This first issue presents a variety of important topics related, on the one hand, to the circular economy in terms of the revaluation of forestry and food industry waste. On the other hand, with the production of biofuels and fatty acids from algal biomass.

The first of the articles in this first issue deals with the reduction of unused residues from eucalyptus plantations, with the aim of introducing an eco-friendly natural and more energy efficient preservative in the food industry. This work presents a case study in which the validation of a preservative product based on eucalyptus essence for use in the red fruit food industry was proposed. In addition, a technological innovation in the analysis of the extraction yield of this essential oil is presented.

The following is an article on the development of new methods for the treatment and utilization of whey since, within the food industry, it is a waste whose volume is of certain relevance. In Spain alone, 1,726,000 tons of this waste are generated annually. Taking into account that whey is considered a highly polluting waste if it is directly discharged into the environment due to its high organic matter content, it is doubly important to convert it into value-added by-products. In this case, the study carried out to obtain a biostimulant liquid for the agricultural sector and a covering liquid for the canning sector from this waste is presented.

The third article remains, like the previous ones, in the context of the circular economy and presents the first phase of a project based on the use of waste from the Cantabrian food sector (cereal waste from the spirits industry and whey) to manufacture an edible, biodegradable, and compostable plastic substrate as an alternative to the current production of plastics. This first study allowed checking both the technical and economic feasibility for its future implementation and, although it is estimated that the business profit margin would not be very high, the positive environmental impacts would be enough to support its implementation.

The first of the articles that deals with the utilization of algal biomass is presented below. This fourth article presents the methodology for obtaining biofuels from the microalgae Chlorella vulgaris. This option, viable to avoid the depletion and environmental problems caused by fossil fuels, requires the capacity to cultivate this raw material. Therefore, this study included the cultivation and reproduction aspects of this microalgae identified as one of the best triglyceride-producing-microorganisms mainly used to obtain biofuels such as biodiesel and bioethanol. In this study, the suitability of this species was demonstrated, although it is necessary to further investigate the best cultivation method in order to obtain a higher yield.

Finally, the fifth article presents the production of biofuels but specifically from fatty acids. Biodiesel, for example, is a mixture of fatty acid methyl esters (FAME) that can substitute diesel and is obtained from different raw materials, such as biomass. In this work, the variation of certain biochemical and FAME properties of Chlorella vulgaris due to the effect of hydrodynamics in column photobioreactors, alternating aeration flows and continuous white light, was analyzed. In addition, the shear rate was analyzed to examine the probable presence of hydrodynamic stress.

Dr. Lázaro Cremades, Dr. María Luisa Sámano
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# RED FRUIT PRESERVATION TECHNOLOGIES BASED ON EUCALYPTUS GLOBULUS RESIDUES

**Lorena Madrigal Hoyos**
Universidad Europea del Atlántico (Spain)
lorena.madrigal@uneatlantico.es · https://orcid.org/0000-0003-1892-8758
**Sandra Sumalla Cano**
Universidad Europea del Atlántico (Spain)
sandra.sumalla@uneatlantico.es · https://orcid.org/0000-0001-9082-7877
**Iñaki Elio Pascual**
Universidad Europea del Atlántico (Spain)
inaki.elio@uneatlantico.es · https://orcid.org/0000-0001-6243-5550

**Abstract.** The environmental interest of this proposal stems from the problem of reducing unused waste from eucalyptus plantations. as well as introducing a natural and more energy efficient "eco-friendly" preservative in the food sector. In short, the project aims to convert the residue of eucalyptus leaves into a value-added raw material that can be used as a food preservative. On the one hand, the validation of a preservative product based on eucalyptus essence for its use in the red fruit food industry was proposed. In particular, for the control of the fungus Colletotrichum acutatum at the post-harvest level, in which, unfortunately, the eucalyptus extract did not show a significant effect, giving values of growth speed of the fungus higher than the use of organic acids (lactic and citric). On the other hand, a technological innovation was proposed in the analysis of the performance of the extraction of essence of Eucalyptus globulus from the forests of Cantabria by different methods. The average yield of essential oil extraction was shown to be higher by the steam distillation method.

**Keywords:** forest residues, eucalyptus, essential oils, extraction, red fruits.

# TECNOLOGÍAS DE CONSERVACIÓN DE FRUTOS ROJOS BASADAS EN RESIDUOS DE EUCALYPTUS GLOBULUS

**Resumen**. El interés ambiental de la presente propuesta parte de la problemática para reducir los residuos no aprovechados de las plantaciones de eucalipto. así como también introducir en el sector de la alimentación un conservante “eco-friendly” natural y más eficiente energéticamente. En definitiva, el proyecto pretende convertir el residuo de las hojas de eucalipto en una materia prima de valor añadido que pueda ser empleado como conservante alimentario. Por un lado, se planteó la validación de un producto conservante basado en esencia de eucalipto para su uso en la industria alimentaria de frutos rojos. En particular, para el control del hongo *Colletotrichum acutatum* a nivel de postcosecha, en el que

lamentablemente el extracto de eucalipto no mostró un efecto significativo dando valores de velocidad de crecimiento del hongo superiores al uso de ácidos orgánicos (láctico y cítrico). Por otro lado, se planteó una innovación tecnológica en el análisis del rendimiento de la extracción de esencia de *Eucalyptus globulus* de los bosques de Cantabria por diferentes métodos. El rendimiento promedio de extracción de aceite esencial demostró ser mayor por el método de destilación por arrastre de vapor.

**Palabras clave:** residuos forestales, eucalipto, aceites esenciales, extracción, frutos rojos.

## Introduction

Eucalyptus is the botanical genus with the greatest diversity of species, all of great environmental value, of which 37 are of interest to the forestry industry and 15 are used for commercial purposes. Among the diversity of species are small shrubs to the tallest trees in the world (Eucalyptus regnans over 100 meters).

Eucalyptus is currently present in more than 90 countries, mostly in tropical and subtropical areas, although there are highly productive plantations in temperate areas of New Zealand, Chile, Argentina, Brazil, Uruguay, South Africa, the Iberian Peninsula, and the United States. It extends over more than 22 million hectares worldwide (0.53% of the world's forest area), although only 13 million hectares are of industrial interest. Plantations with industrial productivity represent 59% of eucalyptus forests. In Spain, eucalyptus plantations represent 3% of the forest area, providing an opportunity for natural and sustainable economic and social development.

As far as eucalyptus is concerned, in Cantabria alone, the felling of these plantations generates a total of 4,000 tons of waste per year. The high cost involved in removing from the forest the branches, stumps, and bark left over from the felling of eucalyptus trees has made it customary to burn these residues in situ, with the consequent risk of causing a forest fire. According to ENCE data, the leaves from which the eucalyptol is extracted are not used in eucalyptus felling: 22% of the cellulose material is used to obtain pulp, while the rest (roots, branches, bark, lignin) is used to obtain energy. But about 2% of the residual material tends to be wasted.

The main objective of this research is to generate knowledge applicable to the food industry for the utilization of *Eucalyptus globulus* residues. The research will evaluate the different alternatives for capturing forest residues generated by the Cantabrian timber industry in the logging of eucalyptus. Then, the industrial process for the extraction of essential oils from the forest residue will be evaluated and optimized. Finally, the product obtained will be characterized and validated for its use as a preservative in the food industry related to red fruits.

The by-product to be obtained from the *Eucalyptus globulus* leaf residues will be a eucalyptus essence to be applied as a preservative.

## Methodology and Results

For the collection of forest residues, an exploration of the companies in the region that offer logging, pruning, and forest residue collection services was carried out, and qualitative information was also obtained regarding the possible willingness of these companies to participate in a circular economy initiative in which they would obtain remuneration for the type of residues they manage. Currently, some of these companies obtain profit from the use of these wastes as biomass for combustion, so the interest in

this alternative would be conditioned by the profitability and practicality that each procedure would offer in comparison.

During the temporary window in which the collection of this waste was carried out, it was not possible to formalize the business collaboration, so collection was carried out at privately owned sites, with prior authorization. However, it was established that the companies involved should comply with the following criteria: report continuous activity during the year, work with trees of the *Eucalyptus globulus species at* least twice a year, manage a volume of waste corresponding to forest plantations and not corresponding to residential areas or public sites.

The forest residues were obtained in Parbayón, municipality of Piélagos, with geographical location 43º 21'44"N, 3º54'09"W (Figure 1). This is an area whose main forest crop species is eucalyptus, specifically the *Eucalyptus globulus* species.




*Figure 1*. Geographical location of Eucalyptus (*Eucalyptus globulus*) residues

The forest residues were obtained "in situ" and then samples of about 5 kg were taken for each lot. A total of 3 batches were evaluated, corresponding to different days of forest residue collection, each of which was subjected to the same technical determinations in order to obtain an average result and statistical estimates of the dispersion of the data obtained.

The dates of the recruitment activity were: 07-28-2019, 08-04-2019, 08-18-2019.

Each batch had to be processed for analysis in the shortest possible time, as the essential oil found in the leaves decomposes rapidly once the leaves are separated from the tree.

The collected residues were subjected to a cleaning treatment to remove impurities that could alter the results of the study. The cleaning was carried out by immersing the eucalyptus leaves in water and making stirring movements to remove impurities (Figure 2), followed by a second washing with distilled water.




*Figure 2.* Washing of the leaves with water

The fresh leaves were then drained (Figure 3) and placed on absorbent paper to remove surface water (Figure 4), which corresponded to the initial drying of the leaves prior to the oven drying process.



Figure 3. Washing and draining of the leaves



*Figure 4.* Initial drying of leaves

Drying was carried out following the optimal conditions determined by the work published by Moreno et al. 2010, corresponding to 20 hours at a temperature of 40 ºC. The samples were cut into small pieces of approximately 1 $cm^2$ (Figure 5) and placed in a Nahita drying oven model 631 (Figure 6).



*Figure 5.* Sample preparation prior to the drying process




*Figure 6.* Oven-dried samples subjected to the drying process

The final stage of the sample preparation phase consisted of grinding the leaves (Figure 7). This is a step that is commonly performed in essential oil extraction processes and has the objective of decreasing the particle size, which increases the contact surface between the sample and the extraction water, thus facilitating the transfer of heat and mass, all with the objective of achieving a better yield of the essential oil extraction operation.



*Figure 7.* Samples before and after the milling process

*Essential oils*

The biophysical phenomenon of essential oil extraction is described as the rupture of the histological structures of the plant (excretory glands), releasing the oil; and it is dispersed in the extraction fluid to be subsequently separated or isolated, usually by decantation (El Asbahani et al., 2015).

The extraction fluid can be in liquid or vapor form, either organic solvents or water. For food, only water can be used as the extraction solvent because organic solvents are toxic, and there is always a risk of finding traces in the essential oil extract (Kumar et al., 2011).

*Hydrodistillation*

Hydrodistillation is one of the oldest and simplest methods for the extraction of essential oils from plants (Meyer-Warnod et al., 1984). In this method (*Figure 8*), the plant material is immersed in boiling water, the vapors are condensed, and the liquid obtained is subsequently decanted to separate the essential oil from the aqueous phase of the condensate. The Clevenger apparatus is the one traditionally used for this type of extraction (Rassem et al., 2016).

This traditional technique has significant disadvantages compared to other conventional techniques, such as steam distillation. Among the main disadvantages are the chemical changes (hydrolysis, cyclization, among others) in the terpene molecules due to prolonged direct contact with boiling water and the loss of polar molecules of the essential oil when trapped in the aqueous phase of the boiling water (Peredo-Luna et al., 2009).

*Steam distillation*

Water vapor distillation (*Figure 9*) is carried out by injecting superheated water vapor directly into the plant sample, causing the breakdown of the plant structure and the release and evaporation of the volatile components. The volatile components are transported together with the water vapor to a condensation stage where a liquid mixture of two phases, aqueous and organic, is obtained, which is separated by decantation to obtain the pure essential oil extract (organic phase).

This method allows the separation of the volatile compounds from the non-volatile ones (selective vaporization) since the latter are not entrained by the steam. This method

also has the advantage that the physical interaction of the superheated water vapor does not produce significant chemical changes in the chemical species of the essential oil.

This technique has the advantage of allowing the use of lower temperatures to extract thermosensitive essential oils. This requires setting up a vacuum generation equipment to perform the extraction process at reduced pressures, and thus the evaporation of volatile compounds will occur at lower temperatures (Peredo-Luna et al., 2009).

*Solvent extraction*

Solvent extraction is also known as Soxhlet extraction as it is the most commonly used method. In this method, the sample, previously dried and ground, comes into contact with organic solvents (alcohol and chloroform among the most used), which are heated to accelerate the process of breaking down the plant structures and solubilization of the essence in the solvent.

One of the most important disadvantages of this method is that all the solvents that can be used are toxic (except ethanol, but this is not used because of its low yield), and therefore cannot be used to obtain essential oils to be added to food products. Another drawback is that the solvent also solubilizes and extracts other substances (creams, fats) and an impure extract is obtained at the end. It is also costly at the industrial level, and there is a risk of explosion and fire due to the flammable nature of organic solvents.

Thus, to obtain the pure essential oil extract, the extraction product must be filtered, and then temperature and pressure conditions are applied to induce volatilization of the solvent, which must also be recovered due to health and safety restrictions and for reuse (Ortuño, 2006).

Solvent extraction by alternative methods to Soxhlet have the disadvantage of requiring longer periods of time and the greater difficulty of separating the essential oil from the organic phase of the extract.

*Evaluation of extraction performance by steam distillation method*

The eucalyptus residues, once conditioned as described above, were subjected to the steam distillation process (Figure 8) at laboratory scale. For this purpose, samples of 500 g, previously weighed on a precision balance (Nahita 5062), were used to obtain experimental data on the weight of the material before extraction, which allowed calculating the yield.

The samples were contained in a flat-bottomed flask of 1000 ml capacity, and the flask for steam generation had a capacity of 2000 ml.



*Figure 8.* Vapor entrainment extraction method performed in the CITICAN laboratory, July-August 2019.

A conventional protocol for extraction was followed under optimal conditions obtained from previous research (Moreno *et al.*, 2010), which corresponds to 120 minutes of extraction time, with leaf extraction conditions of 42 ºC and 20 hours.

Three extraction cycles were performed for each batch of residues collected (three batches, collected at different times and in the same locality) and for each method to be compared. This extraction stage was carried out in approximately three weeks, not including the sample conditioning activities and the subsequent handling and storage of the extract. The extract obtained presented a light-yellow color, was stored at refrigerated temperature (4 °C), and in the absence of light (Figure 9).

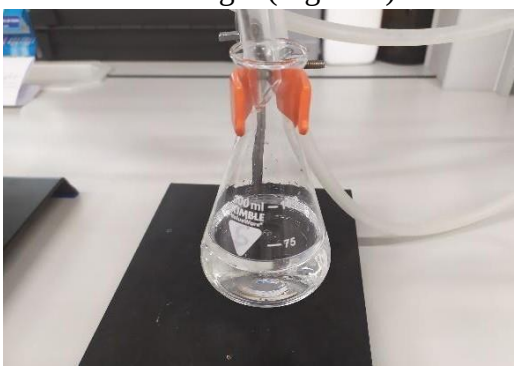

*Figure 9.* Extract obtained by the steam distillation method

Once the extract was obtained, it was separated from the aqueous fraction by decanting (Figure 10), using a separating funnel (emery 19/26, 100 ml).

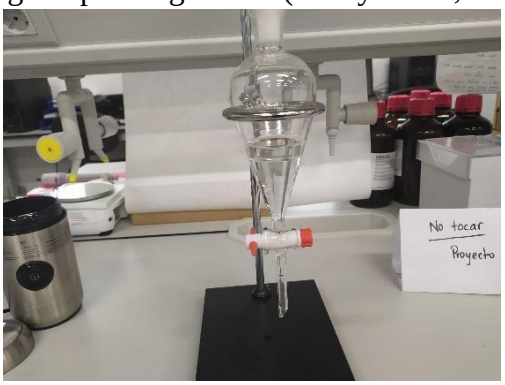


*Figure 10.* Separation of the extract from the aqueous fraction by decantation.

The essential oil extraction yield (*R*), in weight/weight ratio, was determined by the expression:

$$R\ (\%) = \frac{m_a}{m_e} . 100$$

Where $m_a$ is the mass of the extract obtained from the essential oil, and $m_e$ is the mass of the leaves after the drying process, which was weighed before the steam distillation process to obtain the oil.

The average of the values obtained for the yield of this extraction method was 0.84%, with a standard deviation of 0.08% and a coefficient of variation of 9% (Table 1).

A wide range of essential oil extraction yield values using this method is evident in the literature, ranging from 0.35% to 1.30% (Sebei et al., 2015). There are multiple

factors that determine this variability in results, among these: tree location, soil type, tree age, drying method as a pretreatment of leaves, and season of the year (Aziz et al., 2018; Brooker et al., 2006). On the other hand, a coefficient of variation below 10% is usually considered acceptable in this type of research (Rao et al., 2014).

Table 1
*Performance results of the steam stripping method*

| Lot | Yield (%) |
|---|---|
| 1 | 0,85 |
| 2 | 0,91 |
| 3 | 0,76 |
| Media | 0,84 |
| Standard deviation | 0,08 |
| CV (%) | 9,0 |

*Evaluation of extraction performance by hydrodistillation method*

Similar to the previous procedure, the eucalyptus residues were conditioned as previously described before being subjected to the hydrodistillation process (Figure 11). In this case, 500 g samples were also used.

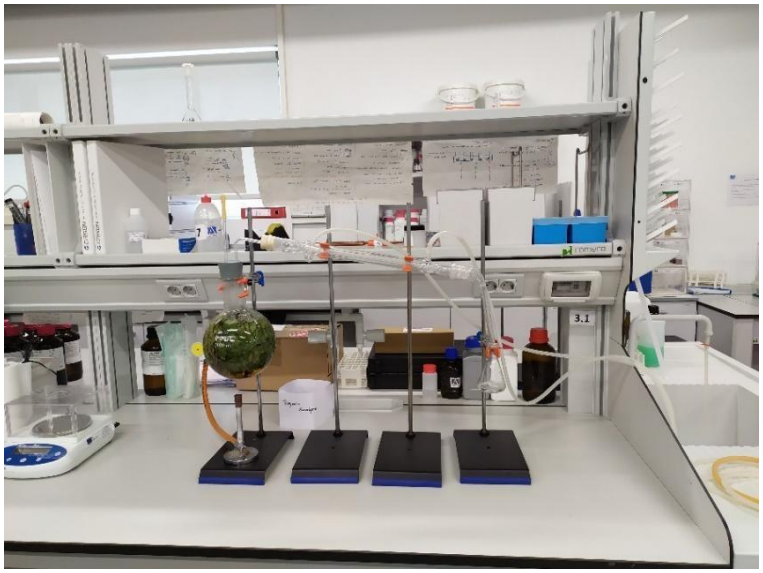

*Figure 11.* Hydrodistillation extraction method performed in the CITICAN laboratory, July-August 2019

The extraction protocol was followed using the same conditions as the comparison method, which were 120 minutes of extraction time and drying of the leaves at 42 ºC for 20 hours.

Similar to the previous method, three batches of leaves were collected in the same locality and three extraction cycles were performed for each batch. This extraction stage was carried out in approximately three weeks, not including the sample conditioning activities and the subsequent handling and storage of the extract. As in the previous method, the extract obtained was stored at refrigerated temperature (4 °C) and in the absence of light, before being subjected to physicochemical analysis. A slightly darker color was observed than the extract obtained by the previous method.

The extract was obtained by decanting separation of the aqueous fraction and the extract obtained was weighed for each extraction batch and, together with the initial mass data of the leaves, the yield was determined for each batch, according to equation 1.

The yield results by the hydrodistillation extraction method are presented in Table 2. An average of 0.78% was obtained with a standard deviation of 0.09% and a coefficient of variation of 10% (Table 2), the latter is considered acceptable in this type of research (Rao *et al.,* 2014).

Table 2
*Performance results of hydrodistillation extraction method*

| Lot | Yield (%) |
|---|---|
| 1 | 0,75 |
| 2 | 0,72 |
| 3 | 0,87 |
| Media | 0,78 |
| Standard deviation | 0,09 |
| CV (%) | 10,2 |

*Comparison of essential oil extraction methods*

As evidenced in the scientific literature, there is a great variability in the results of extraction yield (0.35% - 1.30%) that are not only due to the differences between the different extraction methods but also due to multiple factors related to the characteristics of the tree or leaves, without finding any correlation between at least one of these variables and the yield (Sebei et al., 2015; Brooker et al., 2006). To minimize the effect of these factors or variables, which can influence performance, the same temperature and time conditions were applied in each method for the leaf drying pretreatment, according to the optimal conditions determined by previous studies (Moreno et al., 2010). Likewise, the sample lots were obtained from the same location.

The characteristic of yield variability according to the location of the eucalyptus forest crop makes this study an original research contribution since, in the literature review, no other research work was found that studied the essential oil extraction yield for eucalyptus forest crops in this region of Spain. Thus, experimental data have been generated that may be useful in the future.

In the literature review, multiple references were found on the steam entrainment distillation extraction method, which is pointed out as the most viable procedure from a commercial exploitation point of view and with greater facilities to be scalable at an industrial level (Hesham et al., 2016; El Asbahani et al., 2015; Meyer-Warnod, 2004).

Although the average percentage yield was higher for the steam distillation extraction method, 0.85% ± 0.09%, with respect to the hydrodistillation method, 0.78% ± 0.10%; there are no statistically significant differences between the two values. On the other hand, hydrodistillation extraction is pointed out in multiple studies as a method that presents disadvantages such as chemical changes in the terpene molecules due to the prolonged direct contact with boiling water as well as the loss of polar molecules of the essential oil by being trapped in the aqueous phase of boiling water (Aziz et al.; 2018, Kumar et al.; 2011).

For the above reasons, it is considered that the steam distillation extraction method is the most appropriate for the purposes of this project. Therefore, it is recommended that

this method be chosen for the next phase of research, which consists of the physicochemical characterization of the extract.

*Extraction process proposal*

According to the evaluation of the different methods of extraction of the essential oil, the selected method was steam distillation. This proposal has been substantiated with the experimental results and with the knowledge generated in previous studies obtained from the scientific literature (Hesham et al., 2016; Meyer-Warnod, 2004).

On the other hand, the traditional hydrodistillation extraction method may present the disadvantage of producing chemical changes of the essential oil (Aziz et al., 2018); the organic solvent extraction method carries the risk of finding traces of the solvent in the essential oil extract, which is toxic if found in food (Kumar et al., 2014); while alternative methods represent disadvantages for laboratory scale-up to the industrial level, because they involve greater complexity in design and require higher installation and maintenance costs (El Asbahani et al., 2015; Stateva et al., 2011).

*Physicochemical characterization of the essential oil*

For the characterization of the essential oil obtained by the steam distillation extraction method, the procedures indicated in the Spanish technical standard UNE 84300 for the essential oil of *Eucalyptus globulus* from Spain (Spanish Association for Standardization and Certification [AENOR], 2006) were followed.

For the determination of the relative density of the essential oil at 20 °C, a clean and dry pycnometer (Figure 12) of 3 ml capacity was used, weighed empty on an analytical balance (Nahita 5062), then filled with the essential oil (which was in the bath at 20 °C), the excess sample was covered and cleaned, and the sample was weighed. This procedure was repeated for samples from the three batches of the study. The same procedure was performed with distilled water at 20 °C. The relative density of the essential oil was determined using the following equation.

$$\rho_{2\$^{\circ}C} = \frac{(m_{a.e} - m_p)}{(m_a - m_p)}$$

Where ρ is the relative density at 20 °C, $m_{a.e}$ is the weight in grams of the pycnometer with the essential oil sample, $m_p$ is the weight in grams of the pycnometer without the sample, and $m_a$ is the weight in grams of the pycnometer with distilled water.

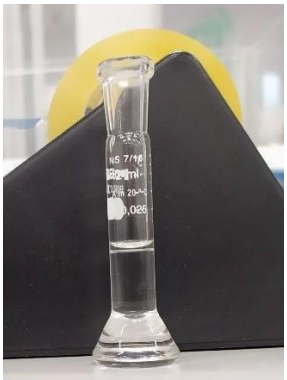

*Figure 12.* 3mL pycnometer for relative density determination.

This procedure was carried out following the guidelines of the corresponding Spanish standard for essential oils (AENOR, 2000), and values were obtained for each batch of essential oil extracted by steam distillation. The average value of relative density was 0.91 ±0.01, which is a value within the range indicated as acceptable by the Spanish standard, being between 0.905 and 0.925.

Table 3
*Relative density obtained for the essential oil samples*

| Lot | Relative density |
|---|---|
| 1 | 0,89 |
| 2 | 0,92 |
| 3 | 0,91 |
| Media | 0,91 |
| Standard deviation | 0,01 |
| CV (%) | 1,4 |

The refractive index is a property defined as the ratio between the velocities of light in two different media, calculated as the quotient of the sines of the angles of incidence (in the first medium) and the angle of refraction (in the second medium). The refractive index is a characteristic property of each essential oil and changes when it is mixed or diluted with other substances, so this is one of the parameters used to evaluate the purity of the essential oil (Ortuño, 2006).

A digital refractometer (Hanna instruments, model HI96801) was used, whose measurements are given in Brix units, and a conversion table corresponding to 20 °C was used.

Table 2 presents the refractive index values obtained for the samples of the different lots. On average, a refractive index value of 1.4601 was obtained, which is within the range of acceptable values, between 1.457 and 1.475, according to the Spanish standard UNE 84300 (AENOR, 2006).

Table 4
*Refractive index of essential oil samples*

| Lot | Refractive Index |
|---|---|
| 1 | 1.4601 |
| 2 | 1.4583 |
| 3 | 1.4597 |
| Media | 1.4594 |
| Standard deviation | 0.0009 |
| CV (%) | 0.0648 |

For the calculation of the ethanol index, the respective procedure according to the Spanish standard (AENOR, 2000) is also followed. In this procedure, ethanol-water mixtures of different volumetric concentrations (70 and 96%) are added to a known volume of essential oil (1ml). When a certain volume of the higher concentration alcohol mixture is added, a momentary turbidity is observed, which disappears by shaking. In a progressive way, the alcoholic mixture continues to be added, while the quantity added is measured with a burette.

The results obtained for the ethanol solubility of the essential oil samples showed complete solubility for the mixture with the highest alcohol concentration, 96%, while for the 70% mixture, a value of 7 parts of ethanol mixture was obtained to obtain a clear solution for each part (volume) of the essential oil.

*Comparison with UNE 84300:2006 standard*

The average value of relative density was 0.91 ±0.01 g/ml, while the standard indicates that values within the range of 0.905 and 0.925 are acceptable. The refractive index value obtained was 1.4594, which is within the range of acceptable values indicated by the standard, between 1.457 and 1.475. The result of solubility in ethanol, 7 volumes or soluble parts of ethanol at 70%, is also within the values indicated in the UNE 84300 standard (AENOR, 2006).

*Preservative design*

The objective of this phase of the research was to inhibit the growth of *Colletotrichum acutatum*. This fungus is responsible for a disease known as anthracnose and has a high incidence in the post-harvest life of all red fruits, causing great losses of commercial product and, therefore, economic losses for the companies.

This purpose is based on the low availability of authorized phytosanitary products available to producers in post-harvest stages, since this is the stage in which the product is acquired by the consumer for consumption.

To perform the test, the methodology proposed by EUCAST (European Committee on Antimicrobial Susceptibility Testing) was used, applied to a commercial eucalyptus extract and the one obtained in this project.

The principle of this methodology is to impregnate discs of laboratory quality paper in a solution of known concentration of the substance to be tested. This paper is placed on an agarified medium that stimulates the growth of the microorganism. This ensures that the agent that inhibits growth is the one that is inoculated on the disc.

If this substance has an antimicrobial property, it will inhibit the growth of the microorganism near it. On the other hand, if it does not have such a property, the microorganism will grow in that area. Since it is a paper disk, the substance diffuses homogeneously, creating a circumference called an inhibition halo, whose diameter is an estimate of the inhibitory power: the larger the diameter, the greater the inhibitory power (Figure 13).




*Figure 13.* Diagram of inhibition halo

To carry out the tests, the anthracnose strain was acquired from the Spanish Type Culture Collection (CECT) but specifically its asexual or imperfect form, *Glomerella acutata*, which is the one certified by CECT, and which it also has the advantage of being easily reproducible under laboratory conditions.

After receiving the fungus, and having acquired the commercial eucalyptus extract, upon reading the label, it was observed that the product was based on an extract concentrate and also had among its ingredients some substance that could also present an inhibitory effect, so it was decided to compare the extract obtained in this project with other frequent agents in the control of pathogens in postharvest: lactic acid and citric acid (Feliziani et al., 2016; Romanazzi et al., 2009).

Once the assay was adjusted, the fungus was replicated in PDA (Potato-Dextrose-Agar) medium, and the paper discs were impregnated with 0.1-5% concentrations of eucalyptus extract, lactic acid, and citric acid. Figure 14 shows the paper discs, the replicated fungus slice, and the mycelium.

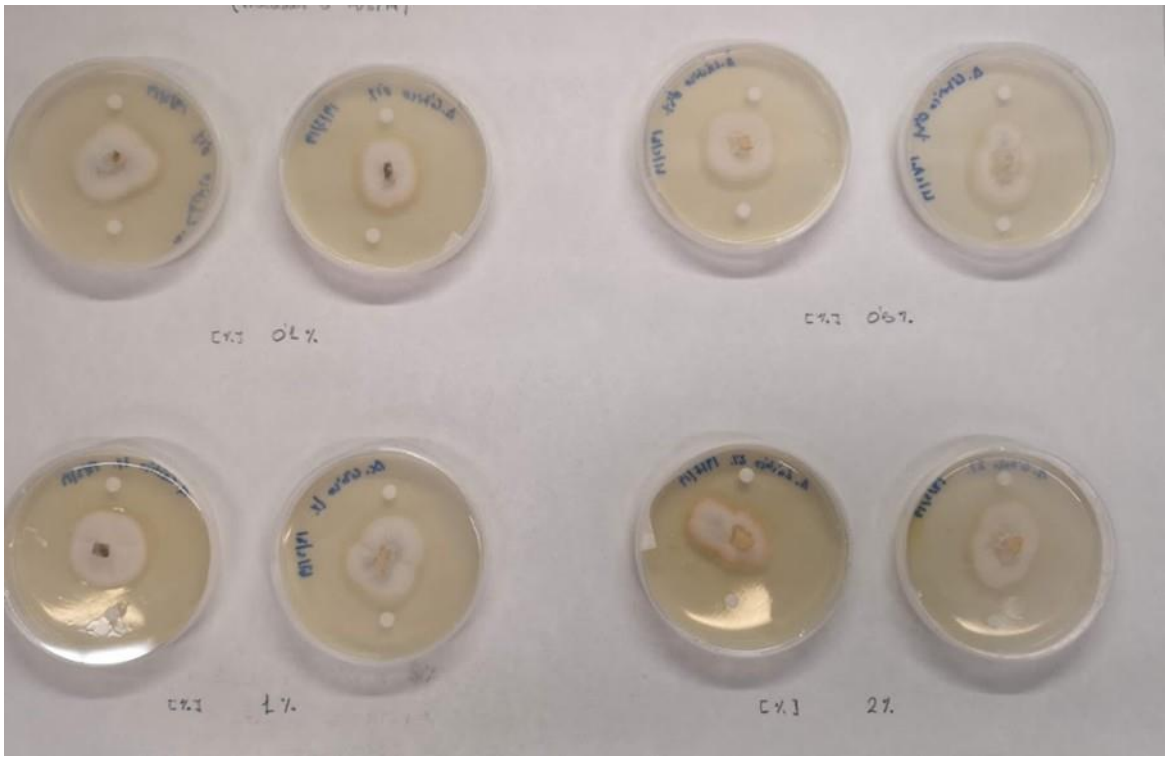

*Figure 14.* Piece of replicated fungus and mycelium.

The growth was monitored daily. As can be seen in the previous figure, the growth shape of the mycelium varied between circular and elliptical. In the first case, the diameter was measured and in the second case the largest diameter. It was observed that in a period of 12-15 days the Petri dish was completely covered by the mycelium of the fungus. It was also observed that the discs did not inhibit the development of the fungus, the mycelium growing on top of the discs.

This result forced to rethink the assay looking for an alternative methodology. It was decided to dilute the eucalyptus extract, citric acid, and lactic acid directly in the PDA medium at the estimated concentrations (0.1-5%) and replicate directly on the fungus. In this case the results were positive, clearly observing how the growth rate of thefungus was modified (Figure 15). The assay was maintained until the mycelium occupiedthe entire plate.

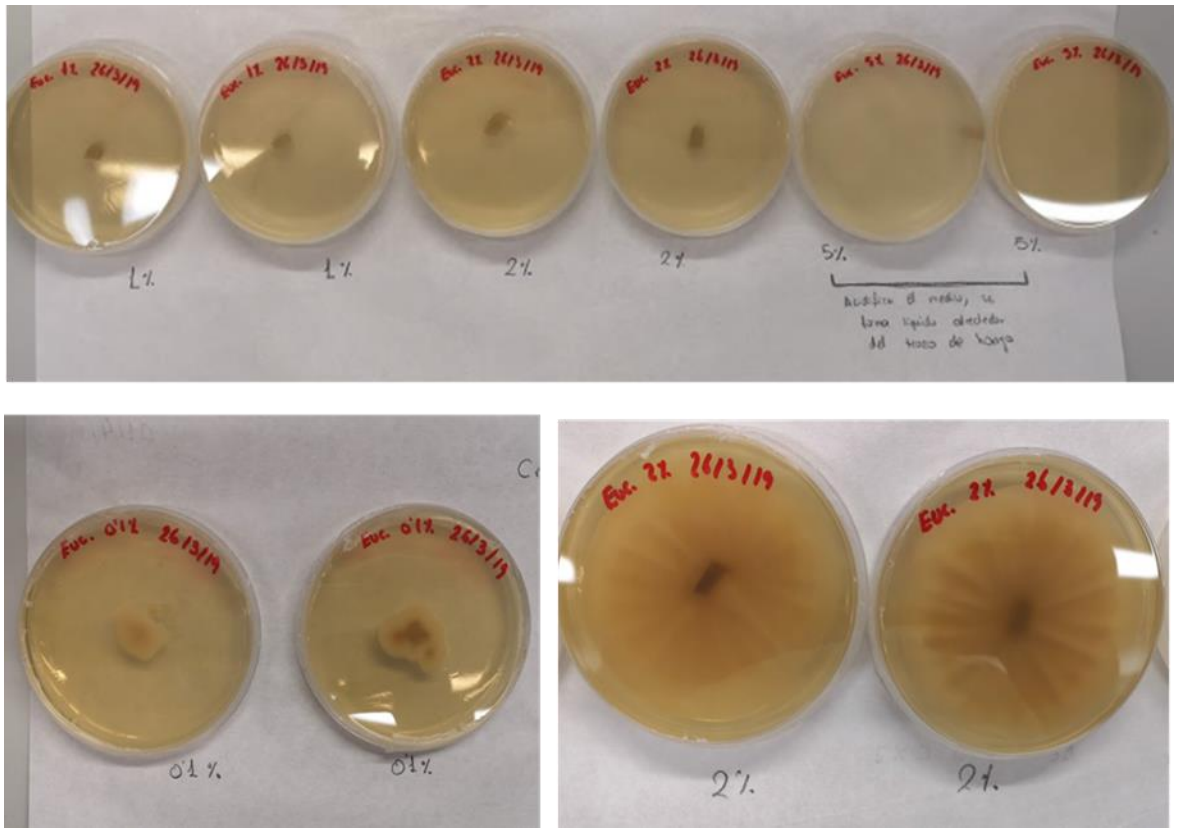


*Figure 15.* Growth rate of the fungus

The concentrations at which the three products were applied were: 0.1%, 0.5%, 1%, 2%, and 5%. In the case of citric and lactic acids, from a concentration of 2%, the fungus was inhibited, and specifically, from 1% the growth rate of lactic acid was much lower than that of citric acid. In the case of eucalyptus extract, the fungus continued to show intense growth activity at 2% and above.

The following figures show the graphs representing the growth rate of *G. acutata*. Each line is defined by an ascending section whose slope is the growth rate, expressed in mm/day, and a plateau or horizontal zone that corresponds to the time when the Petri dish was fully occupied.

In the case of the 0.1% concentration, the fungus grew more slowly with the eucalyptus extract than with the two acids. With the extract, it grew at an average rate of 4.7 mm/day, while for citric and lactic acids it was 6.7 mm/day and 7.1 mm/day, respectively. Despite having a lower rate, from the ninth day onwards, it filled the entire plate. This may be due to the fact that on the fifth and eighth day the fungus had a higher development activity that accelerated the process. However, no documentary references have been found to explain this phenomenon.

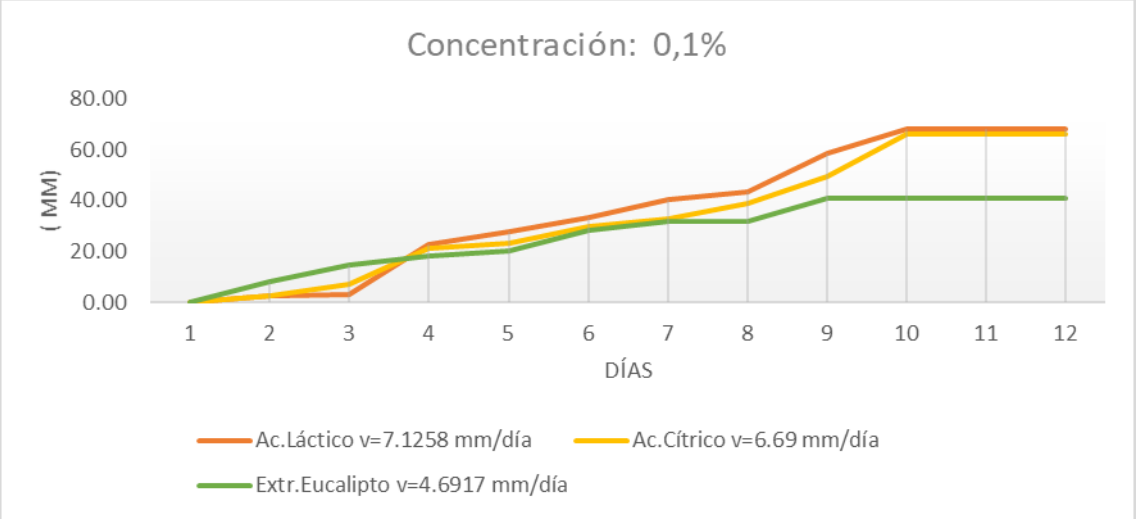


*Figure 16.* Growth rate of *G. acutata* for a concentration of 0.1% of the three control agents tested.

## Discussion and conclusions

Although the average essential oil extraction yield was higher for the steam distillation method compared to the hydrodistillation method, there are no statistically significant differences between the two values. However, hydrodistillation extraction is pointed out in multiple studies as a method that presents disadvantages such as chemical changes in the terpene molecules due to the prolonged direct contact with boiling water, as well as the loss of polar molecules of the essential oil by being trapped in the aqueous phase of boiling water (Aziz et al., 2018; Kumar et al., 2011). Therefore, the steam distillation extraction method is considered to be the most suitable for the purposes of the present project.

Unfortunately, the eucalyptus extract showed no effect for the control of the fungus *Colletotrichum acutatum* at the postharvest level, giving higher values of fungal growth rate than the use of organic acids (lactic and citric).

Comparing the effect on color and composition in compounds with antioxidant activity and pH of the eucalyptus extract, significant differences were only observed in the application of eucalyptus extract in the case of pH and phenolic compounds, being in this case higher than the values found for the treatments with the two acids.

Sensorially, the extract-treated blueberries had the worst rating.

These results, a priori negative, invite further investigation on future occasions focused on:

- Evaluating the effect on different stages of development of *Colletotrichum acutatum.*
- Evaluating the control effect of the extract on bacteria affecting the crop such as the Rust (*Pucciniastrum vaccinii*).
- Evaluating the control effect of the extract on other fungi of interest for blueberry: *Aspergillus, Fusarium, Penicillium,* etc.
-

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# DEVELOPMENT OF TECHNOLOGIES FOR THE SUSTAINABLE REUSE OF WHEY

**Jesus Emilio Rosas**
Universidad Europea del Atlántico (Spain)
jesus.rosas@alumnos.uneatlantico.es · https://orcid.org/0000-0002-8810-4925
**María José Acebo Garfias**
Universidad Europea del Atlántico (Spain)
maria.acebo@alumnos.uneatlantico.es · https://orcid.org/0000-0002-5760-2905

**Abstract**. In Spain, 1,726,000 tons of whey are generated annually. In particular, in Cantabria there is talk of a whey generation of 15,600 tons per year. Whey is considered highly polluting waste if it is dumped directly into the environment given its high content of organic matter. This project sought to develop new methods for the treatment and use of this waste. Separation of the solid fraction from fermented whey can be achieved sustainably and effectively with bentonite. On the other hand, in the composition of the acidified, clarified and sterilized liquid fraction (LCE), no presence of compounds of economic interest for the dairy industry was observed. On the other hand, opportunities were detected to convert it into value-added by-products for the agricultural and canning sector. In the case of agriculture, work was done to obtain a new biostimulant capable of providing minerals, proteins, pH regulation, etc. On the other hand, it is also considered that the food industry market for canned vegetables may be a target market that integrates this by-product as a covering liquid (preservative) and can replace vinegar.

**Keywords:** whey, vegetable preserves, biostimulant, lactic acid.

# DESARROLLO DE TECNOLOGÍAS PARA LA REUTILIZACIÓN SOSTENIBLE DEL LACTOSUERO

**Resumen**. En España se generan 1.726.000 toneladas anuales de lactosuero. En particular, en Cantabria se habla de una generación de lactosuero de 15.600 toneladas al año. El lactosuero se considera un residuo altamente contaminante si se vierte directamente al medioambiente dado su contenido elevado en materia orgánica. Con este proyecto se buscaba desarrollar nuevos métodos para el tratamiento y aprovechamiento de este residuo. La separación de la fracción sólida del lactosuero fermentado se puede conseguir de forma sostenible y efectiva con bentonita. Por su parte, en la composición de la fracción líquida acidificada, clarificada y esterilizada (LCE), no se observaron presencia de compuestos de interés económico para la industria láctea. En cambio, sí se detectaron oportunidades para convertirlo en subproductos de valor añadido para el sector agrícola y conservero. En el caso de la agricultura, se trabajó en la obtención de un nuevo bioestimulante capaz de aportar minerales, proteínas, regulación del pH, etc. Por otro lado, también se considera que el mercado de la industria alimentaria de conservas vegetales puede suponer un mercado

objetivo que integre este subproducto como líquido de cobertura (conservador) pudiendo sustituir al vinagre.

**Palabras clave:** lactosuero, conservas vegetales, bioestimulante, ácido láctico

## Introduction

The environmental challenge assumed by this project is framed in the management of Industrial Waste within the Waste Plan of Cantabria 2017-2023. The project is framed in the reduction of waste from the cheese industry.

Cheese production in Spain is around 500,000 tons per year (Sainz, 2002 and Martín P. 2021), while in the world it reaches the figure of 17 million tons (Martínez et al. 2020). During the process of coagulating milk to obtain cheese, large volumes of cheese whey are generated, which is the main waste given that for every 100 liters of milk that enter a cheese factory, 80 liters of whey may be produced or, in other words, between 9 and 12 liters of whey are generated for every kilo of cheese produced. This means that in the world a production of 190 million tons of whey is generated annually and in Spain 1,726,000 tons of whey are generated annually and up to 190 million tons in the world (Vázquez et al. 2019).

The aim is to develop new methods for the treatment and use of whey to convert it into two value-added by-products for the agricultural and canning sectors. In this case, the commercial opportunity is perceived to transfer the technologies arising from this project to these sectors and the cheese factories.

In the case of agriculture, the aim is to provide the market with a new biostimulant that also uses the water present in whey. This biostimulant market is still in its infancy (3.5% of arable land) but is growing at an annual rate of 12%, favored by the tendency to increase agricultural land.

On the other hand, the market of the canned vegetable food industry is also considered, which is a mature market, with around 1.5 million tons produced annually and a turnover of 7,000 million euros per year.

Finally, it is believed that the cheese production sector can also take advantage of these technologies given that certain cheese dairies can choose to adapt their production plants to generate added value by-products, with commercial interest for third parties. In this case, it is aimed at the cheese production sector, which has a very relevant specific weight in Spain and also in Cantabria, for it has 43 cheese dairies that are not taking economic advantage of the whey that is generated.

The aim of this research is to take advantage of the liquid fraction of the residual whey from the cheese industry either as a biostimulant liquid or as a covering liquid for vegetable preserves.

Among the characteristics of the biostimulant liquid are being a source of minerals and proteins as well as having an acid pH that allows the bioavailability of other minerals. In addition, it allows the reduction of irrigation water expenditure and, therefore, of the water footprint.

In the case of the covering liquid (preservative), the proposal is to use the new by-product obtained from the whey for use in vegetable preserves, which could replace vinegar. This liquid should respond positively to heat resistance tests, be neutral in flavors and aromas and cost 30% less.

In order to achieve the research objectives, it was proposed to evaluate the fermentation conditions of residual whey from the cheese industry to increase its lactic acid content, to design an industrial processing line to separate the solid and liquid fractions of whey, to process and characterize the liquid fraction of whey enriched in lactic acid for its use in the canning industry, and to process and characterize the liquid fraction of whey enriched in lactic acid for its use as a biostimulant for agricultural crops.

## Method

The objective of this research is to chemically and microbiologically characterize the whey to identify if there is any parameter that limits the subsequent development of fermentations.

The whey was supplied by a local cheese company that uses pasteurized cow's milk for the production of fresh cheese (sweet whey). Therefore, it was not possible to perform microbiological characterization from a sterilized product.

For the different trials the company provided 20 liters at two different times. The values shown in the rest of the article correspond to the overall average value.

For the chemical characterization, in addition to some parameters indicated in RD 140/2003 on water for food use (including those of the agri-food industry), other milk quality parameters were measured that could affect subsequent fermentation, such as hydrogen peroxide, the presence of which could be due to a health problem in the cow. The physicochemical parameters considered were:

- Cations and anions: ammonium, nitrite, nitrate, and phosphates.
- Minerals: nitrogen, calcium, potassium, iron, and phosphorus.
- Others: pH, Lactic Acid, and BOD (Biological Oxygen Demand)

The physicochemical methods applied were spectrophotometric, potentiometric, and volumetric. The following table shows in detail the distribution of the methods by parameters.

Table 1
*Characteristics of the methods of analysis (I)*

| Parameter | Method of analysis | Observation |
|---|---|---|
| Ammonium | Spectrophotometric ($\lambda$= 690 nm) | Hypochlorite and phenol |
| Nitrite | Spectrophotometric ($\lambda$= 543 nm) | Sulphanilic acid and N-(1-naphthyl)-ethylenediamine dichlorohydrate |
| Nitrate | Spectrophotometric ($\lambda$= 220 nm) | 2,6-dimethylphenol |
| Phosphate | Spectrophotometric ($\lambda$= 470 nm) | Vanadate-Molybdate |
| Chloride | Spectrophotometric ($\lambda$= 530 nm) | Mercury (II) thiocyanate |
| Nitrogen | Volumetric | Kjeldahl Method |
| Calcium | Spectrophotometric ($\lambda$= 570 nm) | Glyoxal-bis(2-hydroxyanil) |
| Potassium | Spectrophotometric ($\lambda$= 500 n) | Tetraphenyl borate |
| Iron | Spectrophotometric ($\lambda$= 510 nm) | Triazine and thioglycolate |
| Phosphorus | Spectrophotometric ($\lambda$= 400 nm) | Vanadate-Molybdate |
| Hydrogen peroxide | Spectrophotometric ($\lambda$= 528 nm) | Phenanthroline |
| pH | Potentiometry | - |
| Acidity | Volumetry | Valuation up to color change |
| BOD | Spectrophotometric ($\lambda$= 620 nm) | Ramirez's approach (1992) |

Once the methods of analysis had been identified, the spectrophotometric methods were calibrated by determining:

- The linearity range, which will give information on the minimum and maximum concentration of the analyte that the determination method can be applied to (see Figure 1).
- The equation relating absorbance to concentration and its goodness of fit. Goodness of fits close to 1 were sought throughout because this implies that the relationship between concentration and chemical signal is of "high quality."
- The limit of detection and quantification. These were measured from a blank (distilled water) to which the same procedure was applied as if it were a whey sample. With these values, a mean value and deviation were determined, which were used to determine both limits by means of the following expressions (see Figure 1):
  - Detection limit: mean ± 3· deviation
  - Limit of quantification: mean ± 10· deviation

Different solutions were made at the same concentration of each analyte, and their real value was compared with the theoretical value. The ideal is the smallest possible error values. It was decided to set the maximum threshold value for the error to be less than 5%.

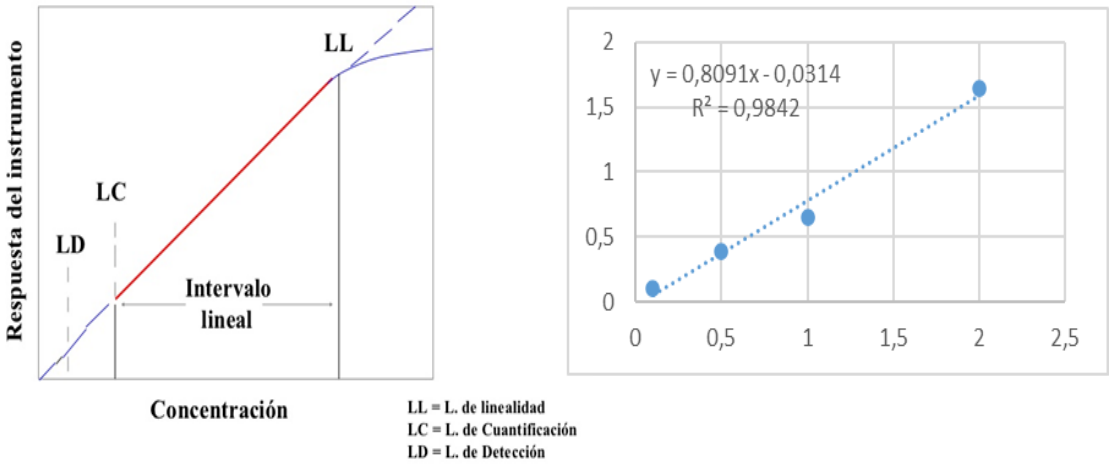


*Figure 1*. (A) scheme of calibration parameters, and (B) Nitrite curve obtained in the project.

## Results

The following table shows the calibration values of the different parameters measured spectrophotometrically:

Table 2
*Characteristics of the methods of analysis (II).*

| Parameter | Equation | Setting | Limits (mg/L) | | Error (%) |
|---|---|---|---|---|---|
| Ammonium | $y = 0.0362x + 1099$ | 0,9996 | 1,01 | 3,50 | 2,24 |
| Nitrite | $y = 0,8091x - 0314$ | 0,9842 | 0,11 | 0,15 | 3,42 |
| Nitrate | $y = 0,0014x - 0111$ | 0,9836 | 154,6 | 398,8 | 0,87 |
| Phosphate | $y = 0.0217x + 0081$ | 0,9981 | 0,17 | 0,89 | 1,38 |
| Chloride | $y = 0,0106x + 2429$ | 0,9951 | 0,06 | 0,11 | 3,14 |
| Calcium | $y = 0.0075x$ | 0,9893 | 3,42 | 7,22 | 1,55 |
| Potassium | $y = 0.0004x + 0095$ | 0,9827 | 26,8 | 55,5 | 7,50 |
| Iron | $y = 0.2701x + 0.036$ | 0,9987 | 0,05 | 0,12 | 1,01 |
| Phosphorus | $y = 0,4474x + 0383$ | 0,9999 | 0,01 | 0,04 | 0,22 |
| Hydrogen peroxide | $y = 0,1717x + 7587$ | 0,9739 | 0,01 | 0,02 | 1,69 |

It can be seen how the goodness of fits are close to 1 and with errors lower than 5%, except for potassium, which had an error of 7.5%. Several attempts were made to adjust the method, but it was not possible to reduce the error, so it was decided not to include this parameter in the successive analyses.

Once the characterization of the starting whey was carried out, the pH of the whey was reduced by means of a fermentation process that increases the lactic acid content. The fermentations were carried out with two bacteria widely used in the dairy industry:

- *L. acidophyllus*
- *E. faecium*

The following tables show the results of the fermentation test in terms of pH and lactic acid evolution. Day 0 corresponds to the day of inoculation, and thus to the initial pH and lactic acid value of the whey. These original pH values are within the range that most of the literature reviews consulted show for sweet whey (Villota et al 2015; Instituto Nacional de Tecnología Industrial - INTI, 2017).

The following tables show the data for both bacteria (with whey supplementation, expressed with the + symbol and without supplementation, expressed with the - symbol). For both bacteria, the pH was reduced more in the unsupplemented medium and between day 2 of fermentation and day 3, the decrease in pH was not as high as between day 1 and day 2. In addition, comparing between bacteria, the lowest pH was obtained for the case of *L. acidophilus*, so it was concluded that the characteristics of the fermentation for the objective sought in this project were inoculation with 2% of *L. acidophilus* directly on the whey and 48-72 hours of fermentation.

Table 3
*pH evolution during fermentation (+: supplemented medium, -: unsupplemented medium).*

| **Fermentation** | **Day 0** | **Day 1** | **Day 2** | **Day 3** |
|---|---|---|---|---|
| *E. faecium* + | 4,93 | 4,46 | 4,33 | 4,3 |
| *E. faecium* - | 5,4 | 4,55 | 4,28 | 4,22 |
| *L. acidophilus* + | 6,21 | 4,2 | 4,09 | 4,03 |
| *L. acidophilus* - | 6,53 | 4,13 | 3,98 | 3,87 |

If the results obtained for acidity are analyzed, it is observed that there are almost zero values from day 0 (because it is a sweet whey), and that its content increases with the days of fermentation until it reaches 12.15 g/L in the case of unsupplemented *L. acidophilus.*

Once the fermentation conditions were identified, a comparison of the chemical composition of the original whey and the same whey fermented with both bacteria for 48 hours was carried out (see Table 4).

Table 4
*Chemical composition of the original whey and after fermentation (mg/L).*

| Parameter | Original whey | *E. faecium* | *L. acidophilus* |
|---|---|---|---|
| Iron | 9,25 ± 1,12 | 1,71 ± 0,37 | 2,55 ± 1,08 |
| Calcium | 85,36 ± 15,11 | 54,18 ± 13,59 | 63,06 ± 22,80 |
| Phosphorus | 14,86 ± 1,63 | 47,73 ± 2,25 | 47,99 ± 5,36 |
| Ammonium | 173,34 ± 19,29 | 439,22 ± 24,41 | 411,94 ± 51,27 |
| Nitrite | 3,08 ± 0,12 | 0,68 ± 0,11 | 1,04 ± 0,35 |
| Phosphate | 235,36 ± 16,05 | 151,03 ± 13,28 | 153,51 ± 16,93 |
| Chloride | 341,34 ± 37,20 | 242,33 ± 9,69 | 255,61 ± 12,09 |
| Nitrogen (%) | 0,14±0,00 | 0,17±0,01 | 0,17±0,01 |
| Hydrogen peroxide | 3,07 ± 1,99 | - | - |

The acidified whey obtained is a mixture of solids (including the remains of microorganisms) and water. The purpose was to separate both fractions so that the liquid part (of interest in this project) could be applied for the generation of a biostimulant liquid or a preservative liquid. For this, we proceeded to the development of two separation tests:

- Test 1: physical filtration by means of membrane and inert earth (such as celite, zeolite, or others of interest).
- Test 2: phase separation using fining agents frequently used in the food industry (bentonite, albumin, etc.).

Before carrying out the tests with a high volume of fermented whey, for the case of test 1, a small filtration rate test was carried out through laboratory filter paper of different thicknesses (similar to a membrane). This paper clogged very quickly, making it almost impossible to improve the process even by applying vacuum filtration. For this reason, the filter paper was replaced by a bed of celite, but it was observed that although initially the process occurred at an adequate rate; the celite bed was compacted preventing the passage of more whey. On the other hand, when trying to separate the fraction of solids from the whey that remained on the top of the celite bed, part of this soil was dragged so it was not possible to obtain a product of a certain purity.

Due to these difficulties, all efforts were focused on the use of clarifying agents such as bentonite and albumin. As a first step, dose-response tests were carried out using as a starting product the whey acidified by L. *acidophiluse* previously pasteurized to stop the fermentation process after 48 hours, destroying all the microbiological load that could cause any problem in the subsequent development of the task.

The dose-response assay was performed as follows. The same amount of this acidified and sterilized whey was placed in a collection of falcon tubes and increasing concentrations of albumin and bentonite were added in triplicate to each tube.

To evaluate the efficiency of one product with respect to the other, the loss of color was measured (see Figure 2) and the evolution of the yellow color at 420 nm, similarto how this color is measured in wine. The process lasted 24 hours and, as can be seen inFigure 2, there was no loss of color with albumin, quite the opposite to what was achievedwith 5% bentonite.

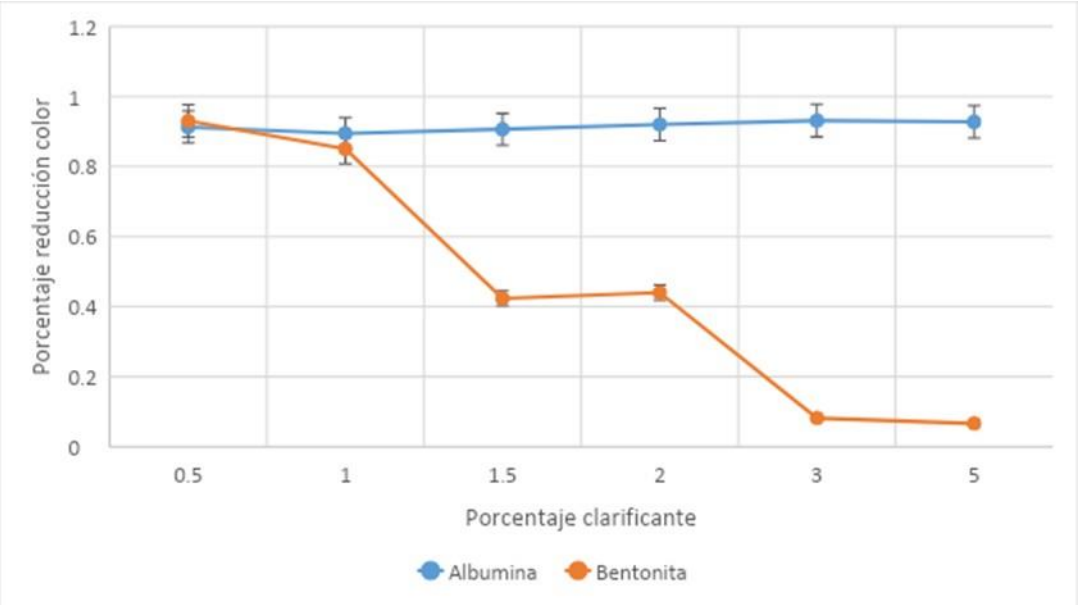


*Figure 2*. Loss of color after clarification.

The amount of precipitated product was also measured as a measure of the efficiency of the degree of separation (see Figure 3). It can be seen that in the case of bentonite, in addition to a greater decrease in color, there is also a higher percentage of solids with respect to albumin.

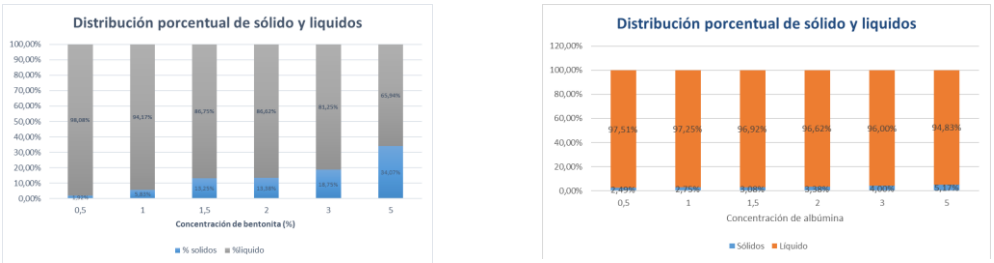


*Figure 3*. Estimation of the percentage of solids and liquids after clarification.






*Figure 4.* Detail of phase separation before clarification (upper image) and after clarification (lower image).

Once the clarified product was obtained, deodorization was carried out in order to eliminate the traces of lactic odor. For this purpose, the fermented whey was passed through a column of activated carbon, although it was observed that part of the activated carbon was dragged and darkened. Given this result, it is proposed that, for future occasions, the best option is to use skimmed whey. This whey skimming process is already common in some dairies to obtain the fat fraction needed to make whey butter. If this equipment is not available, another possible option would be to carry out sequential clarifications to eliminate as much protein as possible from the whey and the fat fraction would go with it.

Subsequently, an analysis was made of the results obtained previously and the uses to which the final product is going to be put: liquid for preserves and biostimulants. The proposal made is shown in the following figure, which includes some results of later tasks:

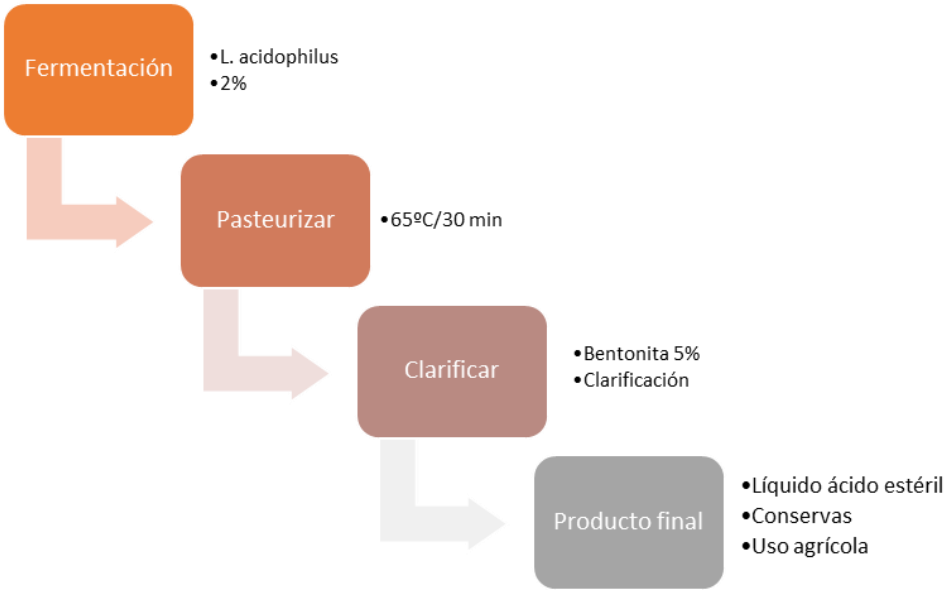


*Figure 5.* Scheme of whey treatment to obtain an acidified liquid fraction.

- STEP 1: Start with pasteurized or sterilized skimmed whey. In this way the fat fraction is eliminated from the beginning and the possible presence of microorganisms that may affect the subsequent fermentation.
- STEP 2: Ferment the whey with *L. acidophilus*, inoculating at a concentration of 2% for 48 hours. Fermentation can be stopped before that time depending on the final pH or lactic acid content.
- STEP 3: Stop fermentation by VAT or LT-LT pasteurization (65ºC for 30 minutes).
- STEP 4: Clarification with bentonite. Although in this project the effective bentonite concentration was 5% for 24 hours, the fact is that the clarification time and the percentage of the product to be used is carried out by means of a dose-response test because it depends on the degree of turbidity of the starting whey.

From this point on, the acidified, clarified, and sterilized whey (hereinafter called LCE) can be put to different uses:

As canning liquid: a double effect of the pH of the medium was observed. On the one hand, a negative effect by increasing the rate of decomposition of borage (product used for the test), but on the other hand, a lower oxidation of the same was observed, which is positive and suggests a different use than the one given in this project.

On the other hand, as a biostimulant: it was observed that it is necessary to raise the pH to avoid stress responses in plants. This can be done by stopping fermentation at pH values close to 5 and increasing the pH with some carbonate or hydroxide. In this case, it can never contain sodium because it would have a detrimental effect on the soil structure. In this project, the pH was increased with KOH but it could be of interest in the future to try other pH regulating agents.

The following table shows the chemical composition of LCE prior to its use as a preserving liquid and biostimulant.

Table 6
*Chemical composition of the original whey after fermentation and after clarification (LCE) (mg/L).*

| **PARAMETER** | **Original whey** | ***L. acidophilus*** | **LCE** |
|---|---|---|---|
| Iron | 9,25 ± 1,12 | 2,55 ± 1,08 | 0,40± 0,03 |
| Calcium | 85,36 ± 15,11 | 63,06 ± 22,80 | 45,27 ± 3,21 |
| Phosphorus | 14,86 ± 1,63 | 47,99 ± 5,36 | 22,75± 4,08 |
| Ammonium | 173,34 ± 19,29 | 411,94 ± 51,27 | 122,47 ± 52,40 |
| Nitrite | 3,08 ± 0,12 | 1,04 ± 0,35 | 0,05±0,03 |
| Phosphate | 235,36 ± 16,05 | 153,51 ± 16,93 | 91,89 ± 0 |
| Chloride | 341,34 ± 37,20 | 255,61 ± 12,09 | 183,93± 1,40 |
| Nitrogen (%) | 0,14±0,00 | 0,17±0,01 | 0,17±0,01 |
| Hydrogen peroxide | 3,07 ± 1,99 | - | 1,30 ± 0,44 |

It is observed that the chemical composition of the LCE with respect to the whey only acidified with *L. acipophilus* decreases, except for the case of nitrogen for which it remains constant. This may be due to the fact that bentonite is a clay with mineral retention capacity (Navarro Blaya and Navarro García, 2003) and when it precipitates

together with proteins and fat, it drags part of these compounds with it. In some cases, the losses are greater than 50% as in iron, phosphorus, ammonium, phosphate, and hydrogen peroxide.

A study was also carried out on the heat resistance and lifespan for its application in the canning industry. The thermoresistance study, aimed at determining which of the thermal sterilization systems currently used in the industry, is compatible with the characteristics of the new governing liquid. It also made it possible to validate whether the composition of the LCE can promote the development of microorganisms and whether the conventional thermal treatments are insufficient, so that some type of adjustment in terms of times and temperatures has to be considered. *St. Aureus* was selected to carry out this test. Three government liquids were also prepared: a standard one (water, sodium chloride, and acidified), LCE, and LCE supplemented with salt. It should be noted that the LCE is already acidified by the fermentation process itself.

Once *the St. Aureus* strains were reactivated, we proceeded to inoculate the three previous types of government liquid, and we determined if the pathogen grew effectively or if, on the contrary, the characteristics of the three liquids had inhibited its development, which could lead us to consider positive results when in fact they are not.

The following table shows that the inoculation with *St. Aureus* was effective, and that the acid character or salt concentration did not inhibit the growth of the bacteria, so the results obtained are only a consequence of the effect of the combination of temperature and time.

Table 7
*St. Aureus count (cfu/100 µL) in the three types of government fluid.*

| **Replica** | **LCE** | **LCE+NaCl** | **Standard** |
|---|---|---|---|
| 1 | $9{,}5\text{-}10^3$ | $2{,}5\text{-}10^3$ | $1{,}05\text{-}10^4$ |
| 2 | $9\text{-}10^3$ | $7{,}5\text{-}10^3$ | $1{,}55\text{-}10^4$ |
| 3 | $1{,}25\text{-}10^4$ | $1{,}35\text{-}10^4$ | $1{,}65\text{-}10^4$ |

For the thermoresistance test, three types of thermal treatments were used in the previous government liquids (confirmed the existence of *St. Aureus*): VAT or LT-LT pasteurization (65ºC/30 min), HT-ST pasteurization (72ºC/15 s), and classic sterilization (120ºC/20 min).

Both for this case and the previous one, specific chromogenic medium was used for *St. Aureus*, whose colonies are shown as pink/purple dots. The green colonies correspond to *St. Epidermidis*, which is not considered a pathogen.

At the end of the thermal treatments, it was observed that the load of this bacterium was practically null in the two pasteurization systems and null in the sterilization one. No color formation was observed as a consequence of caramelization reactions of thepossible residual lactose, which indicates that this sugar was practically transformed intolactic acid. Neither was the appearance of undesirable odors observed. With all this, it was possible to confirm that whatever the heat treatment system used by a canning company, LCE can be used.

The other test that was carried out was the study of the shelf life of the canned products with LCE as the governing liquid, comparing it with the standard governing liquid. It was decided not to use LCE supplemented with sodium chloride because its composition of salts would make the information redundant.

Three temperatures were chosen for the shelf-life study: 5 ºC, 20 ºC, and 5 ºC (with a humidity of 60%). For the first case, the preserves were kept refrigerated; the second case, at room temperature; and for the third case, a thermal cabinet with humidity control was used.

The preserves were made with borage, a typical vegetable from Aragon that was interesting for two reasons. Its green color helped to disguise the initial color of the LCE and, in addition, it slightly reduced the milky smell that could not be eliminated during processing.



*Figure 6*. Borage preserves in LCE (left) and control (right).

Figure 6 shows the slight initial turbidity of the canned water with LCE compared to the control. The test lasted 14 days, and the parameter chosen for monitoring was turbidity measured at 420 nm as is done for drinking water, and because it is a variable that would make the canned product lose commercial value.

Table 8
*Values of the turbidity of the governing liquid on the different days considered.*

| Temperature | Time (days) | Control | LCE |
|---|---|---|---|
| 5ºC | 0 | 0,0583 | 0,0779 |
| | 4 | 0,0746 | 0,1051 |
| | 7 | 0,2869 | 0,1105 |
| | 10 | 0,3142 | 0,1676 |
| | 14 | 0,4722 | 0,2563 |
| 55ºC and 60%. humidity | 0 | 0,0583 | 0,0779 |
| | 4 | 0,4209 | 0,4040 |
| | 7 | 0,4975 | 0,4068 |
| | 10 | 0,6175 | 0,6175 |
| | 14 | 0,6813 | 0,6321 |

Table 8 shows how the turbidity increases are greater in the control than in the LCE for the case of temperature at 5 ºC, while at 55 ºC the values are relatively similar. In addition, it is observed that the values at 55 ºC are higher than at 5 ºC. This last phenomenon is due to the fact that at a higher temperature the biochemical processes are

accelerated, which can be linked to a greater decomposition of the borage due to the effect of the lactic acid, and thus to a greater turbidity.

With respect to the possible nutritional biostimulant effect in plant products for application in the agricultural industry, it was analyzed how the composition of the biostimulant varied throughout the development of the crop and the response of the plant. The test that was proposed for this consisted of adjusting the pH to 5.5, with the addition of potassium hydroxide to the LCE since at these values there could be toxicity problems due to the possible aluminum in the medium. With this information as a starting point, the subsequent trials involved the comparison between LCE without modifying the pH, LCE with the pH adjusted and water as a control. It was observed that the plants irrigated with LCE without adjusting the pH presented symptoms of stress that could be due to the toxicity of aluminum or for developing in acid medium, in addition to the plants entered a stage of non-recovery. This forced to shorten the development of the task. For the test, lettuce seedlings were sown on organic substrate. Water was applied directly at a dose of 30 ml/pot every 4 days until it was considered that the trial should end due to the state of the plants irrigated directly with LCE.

On the other hand, it was observed that the plants irrigated with LCE with adjusted pH responded very positively, which confirmed the biostimulant effect of the liquid fraction of the whey for agronomic purposes. This statement is not only based on observation but also on the mean values of the parameters analyzed in the seedlings.



*Figure 7*. Developmental stages of lettuce seedlings irrigated with LCE without pH adjustment (upper left); with water (upper right); and with LCE with pH adjusted (lower left); and a comparison between them (lower right).

Table 9
*Composition of some nutritional parameters of lettuce seedlings.*

| Sample | Weight (g) | Humidity (%) | Ash (%) | Ascorbic acid (mg/100g) | Nitrogen (%) | Calcium (mg/100g) | Phosphorus (mg/100g) |
|---|---|---|---|---|---|---|---|
| Control | 2,89±0,85 | 94,58±0,42 | 0,85±0,31 | 11,70±1,28 | 0,17±0,02 | 1,72±0,58 | 2,93±0,09 |
| LCE (without pH adjustment) | 5,07±1,31 | 91,41±0,34 | 1,47±0,85 | 7,77±1,11 | 0,27±0,01 | 0,58±0,14 | 2,78±1,07 |
| LCE (pH adjusted) | 2,20±0,73 | 91,75±0,74 | 0,77±0,38 | 12,31±1,42 | 0,14±0,01 | 1,05±0,81 | 2,55±0,27 |

Although at this stage it is not possible to compare these results with those of the bibliography since the plants had not reached commercial maturity, which is the point where they are harvested and their chemical composition is determined, it is necessary to highlight the following results:

On the one hand, it was observed that the mineral content (determined through the ash content) was higher in plants irrigated with LCE of adjusted pH. This suggests a higher utilization of minerals with respect to the other cases, especially the control. Although the latter had higher values of Ca and P. The difference is in the nitrogen content, which was much higher in the case of the pH-adjusted LCE. On the other hand, the ascorbic acid content was lower for the pH-adjusted LCE. This, far from being a negative aspect, is an indicator that the plant was in a situation of lower stress since ascorbic acid (vitamin C), like any other antioxidant compound, is synthesized in greater quantity by plants if they are stressed. These results confirm that the liquid fraction of whey, after processing, can have a positive agronomic use.

## Discussion and conclusions

Based on the results of this project, it can be concluded that among the acid lactic acid bacteria compared to carry out the fermentation of sweet whey from the production of fresh cow's cheese, *L. acidophilus* produced the best result when inoculated at a concentration of 2% and without the requirement of nutritional supplementation. Fermentation and lactic acid production occurred in 48 hours.

On the other hand, the separation of the solid fraction of fermented whey from the liquid fraction can be effectively achieved with bentonite. It is necessary to carry out dosage tests to adjust the exact amount of this clay to be used. In addition, it has been observed that the use of bentonite also implies that the minerals are distributed between the solid and liquid fractions of the fermented whey. The separation of the solid fraction (rich in protein and fats and minerals) from the liquid fraction (water and minerals) through the use of bentonite affirms that this methodology is sustainable (in environmental and economic terms as no chemical processes are used) and easy to apply

by small and medium-sized cheese dairies.

Another thing that has been observed is that in the composition of the acidified, clarified, and sterilized liquid fraction (LCE), no compounds of economic interest for the cheese factory were observed, such as protein or fat, and its composition allows it to be

discharged without producing an environmental problem as it lacks lactose, fat, and protein (compounds responsible for its BOD).

As far as the use of LCE as a governing liquid for vegetable preserves is concerned, it has been shown as a potential agent capable of being compatible with the usual thermal treatment systems present in cheese factories (pasteurization and sterilization). In addition, LCE as a liquid of government for canned vegetables, has been shown as a potential agent able to inhibit the enzymatic oxidation processes in borage, possibly due to polyphenol oxidase. Finally, LCE as a component for the formulation of plant biostimulants has been shown as a potential agent able to improve the response of the plant after a process of pH adjustment towards neutral values.

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# DEVELOPMENT OF AN EDIBLE AND COMPOSTABLE BIOPLASTIC FROM FOOD INDUSTRY RESIDUES

**María Balsa Núñez**
Universidad Europea del Atlántico (Spain)
maria.balsa.n@gmail.com · https://orcid.org/0000-0001-7844-9148
**Jorge Martínez de la Fuente**
Universidad Europea del Atlántico (Spain)
jorge.martinez@alumnos.uneatlantico.es · https://orcid.org/0000-0003-1246-6708

**Abstract**. The project pursues the use of waste from the Cantabrian food sector (cereal waste from the spirits industry and whey), in order to manufacture an edible, biodegradable and compostable plastic substrate, as an alternative to current plastic production, providing a solution in favor of the recovery of industrial waste. For the development of the edible plastic substrate, arabinoxylan and kefiran compounds were used, from cereal residues and milk whey, respectively. Several formulations were developed to create a pre-industrial prototype of the biocomposite for the edible plastic substrate, likewise, a search was made on the use of whey to obtain biofilm.An exploitation plan was drawn up that evidenced the need to sell 1,900 kg of bioplastic pellets per month to ensure the economic viability of the process. This production would have a unit cost of €15/kg, lower than the estimated sale price of €20/kg. Although it is estimated that the business profit margin would not be very high, the positive environmental impacts are good enough to consider the implementation of the developed solution.

**Keywords:** Food waste, plastic, bioplastic, environment.

# DESARROLLO DE UN BIOPLÁSTICO COMESTIBLE Y COMPOSTABLE A PARTIR DE RESIDUOS DE LA INDUSTRIA ALIMENTARIA

**Resumen**. El proyecto persigue el aprovechamiento de los residuos del sector alimentario cántabro (residuos cereales de la industria de bebidas espirituosas y el suero de leche), con el objeto de fabricar un sustrato plástico comestible, biodegradable y compostable, como alternativa a la producción de plásticos actual, aportando una solución a favor de la valorización de residuos industriales. Para el desarrollo del sustrato plástico comestible, se partió de los compuestos arabinoxilanos y kefirán, provenientes de residuos cereales y del suero lácteo respectivamente. Se desarrollaron varias formulaciones para crear un prototipo pre-industrial del biocompuesto para el sustrato plástico comestible, asimismo, se realizó una búsqueda sobre el uso del lactosuero para la obtención de biofilm. Se elaboró un plan de explotación que evidenció la necesidad de vender 1900 kg de pellets de bioplástico al mes para asegurar la viabilidad económica del proceso. Esta producción tendría un coste unitario de 15 €/kg, inferior al precio de venta estimado de 20€/kg. Aunque se estima que el margen de beneficio empresarial

no sería muy alto, los impactos ambientales positivos son suficientemente buenos como para considerar la implantación de la solución desarrollada.

**Palabras clave:** Residuos alimentarios, plástico, bioplástico, medio ambiente.

## Introduction

Due to the increase in population, the excessive consumption of resources, and their negative effects on the environment, in the last 20 years social interest has grown towards a circular economy in which energy, resource management, as well as patterns of production and consumption, must be considered. In January 2018, the European Commission reported that recycling 1 million tons of petrochemical-based plastic is equivalent, in terms of carbon dioxide emissions, to taking 1 million vehicles off the road. Announcements by countries on the need to consider the fight against climate change and pollution, as one of the main current and future challenges of our societies, are multiplying (e.g., the declaration of "climate emergency" by the British Parliament, May 2019.).

In particular, on March 27, 2019, the European Parliament approved the directive banning from 2021 the sale of single-use plastics.

It is known that the solution to plastic pollution is not only to recycle but to reduce the use, consumption, and production of petroleum-based materials (WTO, 2018). The excessive use of unsustainable raw materials, such as petroleum, is one of the main causes of environmental pollution. Consequently, the production of consumer goods needs to be re-evaluated, with a greater focus on food packaging, one of the biggest polluters (they are 18.6% of the plastics present in the sea), that this production consumes less energy, and that plastics continue with a life cycle of minimal waste or different function (Özdamar & Ateş, 2018).

Measures such as reduction, reuse, and recycling of plastics have been taken; however, the increase in production remains constant and reuse and recycling are minimal. A study, carried out by Geyer, Jambeck, and Lavender (2017) shows that of the 6.3 billion tons of plastic turned into waste, only 9% ended up recycled, with 79% accumulated in the environment. This is why alternatives to the use of plastics of petrochemical origin should be raised.

Since the 2000s, bioplastics have emerged from scientific fields. Here we see experiences of manufacturing objects from bio-compostable materials (of vegetable, partially vegetable or fossil origin) such as flexible containers and packaging, catering articles, paper coating, agricultural mulch, shopping bags (Song et al., 2009). Today they constitute in Europe 1% of the total 335 million tons of plastics produced each year, and fortunately there is a growing interest, especially towards new bioplastics (Xu & Yang, 2012).

Bioplastics are considered to be plastics that are bio-based (the material or product is partly derived from biomass, i.e., plants), or biodegradable and/or compostable (materials that can be transformed into natural substances through a process) or both. They are made up of:

(1) biodegradable or non-biodegradable raw materials of renewable origin;

(2) raw material of petrochemical and biodegradable origin.

In the search for better characteristics of these bioplastics, the combination of elements of a biological nature has been explored, generating biocomposites of great interest. Biocomposites are mixtures of two biomaterials and are manufactured to achieve better performance, which is not possible with only one of the components.

Several companies have introduced starch/polyethylene blends as degradable materials for a number of short-life applications, such as beverage bottles, food packaging, and plastic bags. However, the impact on the environment is negative. While the starch component can degrade, the polyethylene residues remain in ecosystems and are not biodegradable.

In order to clearly set apart the different types of bioplastics, we could distinguish three main types of plastics: bio-based or organic-based plastics, biodegradable plastics, and bio-compostable plastics.

Bio-based plastics can be defined as derived from renewable biomass resources, which are largely biodegradable. They can provide functional advantages similar to those of traditional plastics, such as their use in packaging (Song, 2009). They are made of animal or plant organic matter, and very often combined with materials of petrochemical origin. Organic-based plastics depend on crops and scarce resources such as water. In some cases, and only if they do not contain materials from fossil fuels, they are edible.

Biodegradable plastics can be manufactured from renewable resources or from fossil fuels (Bastioli, 2003) since the biodegradability character is related to the chemical structure of the plastic. They also provide similar properties to plastics of petrochemical origin. Biodegradable plastics degrade by the action of microorganisms such as bacteria, fungi, and algae, without requiring human action.

Bio-compostable plastics, unlike biodegradable plastics, imply that the materials that constitute them can be converted into compost (organic fertilizer) through a process in which humans intervene, transforming the plastic through machinery.

Thus, this project was developed to achieve the use of waste from the Cantabrian agri-food sector (cereal waste from the spirits industry and whey from the production of cheese), for the manufacture of an edible, biodegradable, and compostable plastic substrate as an alternative to the production of polluting and non-recyclable plastics, providing a solution in favor of the valorization of industrial waste. Specifically, cereal bran (waste from the production of spirits) and whey (waste from the production of cheese).

## Methodology and Results

First, the characterization of the two wastes involved in the project was carried out. For this purpose, the compounds arabinoxylans and kefiran, from cereal and whey wastes respectively, were analyzed since they are the two main ingredients of the edible plastic substrate proposed to be developed. The type of characterization focused on the integration of the compounds for the creation of the substrate.

As a first approach, a study of the alternatives for the pre-processing of cereal residues was carried out, for which a theoretical research on extractive techniques of compounds of interest from the cereal residue and the corresponding identification of processes and equipment to optimize the extractive process was developed. Of the cereal compounds of interest, this research focused from the beginning on arabinoxylans (AX). AX are polysaccharides present in cereal grains, located in the endosperm and bran (aleurone, cuticle, and pericarp). The interest in these polysaccharides is due to the fact that it has been shown that they can be considered as prebiotics.

AX can be water soluble (WEAX: water extractable arabinoxylans) and water insoluble (WUAX: water unextractable arabinoxylans), and it is this property that has been taken into account to adjust the extractive process.

Since AX are found as part of hemicellulose (Figure 1), we looked for hemicellulose extraction procedures that are easy to apply in the laboratory and that do not involve high reagent use, waste generation, and difficult application in green or sustainable industries.




*Figure 1*. Types of polysaccharides

*Note:* Source: Own elaboration, 2022

Taking into account the above points, the method used is the one proposed by Yadav and Hicks (2017), which is based on the soluble character of hemicellulose and AXs and separates Hemicellulose A from Hemicellulose B, being in the latter the AXs. It was decided not to separate both fractions because no references were found as to which fraction contained the AX. It should be noted that this process requires a centrifugation step as well as the use of reagents such as ethanol and sodium hydroxide. At the industrial level, these requirements are easy to implement. However, the fact that the final product is dissolved in ethanol would make it necessary to implement additional equipment in the industry for the evaporation and subsequent recovery of this solvent.

As a first step of the proposed method, it is necessary to carry out an enzymatic treatment with α-amylase. This is in order to hydrolyze the amylase and facilitate the previous process. But since in this project we start from exhausted cereal (i.e., the residue of the food industry where starch has been extracted as a source of sugar), it was decided to dispense with that process.

Figures 2 and 3 show the cellulose and hemicellulose obtained experimentally.




*Figure 2.* Extracted cellulose and hemicellulose.

*Note:* Source: Own elaboration, 2022

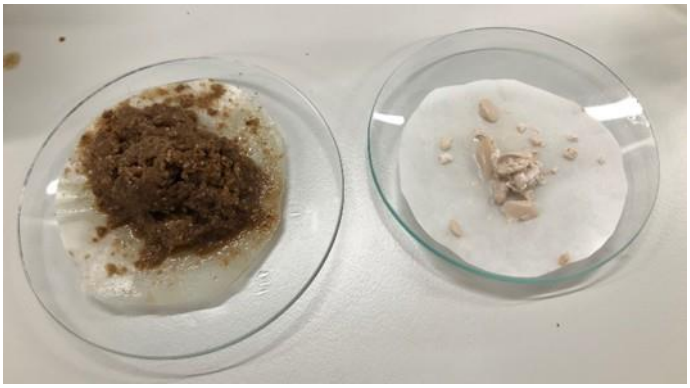

*Figure 3.* Cellulose (left) and hemicellulose (right) before drying.

*Note:* Source: Own elaboration, 2022

The characterization of kefiran was obtained from fermentation and whey purification processes by means of a laboratory-scale production protocol from whey and obtaining fresh kefir granules by growth in sterilized whey supplemented with $KH_2PO_4$ salts and sucrose, followed by homogenization, centrifugation, and precipitation of kefiran with ethanol.

Kefiran is the name given to a water-soluble gelatinous gum or polysaccharide released from kefir grains, formed by the monosaccharide's glucose and galactose, which is inside the matrix of the kefir granule (Piermaria et al, 2009). This kefir granule is an ecosystem inhabited by bacteria and other microorganisms responsible for the fermentation of lactose to lactic acid.

The methodology for obtaining it is that described by Joe Dailin et al. (2016), slightly modified. Said methodology is based on growing kefir in milk and identifies kefiran as the gelatinous polysaccharide that coats each granule, subsequently making several proposals for its isolation and purification.

After an analysis of this process, it was observed that it requires some factors that make the scaling up of the methodology towards a green or sustainable industry complicated. These factors were:

- Milk is required, which is not a by-product.
- Large volumes of solvents are required for kefiran washing and isolation.
- The purification process also requires a methodology and technology that do not justify the amount of waste generated and its subsequent management.
- These being the limiting factors, the following modification was proposed to overcome these drawbacks:
- Instead of using milk, residual whey from the cheese industry was used, thus working with a by-product. This whey was enriched with lactose so that fermentation could take place, the step from lactose to lactic acid.
- Instead of isolating the kefiran, the gel formed by the fermentation process itself was used as part of the biofilm. In this way, no waste is generated (Figure 4).

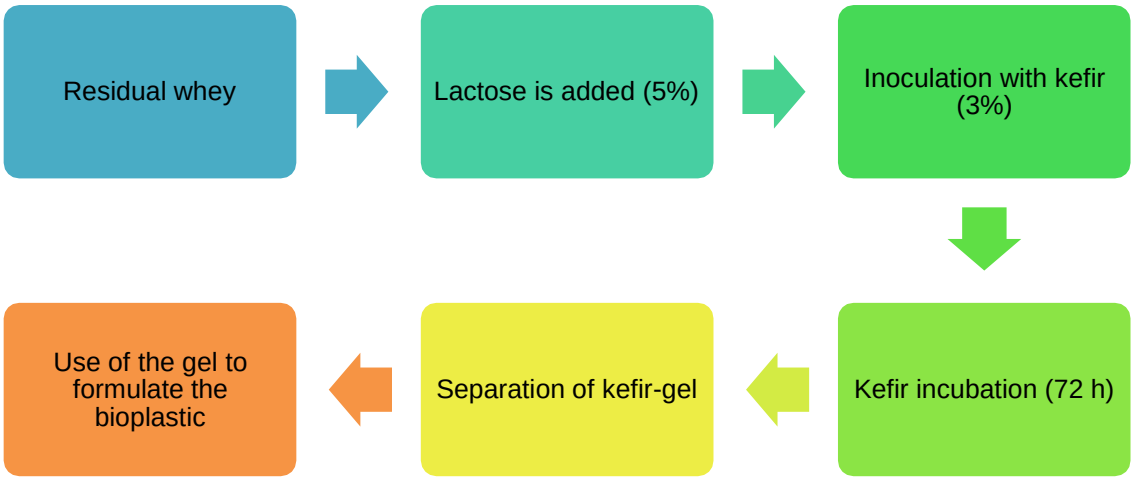


*Figure 4.* Process for obtaining biofilm gel at laboratory scale.

*Note:* Source: Own elaboration, 2022

Once the kefir is separated from the gel, it can be used for another inoculation and subsequent fermentation process, so it is a cyclic process that does not generate waste. As for lactose, it is a sugar that is easy to acquire in industrial doses. Figure 5 shows the final gel obtained after 72 hours of fermentation.

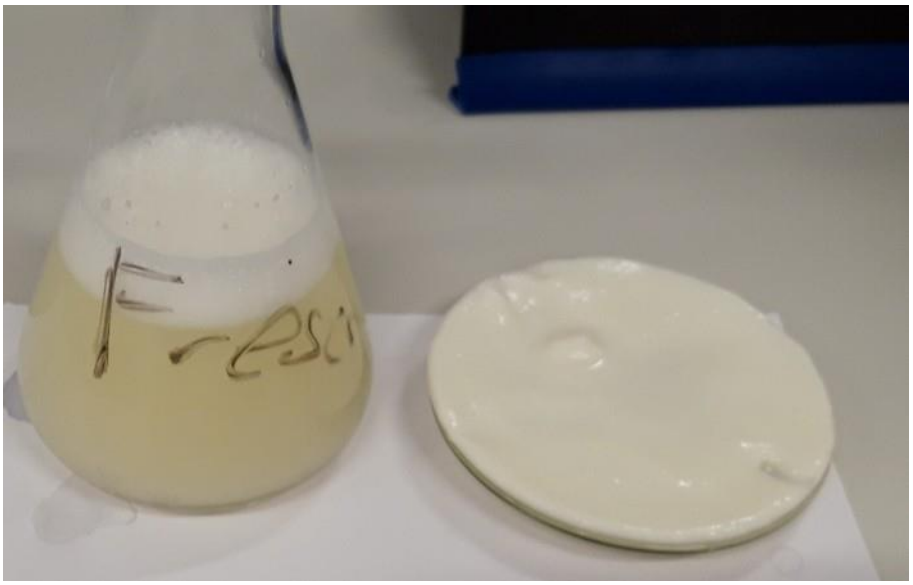

*Figure 5*. Kefir obtained from fermented whey.

*Note:* Source: Own elaboration, 2022

Kefir was inoculated at 3% on whey enriched with 5% lactose. From previous experiences of projects carried out with whey, it is known that its composition in protein and non-protein nitrogen, as well as its mineral composition, does not require adjustment of the chemical composition to facilitate the development of any microorganism. Table 1 shows the results of its analysis, which was performed on the original whey. After the fermentation process, the residual lactose, pH, and lactic acid content were measured, parameters that can have the greatest influence on subsequent use (Table 2).

Table 1
*Initial composition of whey*

| **Parameter** | ***L. acidophilus*** |
|---|---|
| Phosphate (mg/L) | 154 |
| Protein (mg BSA/mL) | 0,7 |
| Calcium (mg/L) | 62 |
| pH | 6,9 |
| Lactose (%) | 0,38 |

*Note:* Source*:* Own elaboration, 2022

Table 2
*Final composition of fermented whey with kefir*

| **Parameter** | **Value** |
|---|---|
| *pH* | 4,39 |
| *Lactic (%lactic acid)* | 1,50 |
| *Lactose (%)* | 0,20 |

*Note:* Source: Own elaboration, 2022

The separation of kefir from the gel was carried out by sequential filtration. First through larger diameter filters and then through smaller diameters. In this case, the microbiological characterization was not considered relevant because it is a pasteurized whey. It should be noted that we always worked with sweet skimmed whey.

The second characterization was of the raw cereal residues. For this purpose, the cereal residue was washed, dried, and crushed to obtain arabinoxylans. In this phase, the methodology described above was applied, and the volume of cellulose/hemicellulose was quantified, and the degree of viscosity-polymerization obtained in each working condition was characterized with a hedonic scale (Table 3). The experimental design consisted of modifying the temperatures and alkaline treatment times to separate cellulose from hemicellulose from the method described by Yadav and Hicks (2017).

The tests were carried out with 0.5 g of degassed cereal (Figure 6) that was dissolved in 10 ml of 2.3 N NaOH and subjected to heat treatment (70ºC and 40ºC in different tests). It was decided not to precipitate the hemicellulose in order to avoid a new residue and because being in a liquid medium would allow to see if it has thickening capacity or not. The thickening capacity was determined by allowing the hemicellulose suspension to cool to room temperature and observing the final result. The results are shown in Table 3.

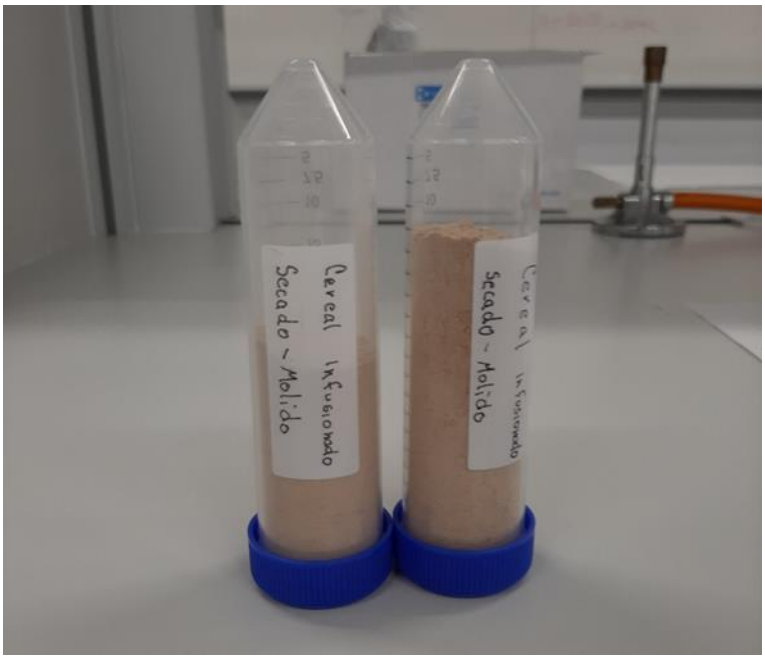


*Figure 6*. Degraded cereal flour
*Note:* Source: Own elaboration, 2022

Table 3
*Results of initial alkaline treatment*

| **Temperature (ºC)** | ***Time (min)*** | ***Cellulose (g)*** | ***Hemicellulose (ml)*** | ***Thickening capacity*** |
|---|---|---|---|---|
| 70 | 60 | - | - | Very high |
| 40 | 60 | 0,3 | 8 | Medium |

*Note:* Source: Own elaboration, 2022

At 70ºC, a highly viscous mass was formed that did not allow separation of the two fractions, so it was decided to repeat the test at 40ºC, modifying the duration of the alkaline treatment. The results are shown in Table 4.

Table 4
*Treatment results at 40ºC*

| ***Time (min)*** | ***Cellulose (g)*** | ***Hemicellulose (ml)*** | ***Thickening capacity*** |
|---|---|---|---|
| 60 | 0,3 | 8 | Medium |
| 120 | 0,4 | 7 | High |
| 180 | 0,5 | 6 | High |

*Note:* Source: Own elaboration, 2022

After 12 and 180 min, the final supernatant thickened upon cooling, without reaching the level observed when the temperature was 70ºC. On the other hand, the 40ºC/60 min treatment allowed a very good differentiation between the two fractions and no solidification effect on cooling, which allowed the samples to be preserved and subsequent concentration and purification treatments to be carried out.

Finally, the selection and evaluation of the biocomposite was carried out in three stages. First, different formulations adapted to the observations collected in the previous activities were carried out. As a result, no isolation of the arabinoxylans was performed, instead the hemicellulose suspension was used where these compounds and others that can facilitate the formation of the gum or biofilm are found. In this way, no waste is generated. Similarly, instead of using kefiran, the gel formed by the fermentation of whey was used.

Specifically, the experimental design consisted of comparing the following formulations:

Control: starch as structuring polysaccharide, acidification with 0.1N HCl, and viscosity adjustment agent consisting of a mixture of 50% glycerin and 2M NaOH 2M in a ratio of 1:1.25.

Formulation 1: hemicellulose from spent cereal flour, fermented whey (being acidified it replaces HCl), and 50% glycerin. NaOH 2M is not applied because it is already present in the hemicellulose suspension.

After mixing, a microwave heat treatment was applied. The heat allows the formulation to polymerize and a film is obtained. The results obtained are shown in Table 5.

Table 5
*Comparison of formulations (I)*

| ***Film*** | ***Polymerization*** | ***Viscosity*** |
|---|---|---|
| Control | Yes | Adequate |
| Formulation 1 | Very low | - |

*Note:* Source: Own elaboration, 2022

After a first test, Formulation 1 was observed to have a very low degree of polymerization (Figure 7).

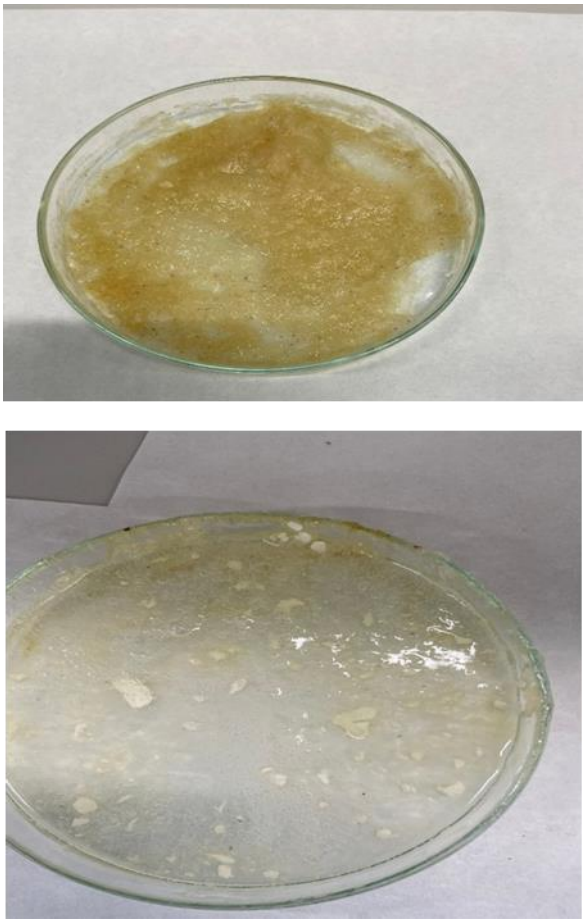

*Figure 7*. Comparison of the control biofilm (upper) with that of formulation 1 (lower)
*Note:* Source: Own elaboration, 2022

An analysis of the results led to the theory that the amount of hemicellulose is insufficient to form the film, so two possible solutions were proposed: a) enriching the formulation with starch, and b) increasing the hemicellulose content.

Choosing this second option has a practical disadvantage when it comes to implementing the process in an industry, which is the reagent and heat application requirements necessary to obtain hemicellulose, in addition to the need for an industrial centrifuge to separate

it from cellulose. For all these reasons, the first route was chosen by means of starch supplementation (Figure 8).

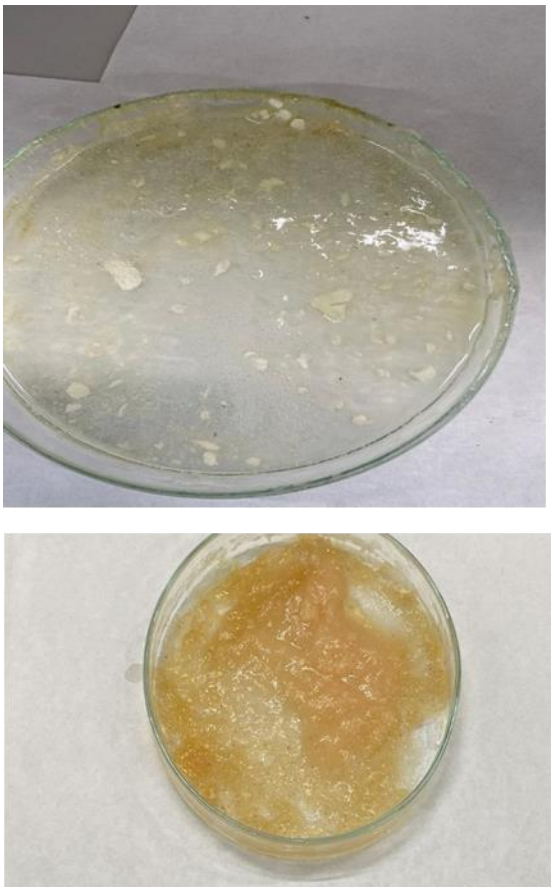

*Figure 8.* Comparison of formulation 1 (top) with the same formulation supplemented with 2% starch (bottom)

*Note:* Source: Own elaboration, 2022

Starch is a readily available product, very common in the food industry and low cost, and it would not have any technological impact or waste generation. After this first test, 2% starch was added to the new formulations. The results obtained showed a very good structure and the same behavior as the control after a few days, i.e., its consistency was maintained.

Based on these results, it was proposed to obtain bioplastic pellets since this is the usual form in which the plastics industry receives its raw material and, after an extraction process, gives it the desired shape.

Of all these compounds, the fermented whey fraction provides calcium (by its own composition), which is a structuring element that in combination with alginate has the capacity to form spheres, i.e., the desired pellets. A dose-response trial-error test was performed to determine how much alginate to add to the formulation, making the decision based on the final characteristics of the pellet, as shown in Table 6:

Table 6
*Pellets characteristics at different alginate doses*

| ***Alginate (%)*** | ***Biofilm characteristics*** |
|---|---|
| 0,5 | No pellets are formed. |
| 1 | Pellet formation is observed but in a very limited quantity. |
| 1,25 | Unstable pellets are formed with time. As they lost their structure, it was not possible to evaluate whether or not microorganisms were growing. |
| 1,5 | Good density, good pellet formation, and stable over time. No proliferation of microorganisms. |

*Note:* Source: Own elaboration, 2022

To obtain the pellets, a burette was loaded with the working formulation and dropped onto a bed of alginate with agitation and heat to promote the solidification process of the spheres to pellets (Figure 9).

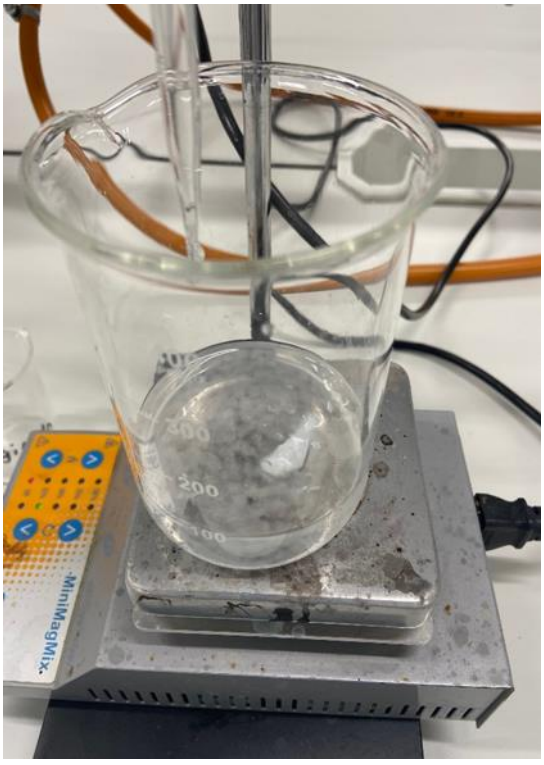


*Figure 9*. Formation of spheres or pellets in the laboratory
*Note:* Source: Own elaboration, 2022

Thus, Figure 10 summarizes the final protocol designed to obtain bioplastic pellets.

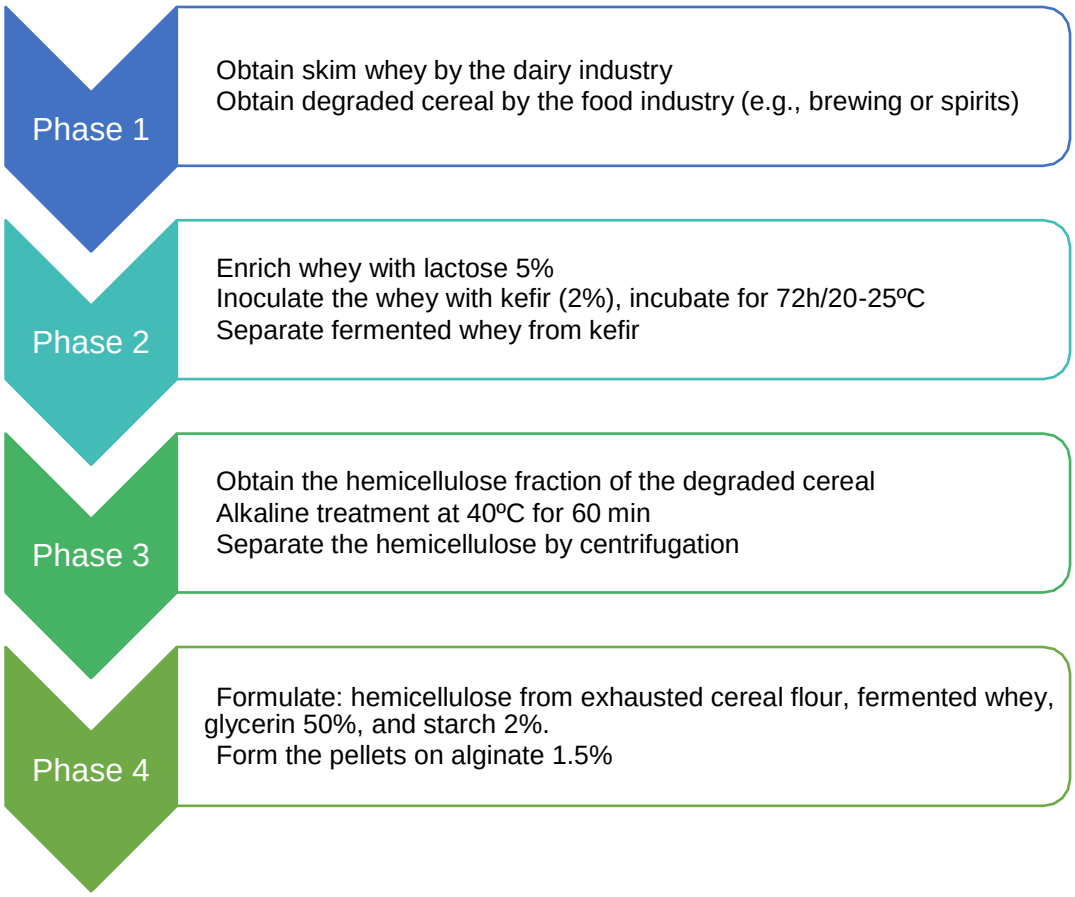


*Figure 10*. Bioplastic pellet production process
*Note:* Source: Own elaboration, 2022

A preliminary business plan was made by means of an economic estimation considering the most important costs if a start-up is created to process the industry waste to form the biofilm. Assuming that the bioplastic pellet should not exceed the price of 20 €/kg, it was estimated the quantity that should be produced monthly so that the unit cost is compensated with the selling price. The results showed that 1900 kg of bioplastic pellets should be sold per month. This production would have a unit cost of €15/kg, lower than the estimated selling price but with a low profit margin for the company.

## Discussion and conclusions

As a result of this project, an edible plastic substrate was created, providing a possible solution for the valorization of industrial waste through the use of waste from the Cantabrian agri-food sector, specifically cereal waste from the spirits industry and whey from the dairy industry.

For the development of this substrate, the compounds arabinoxylans and kefiran from cereal residues and whey, respectively, were obtained by means of experimental protocols that were the result of an investigation of methodologies adapted to the objectives of the study.

Specifically, it was observed that the pellet manufacturing process should consist of the following tasks:

- Obtain the necessary components (skimmed whey and degraded cereal) from food industry waste.
- Enrich whey with lactose 5%.
- Inoculate the whey with kefir (2%), incubate for 72h/20-25ºC.
- Separate fermented whey from kefir
- Obtain the hemicellulose fraction of the dehulled cereal
- Provide an alkaline treatment at 40ºC for 60 min
- Separate the hemicellulose by centrifugation
- Formulate: hemicellulose from exhausted cereal flour, fermented whey, glycerin 50%, and starch 2%
- Form the pellets on alginate 1.5%

It should be noted that in this first stage of research, although the bioplastic generated has been obtained from waste already suitable for consumption, the necessary aseptic conditions have not been taken into account, since what was sought in this initial phase was to determine the possibility of obtaining this material. Subsequent research will include the appropriate considerations to make it edible.

With respect to economic feasibility, a business plan was developed, which led to the conclusion that 1900 kg of bioplastic pellets should be sold per month. This production would have a unit cost of 15 €/kg, which is lower than the estimated selling price of 20 €/kg. These data can be considered as the minimum reference value.

## Acknowledgments

The authors wish to express special thanks to Dr. Marcos Hernández Suárez for his support and advice in this project, making available his knowledge and dedication. The authors would also like to thank the Society for the Development of Cantabria (SODERCAN) for the co-funding of the project EC19-XX-001 belonging to the Circular Economy Call.

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# OBTAINING BIOFUELS FROM *CHLORELLA VULGARIS* BIOMASS

**Celia Verenice Blanco Cornelio**
Universidad Autónoma del Carmen (Mexico)
ailecverenice72@gmail.com · https://orcid.org/0000-0002-8302-1629
**Asteria Narvaez Garcia**
Universidad Autónoma del Carmen (Mexico)
anarvaez@pampano.unacar.mx · https://orcid.org/0000-0001-6484-6062
**Juan Carlos Robles Heredia**
Universidad Autónoma del Carmen (Mexico)
jrobles@pampano.unacar.mx · https://orcid.org/0000-0003-2591-6528

**Abstract**. The objective of this work is to analyze the importance of obtaining biofuels through the microalgae Chlorella vulgaris as a raw material for obtaining fuels such as biodiesel and bioethanol. To cultivate microalgae, light, water, nutrients and a minimum amount of land are required to install the cultivation area. These organisms, like plants, are capable of using $CO_2$ and sunlight to generate complex biomolecules that are necessary for their survival. From the hydrodynamic effect of aeration and continuous white light conditions in bubble column photobioreactors; The different methods and sources for obtaining renewable fuels are analyzed. The microalgae will be in a culture preserver for the reproduction of more cells in acclimatization for 30 days in 250 ml Erlenmeyer flasks with constant lighting that will help motivate the reproduction of the algae. In relation to the analysis of the methodology, it is obtained that the exempted microalgae are identified as one of the best triglyceride-producing microorganisms mainly used to obtain biodiesel and bioethanol. However, more research is currently needed to determine the best culture method and thereby obtain a higher balanced yield of biomass and lipids.

**Keywords:** Aeration, Microalgae, Chlorella vulgaris, biodiesel, bioethanol.

# OBTENCIÓN DE BIOCOMBUSTIBLES A PARTIR DE BIOMASA DE CHLORELLA VULGARIS

**Resumen**. El presente trabajo, tiene como objetivo analizar la importancia de la obtención de biocombustibles mediante la microalga *Chlorella vulgaris* como materia prima para la obtención de combustibles como biodiésel y bioetanol. Para cultivar microalgas se requiere de luz, agua, nutrientes y una mínima extensión de tierra donde instalar el área de cultivo. Estos organismos al igual que las plantas, son capaces de utilizar el $CO_2$ y la luz solar para generar complejas biomoléculas que son necesarias para su

supervivencia. A partir del efecto hidrodinámico de aireación y en condiciones de luz blanca continua en fotobiorreactores de columna por burbujeo; se analizan los diferentes métodos y fuentes para la obtención de combustibles renovables. Las microalgas se encontrarán en un conservador de cultivos para reproducción de más célula en aclimataciones por 30 días en matraces Erlenmeyer de 250 ml con una iluminación constante que ayudara a la motivación de la reproducción de las algas. En relación al análisis de la metodología, se obtiene que las mciroalgas eximidas se identifican como uno de los mejores microorganismos productores de triglicéridos principalmente empleados para la obtención de biodiésel y bioetanol. Sin embargo, actualmente es necesario una mayor investigación para determinar el mejor método de cultivo y con ello obtener un mayor rendimiento equilibrado de biomasa y lípidos.

**Palabras clave:** Aireación, Microalga, Chlorella vulgaris, biodiesel, bioetanol.

## Introduction

Currently, fossil fuels are one of the main factors of various environmental problems, this resource obtained from the natural decomposition of organic matter is depleting day by day, causing the prices of oil reserves to rise, making it difficult to access them. A viable option to avoid these problems of depletion is the alternative of elaborating new fuels by means of other compounds.

Alternative methods and compounds for the production of fuels mainly involve reducing the environmental impact and being able to prevent the scarcity of fossil fuel reserves. Microalgae is one of the fundamental alternatives to the production of biodiesel as well as for obtaining other energy products such as bioethanol, biomethane, and biohydrogen (Heredia et al. 2019) The present work, aims to analyze the importance of obtaining biofuels through the microalgae *Chlorella vulgaris* as raw material, in order to give a better ideology to the different methods and sources for obtaining renewable fuels that do not affect the environment.

In addition, they can be used in areas unsuitable for traditional cultivation and achieve high growth rates (0.5 to 1.2 $d^{-1}$). However, many microalgae cultivation technologies involve the use of uncontaminated water, fertilizers, and CO2 injection for growth, which raises the cost of algal biomass production and reduces its attractiveness as a technology. To minimize these drawbacks, one cultivation alternative is to use municipal, agricultural, and livestock wastewater, where microalgae can grow by taking advantage of the nutrients in these types of discharges. This integration allows:

1. Treat wastewater.
2. Obtain a high-quality effluent.
3. Generate useful algal biomass to produce biodiesel or other types of biofuels.

Therefore, algae are photoautotrophic organisms and present a rapid growth in a short time, which allows them to be cultivated in this type of water; since the absorption of nutrients by microalgae uses a high content of nitrogen, silicon, phosphate, and sulphate from human or animal waste; in addition, they can retain carbon dioxide ($CO_2$) from industrial sources. Thus, these reasons support the research on microalgae *Chlorella vulgaris* since obtaining them is not an obstacle.

In a study carried out in 2006 by the Mexican Ministry of Energy (SENER), it is mentioned that the production of biodiesel on a commercial scale can be feasible in the medium term if comprehensive actions are carried out, including technical, economic, and environmental aspects with the agricultural and agro-industrial sector, combining efforts in research and technological development. They allude that Mexico requires 10 industrial plants with a capacity of 100,000 tons/year each, just to replace 5% of

petroleum diesel used each year and recommend that the production and processing be carried out with technologies designed and built in the country itself. They also argue that the use of biodiesel would reduce hydrocarbon emissions by 45%, 47% of $CO_2$, and up to 66% of particulate emissions into the environment (Escalante 2019).

In this sense, long-chain fatty acids obtained from renewable biomass (vegetable oils, animal fats, and microalgae oils) represent the main raw material for the production of liquid biodiesel obtained in the form of alkyl esters of short-chain alcohols such as ethanol and methanol. The most commonly used processes for obtaining biodiesel are pyrolysis and transesterification; however, in the first case, the method is expensive and offers low yields, while the second is presented as the most viable method for obtaining biodiesel. This process of converting oils to biodiesel is necessary because vegetable oils or oils extracted from microalgae have high viscosity and low volatility, causing incomplete combustion and the disposal of carbon deposits (Escalante 2019).

Large-scale cultivation of microalgae seeks to obtain significant amounts of a valuable product; therefore, the productivity of the system must be maximized. Understanding the factors that determine optimal growth is fundamental (Robles et al 2019):

- Culture medium.
- Gas exchange.
- pH.
- Temperature
- Lighting regime.

Temperature is a very important environmental factor due to its great influence on the development of microalgae. The effect of temperature on biochemical composition mainly affects two different mechanisms: the temperature dependence rate of chemical and biochemical reactions, and the temperature dependence for photosynthetic carbon fixation in various types of macromolecules, such as proteins, carbohydrates, and lipids.

This type of microalgae has an impact especially on the ecology since through them is a very important advantage from the energy and ecological point of view, since they have a minimum level of emission of harmful gases such as carbon dioxide ($CO_2$), the main compound of the greenhouse effect (Gonzalez 2015). In addition to reducing other amounts of pollutants, thus defining biodiesel as a biodegradable product, which from this can also be obtained vegetable oils such as soybean, palm, sunflower, and others.

## Method

### *Microalgae Chlorella vulgaris*

Microalgae are microscopic photosynthetic and unicellular microorganisms; they are classified into prokaryotes and eukaryotes that can grow autotrophically or heterotrophically. They are divided into different groups depending on their taxonomy. *Chlorella vulgaris* is a unicellular algae with green pigmentation and spherical shape; considered as an option for the production of biodiesel and bioethanol because of its high content of lipids and oils; however, they have the ability to produce biomass quickly compared to other energy crops. These organisms, like plants, are able to use $CO_2$ and sunlight to generate complex biomolecules that are necessary for their survival. In general, they are photoautotrophic organisms, that is, they obtain energy from light coming from the sun and develop from inorganic matter. However, some species are able to grow using organic matter as a source of energy or carbon. The selection of microalgae

is the first step in the development of a production process, they must have the appropriate characteristics for the specific cultivation conditions in order to achieve a certain product (Table 1) (Escalante 2019).

Table 1
*Lipid percentage and biomass and lipid productivity of different microalgae*

| Species | Accumulated lipids (%) | Biomass productivity ($g\text{-}L^{-1}\text{-}d^{-1}$) | Lipid Productivity ($g\text{-}L^{-1}\text{-}d^{-1}$) | Reference |
|---|---|---|---|---|
| Anabaena variabilis Kützing ex Bornet & Flahault | 46.90 | 0.1156 | 0.0542 | Han et al. (2016) |
| Ankistrodesmus falcatus (Corda) Ralfs | 59.60 | 0.1246 | 0.074 | Singh et al. (2015) |
| Chaetoceros muelleri Lemmerman | 43.40 | 0.2720 | --- | Wang et al. (2014) |
| Chlamydomonas reinhardtii P.A. Dangeard | 25.25 | 2.0 | 0.505 | Kong et al. (2010) |
| Chlamydomonas sp. | 33.10 | --- | 0.169 | Nakanishi et al. (2014). |
| Chlorella sorokiniana Shihira et R. W.Krauss | 31.50 | 12.2 | 2.9 | Li et al. (2013) |
| Chlorella minutissima Fott et Nováková (UTEX2341) | 62.97 | 1.78 | 0.29 | Li et al. (2011) |
| Chlorella pyrenoidosa H. Chick | 24.25 | 0.144 | 0.02685 | Tang et al. (2011) |
| Auxenochlorella protothecoides (Krüger) Kalina et Puncochárová (= Chlorella protothecoides Krüger) | 51.50 | --- | 1.19 | Mu et al (2015) |
| Chlorella vulgaris Beyerinck [Beijerinck]. | 22.80 | 0.0848 | 0.01043 | Frumento et al (2013) |
| Chromochloris zofingiensis (Dönz) Fucíkocá et L.A. Lewis (= Chlorella zonfingiensis Dönz) | 54.50 | 0.0584 | 0.0223 | Mu et al. (2015) |
| Desmodesmus abundans (Kirchner) E. Hegewald | --- | 0.27008 | 0.06708 | Xia et al. (2014). |
| Dunaliella tertiolecta Butcher | 11.44 | 0.42 | 0.0164 | Sidney et al. (2010) |
| Nannochloropsis oculara (Droop) D.J. Hibberd | 50.40 | 0.497 | 0.151 | Sirin et al. (2015) |
| Neochloris oleoabundans S. Chantanachat et Bold | 29.00 | 0.98 | 0.1124 | Santos et al. (2013) |
| Tetradesmus obliquus (Turpin) M.J. Wynne (= Scenedesmus obliquus (Turpin) Kützing) | 49.60 | 0.45-0.55 | 0.151-0.193 | Feng et al. (2014). |
| Scenedesmus sp. | 16.60 | 0.174 | 0.0195 | Taher et al. (2014) |
| Tetraselmis sp. | 30.50 | 0.130 | 0.047 | Kim et al. (2016) |

***Bioreactor***

*Chlorella vulgaris* microalgae are a type of algal cells from a genus of green algae of the *Chlorococcales* class. To carry out the production of biodiesel and bioethanol, open and closed systems are used by means of photobioreactors (PBR), where different

containers and configurations can be used. Closed systems are those that are found as

tubular (Figure 1a) and column (Figure 1b) PBR. However, they have a high cost in their construction and operation, but they have the advantage of controlling and improving the culture conditions, besides reducing the risks of contamination by other microorganisms; and providing a higher biomass productivity and increasing the efficiency in the use of light.

a)  b) 

*Figure 1.* a) Tubular Photobioreactors, b) Column Photobioreactors.

While open systems are more commonly used for commercial production of biomass from microwaves because of their speed and ease of maintenance. These types of systems operate at a low cost; however, their disadvantage lies in water losses due to evaporation, limited light penetration, high production periods, limited control of growing conditions, and limited $CO_2$ transfer due to its low concentration in the air.

However, there is an alternative for biofuel production by *Chlorella vulgaris*, where even 2 L plastic bottles can be used. As well as glass PBR of larger volume, (5, 10, 20) L, for extensive processes in the creation of biofuels (Figure 2).

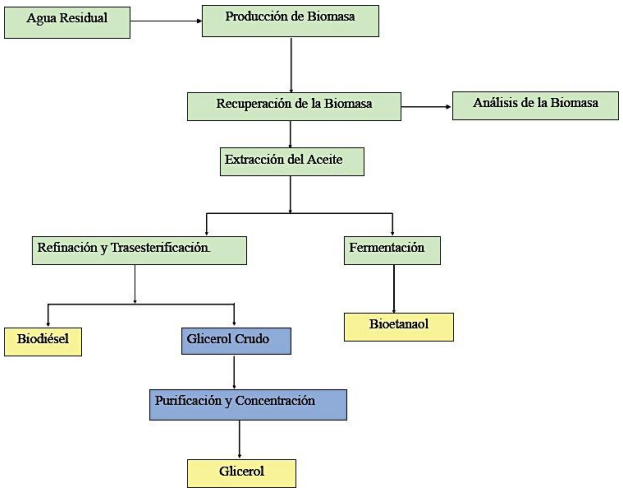


*Figure 2.* General Operation Methodology for the Production of Biodiesel and Bioethanol.

### *Aeration Process*

Aeration is of great importance for the production of microalgae, in this process all nutrients are homogenized. Therefore, a well distributed mixing must be carried out, and sedimentation of the algae cells must be avoided as excessive agitation can cause hydrodynamic stress and as a consequence a decrease in the growth rate. This water treatment consists of requiring an oxygen source, commonly known as aerobic biological water purification (Plata, Kafarov and Moreno 2009).

For the realization of biofuels such as bioethanol and biodiesel, any kind of electric light is needed for the motivation of algae reproduction, so the process is shaped by rotameters that control the aeration without affecting their growth. Therefore, the mixing is done by injecting air while stirring by bubbling, where by gravity the cells descend and by the injection of air rise. The mixing is continuous during the cultivation process, ensuring the homogeneity of the cells and nutrients that are inside the culture; in such a way that the gradients of light, nutrients, and temperature are eliminated.

***Culture Medium***

There are several factors that influence the cultivation process of the microalgae *Chlorella vulgaris*, so it is important to know and identify the optimal conditions both individually and as a whole that have tolerance microalgal strains. Therefore, to achieve an actively growing microalgae culture, it is necessary to have a viable inoculum, a minimum supply of nutrients, and adequate chemical and physical conditions. These conditions are:

Illumination is divided into two important components: irradiance and photoperiod. The former refers to the flux of light in which the microalgae are exposed to, while the latter term indicates the number of hours in which the microalgae are subjected to irradiance. Algae adapt to changes in light by varying the chlorophyll content of their cells, so that algae adapted to low light levels have a faster response to changes in light intensity because they have more chlorophyll than those adapted to high light intensities (Plata, Kafarov and Moreno 2009). Photosynthetic organisms only use the fraction of the sunlight spectrum that is photosynthetically active, i.e., between 350 and 700 nm. This photosynthetically active fraction accounts for 40% of the total radiation from the Sun. Most natural plant ecosystems have an efficiency of about 1% in terms of conversion of light energy into biomass. Light-biomass conversion efficiencies between 1 and 4 % have been demonstrated for microalgae in open systems and even higher in closed photobioreactors (González et al 2019).

Another influential factor is the temperature. During the cultivation process, three types of temperature are considered. The minimum temperature is the one that is below the optimum; therefore, it is not possible for growth to occur as well as the maximum which is above and is around 35ºC. While the optimum temperature is between 16 and 27ºC; however, this varies depending on the type of microalgae. Nevertheless, temperature changes can cause alterations in metabolic pathways, affecting the growth and development of crops as it dissociates the carbon molecules of the medium, making them available for photosynthesis.

Also, microalgae need a pH for their growth, the pH range for most microalgae cultures is between 7 and 9. An optimal pH in the culture is usually maintained by aeration with $CO_2$-enriched air. In the case of high cell density cultures, the addition of carbon dioxide corrects an increase in pH, which can reach a limit value of 9 for microalgae growth. A high or low reduction of pH decreases microalgae growth by the breakdown of many cellular processes. The optimum range can be between 8.2 to 8.7. The pH can be controlled by addition of $CO_2$. There is also an increase in pH with age or time of culture, and the photosynthetic process of $CO_2$ fixation causes a gradual increase in pH in the medium due to accumulation of $OH^-$ (Gonzalez et al 2019).

Therefore, the microalgae will be in a culture preservative for reproduction of more cells (Figure 3a), and by means of cells take inocula from 500 mL of solution with constant agitation for 5 days in order to determine certain amounts of cells (Santos,

Gonzalez and Martin 2014). At the end of the process, the *Chlorella vulgaris* undergoes transesterification and fermentation by aeration until it becomes biodiesel and bioethanol, substances used by society as fuel in cars, collection in household items among infinite actions of human daily life (Figure 3b).

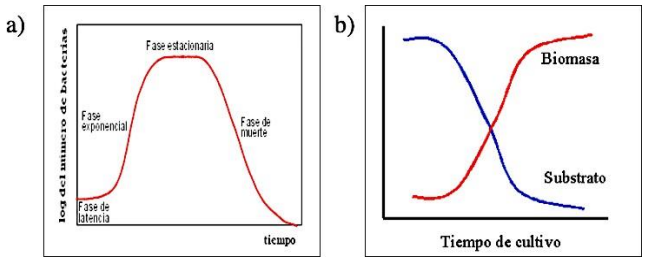


*Figure 3.* a) Growth Diagram of Microorganisms, b) Growth and Substrate Decrease.

***Transesterification or alcoholysis***

Transesterification or alcoholysis is the chemical reaction that occurs between oils and an alcohol (commonly methanol or ethanol) to produce glycerol and alkyl fatty acid esters, which are known as biodiesel. The main factors influencing the process are the alcohol: triglyceride molar ratio, the type of catalyst (alkali, acid, lipases), the temperature, the reaction time, and the content of water and free fatty acids in the feedstock. Currently, most biodiesel is produced by alkaline transesterification because of its speed and moderate operating conditions (Gonzalez 2015).

The use of a catalyst is required to improve the conversion, which can be acidic or basic, homogeneous or heterogeneous. Homogeneous catalysis has so far been the most widely implemented industrially. H2SO4 is most frequently used in acid catalysis; however, in addition to the difficulties arising from corrosion of the equipment involved in the process, high molar ratios are needed to achieve significant conversions [Robles et al. 2019, Conde et al. 2015]; therefore, basic catalysts are preferred. Among them, the most commonly used is NaOH at levels ranging from 0.75 to 1.5% w/w based on oil weight. However, in the particular case of ethanolysis, the recommended levels range from 0.4 to 0.8% (Figure 4), (Robles et al. 2019).

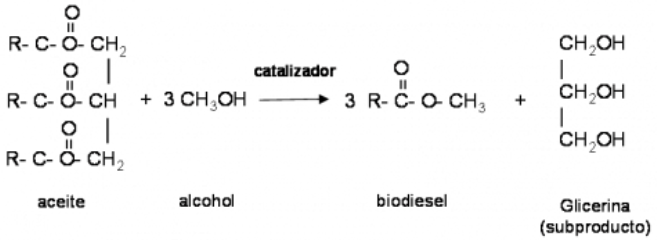


*Figure 4.* Chemical reaction of the transesterification process.

***Fermentation***

Microalgae are rich in carbohydrates, by this means they are used as carbon sources to obtain one of the biofuels such as bioethanol. By fermenting the biomass these algal organisms can be converted favorably with biomasses obtained from food crops such as sugar cane or corn. Bioethanol from microalgae can be obtained by two technologies: fermentation, which involves the conversion of biomass materials containing sugars into ethanol by yeasts; and gasification, which involves converting the

biomass into a synthesis gas, which is then converted into ethanol by catalysis. A simplified diagram of the bioethanol production process, with fermentation and distillation as key steps, is shown in Fig. 5.

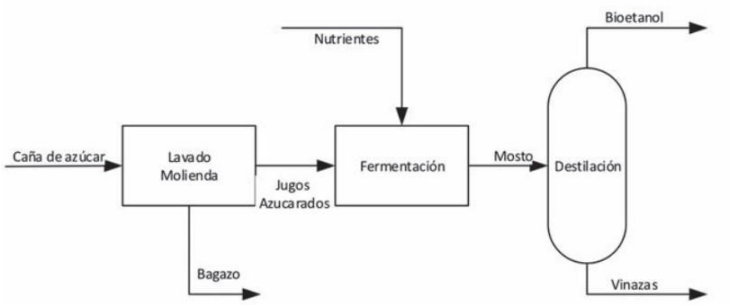


*Figure 5.* Diagram of the bioethanol production process.

## Results

Currently, the economic and environmental factors are of high indexes; therefore, an alternative fuel of low environmental impact without affecting the economy is sought. Fuels such as biodiesel and bioethanol from microalgae, are emerging as substitutes for fossil fuels since they have advantages mainly due to their biodegradability and minimal toxicity. In addition, during their combustion, they produce components of lower emissions such as sulfates, aromatic compounds, and carbon dioxide.

*Chlorella vulgaris* strains were analyzed to obtain biodiesel and bioethanol in the Biotechnology laboratories of the Universidad Autónoma del Carmen (UNACAR), using an ecological PBR operation system. The bubbling column was replicated using PET bottles with a volume of 3 L, in addition to a bottle with a volume of 1 L for the distilled water used to hydrate the air by means of agitation and avoid evaporation of the culture medium (Figure 6a). In the same way, two other bottles with 20% chlorinated water were used to avoid possible contamination to the outside.

The function of the system in general had a plastic hose to a blower and at the other end a rotameter at the bottom. While at the top of the rotameter was connected another plastic hose 1 m long and its end was introduced into one of the holes of the bottle with distilled water; then a segment of plastic hose 0.7 m long was used and introduced into the second hole of the bottle of distilled water with a depth of 3 cm inside the container, the other end of the 0.7 m hose was inserted into the PBR with the culture medium; another 0.7 m long hose was taken and inserted through one of the holes in the lid of the PBR to capture the expelled air and transferred to a chlorine solution contained in a second bottle.

The cultures were kept in acclimatization for 30 days in 250 ml Erlenmeyer flasks with a constant illumination of cold white, fluorescent light lamps that helped the motivation of algae reproduction (Figure 6d), in such a way that the process is conformed by rotameters that control the aeration without affecting their growth (Figure 6c). Enriched culture media were prepared at 90 mg L-1 (C90): 4 L of fresh medium were prepared, 3 mL of salts were added, 3 mL of trace metals (f/2 medium of Guillard and Ryther) per L of solution; the medium was sterilized in autoclave at 120ºC and 30 atm, it was left to cool to add 3 mL of vitamins per liter of water.

During the process the exhaust air is bubbled in chlorinated water in order to avoid contaminating the exterior (Figure 6b). Once the biomass is obtained, the product is recovered for oil extraction. When the oil is extracted, a certain percentage of solvent

is added to perform a transesterification process in order to obtain biodiesel, where a chemical reaction occurs between vegetable oil and alcohols, mainly influenced by the alcohol molar ratio factor such as triglycerides generated by microalgae; once the substance is catalyzed, alkyl esters of fatty acids and glycerol are obtained. While by a fermentation process, bioethanol is obtained as a result with the help of yeast as sugars from the biomass of microalgae. The world's population must solve problems related to energy shortages, hence the importance of exploring new sources of renewable energy. Thus, algal biomass could satisfy about 25% of the world's energy needs, also providing other biotechnological products (Santos, González and Martín 2014).

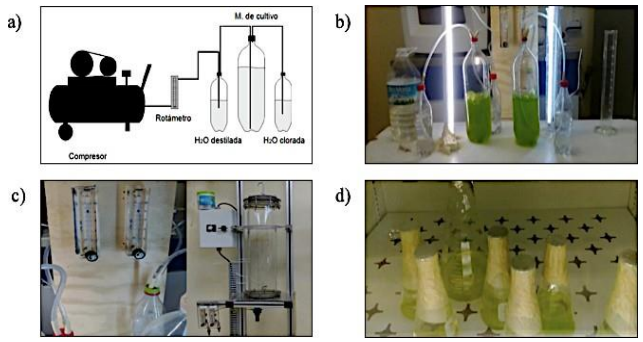


*Figure 6.* a) Mode of operation of a PBR for the culture system; b) *Chlorella vulgaris* transesterification method; c) Rotameter and Reactor equipment; d) *Chlorella vulgaris cultures.*

## Discussion and conclusions

The analyses obtained from the microalgae *Chlorella vulgaris,* examined in the UNACAR facilities, are identified as one of the best microorganism producers of triglycerides mainly used to obtain biodiesel and bioethanol. However, nowadays, more research is necessary to determine the best cultivation method and, with it, to obtain a higher balanced yield of biomass and lipids, as a base to obtain these bi-fuels, as well as to improve the technology in order to carry out an ideal harvesting process, which is the point of higher cost in relation to the cultivation processes of these microorganisms.

At the industrial level, large-scale microalgae cultivation has proven to be effective and efficient. Therefore, the dissemination of this technology is of a scientific scope and fundamental to continue its development. However, there are certain limitations, such as the laws that allow the use of wastewater for cultivation. Another influencing factor is land use since it is of utmost importance to determine the location of the crop plant.

Microalgae are the main source of renewable energy as in human and animal nutrition. There are several extraction methods used in microalgae such as physical, mechanical, and chemical. However, the chemical extraction method is one of the fundamental processes for microalgae, since a higher yield is obtained due to the presence of organic solvents that offer a higher extraction of lipids present in the microalgae *Chlorella vulgaris*.

Unlike fossil fuels, fuels obtained from microalgae, or also defined as biofuels, have a high capacity to capture greenhouse gases, the main problem that currently affects the environment. Carbon dioxide (CO2) is one of the components that affects the ozone layer, and that most of the items or products used by humans produce it; therefore, microalgae are excellent biomitigators of CO2 and wastewater treatment.

In the case of biodiesel and bioethanol production, it has been of great help in reducing greenhouse gas emissions produced during the combustion of fossil fuels. In addition to avoiding the use of crops traditionally used for human consumption as raw material. In spite of the current adversities to acquire this type of oil, the development of a well elaborated transesterification or fermentation methodology allows the obtaining of oil from *Chlorella Vulgaris* to be encouraging.

In Mexico, this type of productions to obtain biodiesel and bioethanol from microalgae is of great importance if they are considered a good development of technology and energy. Therefore, in the future, these factors will give satisfactory answers to the ecology and potentially sustainable to the requirement of liquid fuels produced, increasing the economy in our country.

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# OBTAINING FATTY ACIDS OF METHYL ESTERS IN ALGAL BIOMASS AT DIFFERENT AERATION RATES IN COLUMN FBR

**Juan Carlos Robles Heredia**
Universidad Autónoma del Carmen (Mexico)
jrobles@pampano.unacar.mx · https://orcid.org/0000-0003-2591-6528
**Asteria Narvaez Garcia**
Universidad Autónoma del Carmen (Mexico)
anarvaez@pampano.unacar.mx · https://orcid.org/0000-0001-6484-6062
**Alejandro Ruiz Marin**
Universidad Autónoma del Carmen (Mexico)
aruiz@pampano.unacar.mx · https://orcid.org/0000-0001-5075-9611

**Abstract**. Fossil fuels contribute to air pollution, due to the compounds that are released into the atmosphere during combustion; For this reason, they have been proposed to replace them with so-called biofuels, such as biodiesel, which is the mixture of fatty acid methyl esters (FAME) for its acronym in English, which can replace diesel and is obtained from different raw materials, such as biomass. . The variation of cell growth, nitrogen consumption, lipid productivity and biodiesel quality was analyzed depending on the type of FAME of Chlorella vulgaris due to the effect of hydrodynamics in column photobioreactors (FBRC), varying aeration flows at (0.75, 1.25, 1.75, 2.25) vvm and continuous white light; the shear rate was analyzed to verify the probable presence of hydrodynamic stress. The shear rate data were low (26.34 to 45.60) $s^{-1}$ , while the maximum values of cell growth and specific growth rate (μ) were (6.80 x $10^6$ cell $mL^{-1}$ and 0.023 $d^{-1}$ ), respectively, on the other hand, nitrogen consumption was 65% at 0.75 vvm and lipid productivity was 15.92 mgL-1d-1 at 1.25 vvm. In relation to the FAME, a greater presence of polyunsaturated fatty acids (PUFA) was observed at 0.75 vvm, 1.75 vvm and 2.25 vvm; while at 1.25 vvm, they were saturated (SFA); the highest amount of monounsaturated (MUFA) was at 0.75 vvm. The components with the greatest presence were C12:0; C20:5N3; C24:1; C22:0; C22:2.

**Keywords:** FAME, aeration, Chlorella vulgaris photobioreactor, hydrodynamics

# OBTENCIÓN DE ACIDOS GRASOS DE METIL ESTERES EN BIOMASA ALGAL A DIFERENTES TASAS DE AIREACION EN FBR DE COLUMNA

**Resumen**. Los combustibles fósiles contribuyen en la contaminación del aire, por los compuestos que se liberan a la atmosfera durante la combustión; por esta razón se han propuesto reemplazarlos por los llamados biocombustibles, como el biodiesel que es la mezcla de esteres metílicos de ácidos grasos (FAME) por sus siglas en inglés, que puede sustituir al diésel y se obtiene de diferentes materias primas, como la biomasa. Se analizó la variación de crecimiento celular, consumo de nitrógeno, productividad de lípidos ycalidad del biodiesel en función del tipo de FAME de *Chlorella vulgaris* por efecto de la hidrodinámica en fotobiorreactores de columna (FBRC), variando flujos de aireación a (0.75, 1.25, 1.75, 2.25) vvm y luz blanca continua; además de la tasa de corte para comprobar probable presencia de estrés hidrodinámico. Los datos en tasa de corte fueron bajos (26.34 a 45.60) $s^{-1}$, mientras que los máximos valores de crecimiento celular y tasa de crecimiento específico (μ) fueron de (6.80 x $10^6$ cel $mL^{-1}$ y 0.023 $d^{-1}$), respectivamente; por otra parte, el consumo de nitrógeno fue de 65% a 0.75 vvm y productividad de lípidos de 15.92 $mgL^{-1}d^{-1}$ a 1.25 vvm. En relación a los FAME, se observó mayor presencia de ácidos grasos poliinsaturados (PUFA) a 0.75 vvm, 1.75 vvm y 2.25 vvm; mientras que a 1.25 vvm, fueron saturados (SFA); la mayor cantidad de monoinsaturados (MUFA) fue a 0.75 vvm. Los componentes con mayor presencia fueron C12:0; C20:5N3; C24:1; C 22:0; C22:2.

**Palabras clave:** FAME, aireación, Chlorella vulgaris fotobiorreactor, hidrodinámica

## Introduction

Reducing the use of fossil fuels is very important to reduce the environmental pollution problem they represent (Castillo et al., 2017). It is important to reduce the use of fossil fuels as they cause emissions of $CO_2$ , $CH_4$ , $N_2O$, nitrogen oxides (NOx), carbon monoxide (CO), non-methane volatile organic compounds (NMVOCs), as well as sulfur dioxide ($SO_2$), causing damage to the atmosphere, (Chandrasekhar et al., 2015; Kumar et al., 2017; Kumar-Enamala et al., 2018). Hence, the importance of looking for other renewable energy alternatives (Castillo et al., 2017; Anto et al., 2020).

Biofuels obtained from biomass, such as biodiesel, are alternative energy sources that have received more attention for research and use (Qaria et al., 2017; Ashok et al., 2019). Globally, microalgae biomass could satisfy up to 25% of energy needs due to the characteristics of the methyl ester fatty acid compounds they can produce (Castillo et al., 2017). Because of the applications for $CO_2$ sequestration, producing biofuels, human, and animal food, in addition to the use in the production of high-quality biomolecules, the use of microalgae is to be taken into account (Posten and Feng-Chen, 2016; Alishah et al., 2019; Gomez-Luna et al., 2022).

Despite the considerable number of microalgal species existing in different habitats, approximately 30,000 species have received more attention (Richmond 2004; Agarwal et al., 2018; Chew et al., 2018). Being photosynthetic microorganisms, microalgae can coexist in diverse natural habitats; however, some microalgae can reproduce mixotrophically or heterotrophically (Castellanos et al., 2020). Certain microalgae contain large amounts of lipids, which can be increased by varying different factors such as light intensity and type, temperature variations, salinity, agitation intensity, etc. (Posten and Feng-Chen 2016, Basto-Flores et al., 2022).

Some microalgae can accumulate high amounts of triglycerides (TAG), Kumar et al., (2018), which are the feedstock for producing biodiesel. The amount of biodiesel

using microalgae biomass does not only depend on the amount of biomass, but also on the amount of oil contained per cell (Wu et al., 2017; Chew et al., 2018). Due to its high adaptability to different conditions during cultivation, including wastewater, the microalga *Chlorella vulgaris* has been used for experimentation in the production of biodiesel and other widely used compounds (Li et al., 2012; Zhan et al., 2016).

For the cultivation of microalgae, open systems can be used in the form of ponds or closed systems or photobioreactors (FBR) to better control the cultivation process, amount of lipids, and other products for use in other areas (Kumar et al., 2018). Both types of culture systems present advantages and disadvantages, thus, of the main advantages of open systems is their low cost due to the materials used for their construction. However, the main problem they present is contamination by microorganisms due to their exposure to the environment; while FBRs present other characteristics, internal or external lighting, different configurations, better control of the variables of the type of culture process, and exhibit higher productivities (Ashok et al., 2019); due to these characteristics their main disadvantage is their cost; hence, the need to improve their design. Different aeration rates can be used in FBRs that can influence the growth of microalgae during the cultivation process, mixing helps the cells to have access to the light source and avoids oxygen accumulation in the medium, preventing microalgae from adhering to the walls or precipitating. (Deconinck et al., 2018; De jesus et al., 2019). Some considerations in FBR design are efficiency to harness light energy, easy scale-up, efficient mixing, and better control of side reactions, suggested under cellular hydrodynamic stress.

Among the hydrodynamic conditions of importance for a good performance of column FBRs are gas hold up (remaining gas), liquid and gas surface velocities inside the reactor, in addition to estimating the shear rate (Beal et al., 2015; Gonzalez-Lazo et al., 2019). An adequate aeration rate is necessary, with the idea of avoiding cell sedimentation and cell death due to the absence of light. Similarly, there is an upper limit to the acceptable level of turbulence since hydrodynamic forces have a stimulating effect on the physiological processes of algal cells. A progressive increase in turbulence, in some microalgae favors an increase in growth rate as agitation favors the supply of light and $CO_2$. However, with high levels of turbulence, growth is decreased, in addition to simultaneously increasing the surface gas velocity causing possible cell damage (Trivedi et al., 2015; Gonzalez-Lazo et al., 2019). Shear stress is one of the main problems in microalgae cultivation due to cell damage. Excessive agitation causes turbulence, affecting cell structure, decreasing growth, and metabolite production. Conversely, insufficient agitation causes sedimentation and cell death (Robles-Heredia 2014, Montoya 2021). Hydrodynamic stress due to mechanical agitation and bubble rupture in FBRs can affect the growth and metabolism of microalgae (Arguelles et al., 2018; Alishah et al., 2019).

In this work, the variation of cell growth, nitrogen consumption, lipid productivity, and biodiesel quality was analyzed as a function of the type of FAME of *Chlorella vulgaris*, by the effect of hydrodynamics in column photobioreactors (FBRC), varying aeration flows at (0.75, 1.25, 1.75, 2.25) VVM and continuous white light; in addition to the cut-off rate to check for the probable presence of hydrodynamic stress. Hence, the interest in experimenting if at higher aeration flow and with discrete illumination, greater amounts of biomass and lipid production could be achieved in the microalgae.

## Method

With the following methodological process, different determinations were carried out to know the variation of cell growth, nitrogen consumption, lipid productivity, and biodiesel quality as a function of the type of methyl ester components present FAME of *Chlorella vulgaris* in column photobioreactors (FBRC), alternating aeration flows of (0.75, 1.25, 1.75, 2.25) VVM and continuous white light.

### *Strain adaptation*

The strain comes from the Ensenada Center for Scientific Research and Higher Education (CICESE), Mexico. It was acclimatized for 30 days in 250 mL Erlenmeyer flasks, for illumination cold white light was used with fluorescent lamps, with emission at 100 µmol $m^{-2}$ $s^{-1}$, continuously. Transfers were made to 250 mL Erlenmeyer flasks.

### *Culture medium*

For the medium at 90 mg $L^{-1}$ of $N\text{-}NH_4^+$ ($C_{90}$), 3 mL of nutrients were added per L of water with the following composition: 7 mg NaCl, 4 mg $CaCl_2$ , 2 mg $MgSO_4$ -$7H_2O$, 15 mg $KH_2PO_4$ , 115.6 mg $NH_4Cl$, dissolved in 1L of water, similarly trace metals and vitamins were added considering the technique for f/2 culture medium (Guillar and Ryther 1962), sterilization was in autoclave at 120º C and 30 atm; once the culture medium was cold, 4 mL of vitamins were added per liter of medium (Robles-Heredia, 2014).

### *Inoculum*

For the inoculum, 500 mL of solution were used at $C_{90}$. Maintaining constant agitation for 5 days to obtain a concentration of $1x10^6$ cells $mL^{-1}$ (cell x mL).

### *Cell growth*

To verify cell development, every 24 h a 0.1 mm Neubauer Hematocytometer chamber was used to count the cells; with high cell densities, the sample was diluted 1:10 mL to facilitate counting, then the total number of cells counted was multiplied by a factor of 10 according to the dilution, taking into account equations (1 and 2) as appropriate (Ruiz-Marín et al., 2010):

$$Cc = \text{Cells counted} \times 10{,}000 \quad (1)$$

$$Ccd = \text{Cells counted} \times 10 \times 10{,}000 \quad (2)$$

Where: Cc represents the number of cells counted and Ccd represents the number of cells in dilution.

### *Biomass dry weight*

Ten mL of the medium with microalgae were filtered using a constant weight filter, the filter with the biomass was introduced in an oven for 24 h at 130º C. Afterwards, the filter was placed in a desiccator to cool, the weight of the dry sample was obtained by weight difference, considering the sample volume used. The process was performed every 24 h until the end of the process (Robles-Heredia, 2014).

### *Nitrogen consumption*

A 50 mL sample of culture medium was taken every 24 h, filtered, and 5 drops of $H_2SO_4$ were added to fix nitrogen, then the sample was divided into 2 Erlenmeyer flasks with 25 mL each, 5 mL of borate buffer, and 4 drops of NaOH 6N were added. Boric acid indicator solution was added in 2 flasks, each of 20 mL and 3 drops of Shiro Toshiro indicator were added. Subsequently, the samples were distilled in Buchi micro Kjeldahl equipment and 50 mL were collected in the flasks with the boric acid solution and titrated

with $H_2$ $SO_4$ 0.02 N until the solution turned from green to purple. The concentration of N-$NH_4^+$ was determined with the following equation (3) (Robles-Heredia, 2014):

$$N - NH_4^{"} = \frac{Volume\ o^*\ acid\ spent\ x\ 4.46Nx84}{Sample\ volume} x1000 \quad (3)$$

Where: N-$NH_4^+$ represents ammoniacal nitrogen concentration; 0.02N represents normal sulfuric acid; 14 and 1,000 represent constant values.

***Harvest***

After the cultivation process, the remaining volume was centrifuged at 10,000 rpm for 10 min to concentrate the microalgal biomass. The recovered biomass was frozen at -4.0°C for preservation, then freeze-dried for 3 to 5 days (Ruiz-Marín et al., 2010).

***Oil production***

The modified method of Bligh and Dyer (1959) was used: 10 mg of lyophilized biomass were placed in tubes with thread, 4 mL of methanol, 2 mL of chloroform, and 0.5 mL of distilled water. This mixture was subjected to ultrasound for 15 minutes to break the cell wall and covered with aluminum foil, incubated for 24 h at 4 ºC. Afterwards, the aluminum foil was removed to submit them to ultrasound for 5 min, and they were centrifuged at 4,000 rpm for 10 min, the remaining liquid was transferred to new tubes with screw cap, and 4 mL of water were added for washing, The water of the tubes was removed with a Pasteur pipette, the chloroform was vaporized in a water bath, and 2 mL of 95% hydrochloric acid-methanol mixture was added. They were placed in a Hach DRB 200 digester for 1 h at 100ºC. Afterwards, they were wrapped again in aluminum foil to be kept refrigerated for 24 h at 4 ºC. Then, 3 mL of hexane were added and shaken to form a bi-phase from which the lower part was extracted with a Pasteur pipette. 4 mL of water were added to the tubes and shaken again. The water was extracted using a Pasteur pipette. They were wrapped again in aluminum foil and kept refrigerated for 24 h at 4 ºC. After the estimated time, they were vaporized in a water bath. 3 mL of hexane were added during the vaporization process before completing vaporization. The samples were transferred to vials and left to rest for 24 h. The lipid content was translated into lipid content in the tubes. Lipid content is translated to lipid composition (% $ww^{-1}$) on a dry w basis. And this, in turn, is translated to lipid productivity $P_L$ (in mg $L^{-1}$ $d^{-1}$) with the following equation (4):

$$P_L = \frac{\leq_2 = _2 \geq \leq_1 = _1}{t_2 > t_1} \quad (4)$$

Where: $X_1$ and $X_2$ is the mass concentration of dry biomass in the medium at time $t_1$ and $t_2$ (initial and final). w lipid content on a dry basis. $P_L$ lipid productivity in units of mg $L^{-1}$ $d^{-1}$

***Obtaining fatty acid methyl esters.***

The profile of fatty acid methyl esters (FAME) was obtained using an Agilent Technology 7890 gas chromatograph. 1 µL of the hexane-lipid solution was injected into the chromatograph with a flame ionization detector (FID) and a DB-23 separation column (60 m length, 0.32 mm ID, 0.25 µm thickness). Chromatographic conditions: T of the detector: 250 ºC; T of the injector: 250 ºC; Oven temperature program: 120 ºC for 5 min, increase the temperature at a rate of 10 ºC min up to 180 ºC, hold for 30 min, increase the temperature again at a rate of 10 ºC min up to 210 ºC and hold for 21 min (total 65 min); Carrier gas flow: 15 psi; Split: 1:100; Carrier gas: He high purity. A fatty acid methyl ester mixture standard (weight %). SupelcoTM 37 FAME component was used to identify the FAME components. The results of the experimental design were estimated with full

factorial analysis of variance (ANOVA) (α: 0.05), using STATISTICA V7 software.

***Assembly of the Column Photobioreactors (FBRC)***

For the FBRC system, PET bottles with 2 L operating volume and others with 1 L volume were used; to avoid evaporation of the medium, distilled water was used to hydrate the injected air. The outgoing air was bubbled in water-chlorine to avoid external contamination; ¼ inch hoses were used for air injection. Cole Parmer vertical, transparent acrylic flowmeters for air control, Model ACRY-010052, air level 2-8 $Lmin^{-1}$, and strip-type dial; compressor of 2.5 Hp of power and 8.5 kg $cm^{-2}$ of pressure to inject air. External illumination with cold white fluorescent light at 100 µmol $m^{-2}$ $s^{-1}$. Figure 1 shows certain variables for hydrodynamic calculations.

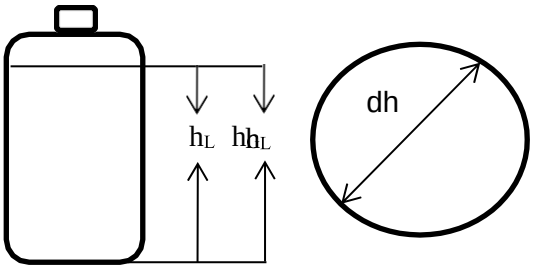


*Figure 1.* Bubbling column equipment (FBRC)

Where the indicated variables are as follows: $h_L$ is the height of the liquid at rest without air inlet (m); $h_G$ is the height of the column including gas retention (m); dh is the diameter of the bubbling column (m); Ac is the cross-sectional area of the column ($m^2$); and ρL is the density of the liquid.

***Aeration***

Four aeration rates (0.75, 1.25, 1.75, 1.75. 2.25) vvm per experiment were considered, with one response variable (lipid productivity), one configuration (FBRC), and two replicates.

To calculate the shear rate the column height data ($h_G$) were (0.21, 0.22, 0.23, and 0.24) for each aeration flow, respectively; the following data were considered as constants for all experiments: $h_L$ = 0.205 m; do = 0.12 m; $Ac$ = 0.036 $m^2$; ρL = 998kg / $m^3$.

Equation (5) is considered to calculate the total air flow corrected by the absolute pressure at the bottom of the reactor (Robles-Heredia, 2014).

$$F_g = F_a \frac{*8}{@4} + \frac{*8}{8444} + \qquad (5)$$

Where: Fg is the total air flow expressed in ($m^3$ $s^{-1}$) and Fa the supplied air flow in (L $min^{-1}$)

Equations (6) and (7) were used to calculate the column sectional area (Ac) in ($m^2$) and the gas surface velocity (Ug), which is the gas flow per unit area within the system in ($ms^{-1}$), according to Babcock et al, (2002).

$$Ac = b \; x \; h \qquad (6)$$

$$U_g = \frac{Fg}{Ac} \qquad (7)$$

With $Ug$ for bubble column, the shear rate ($\gamma$) expressed in ($s^{-1}$) is calculated with equation (8) valid in the range of 0.008 < $U_g$ <0.09 $ms^{-1}$. (Cerri et al., 2008).

$$\gamma = 1000 U_g^{4.C} \qquad (8)$$

The retained gas ε is calculated with equation (9); it determines the percentage of gas or air retained inside the equipment by the increase in air volume when air is injected (Doran, 1995).

$$\varepsilon = \frac{D_G \% D_L}{D_G} \quad (9)$$

Equation (10) is used to calculate the surface velocity of the liquid, which is the liquid flow per unit area within the system. It can be calculated for bubbling columns with a diameter between 0.1 and 7.5 m and $0 < U_g < 0.4$ ms$^{-1}$ (Doran, 1995).

$$U_L = 0.95 g d o U_g 9^{4.EE} \quad (10)$$

Where $U_L$ is the surface velocity of the liquid in (m s$^{-1}$), g is the acceleration of gravity in (ms$^{-2}$), do is the diameter of the column in (m), and $U_g$ is the surface velocity of the injected gas or air.

Equation (11) calculates pneumatic power, power generated by the air injected to the equipment for agitation of the fluid inside the FBRC. (Doran, 1995):

$$\frac{PG}{VL} = \rho_L g U_g \quad (11)$$

Where: PG/VL is the pneumatic power in (Wm$^{-3}$), calculated with ρL, which is the density of the liquid in (kg / m$^3$), g is the gravity in (ms$^{-2}$), and $U_g$ the surface velocity of the gas or air injected. The use of different aeration flows was with the purpose of establishing the best aeration conditions, defining Cutoff Rate ($\gamma$) in order to verify the effect on methyl ester fatty acids, cell development, biomass production, nitrogen depletion, and lipid productivity.

## Results and discussion

The results obtained from this work are shown below.

Table (1) shows the hydrodynamic calculations of the FBRC at the proposed aeration rates.

Table 1
*Hydrodynamic data*

| Team | Aeration rate (vvm) | $F_a$ (Lm$^{-1}$) | $F_g$ x 10$^{-5}$ (m$^3$ s$^{-1}$) | $U_g$ x 10$^{-3}$ (ms$^{-1}$) | ε (%) | $U_L$ x 10$^{-2}$ (ms$^{-1}$) | PG/VL (Wm$^{-3}$) | γ (s$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **FBRC** | 0.75 | 1.5 | 2.50 | 0.694 | 2.38 | 9.57 | 6.80 | 26.34 |
| | 1.25 | 2.5 | 4.16 | 1.150 | 6.81 | 10.18 | 11.26 | 33.91 |
| | 1.75 | 3.5 | 5.83 | 1.620 | 10.86 | 11.40 | 15.86 | 40.25 |
| | 2.25 | 4.5 | 7.50 | 2.080 | 14.58 | 12.40 | 20.36 | 45.60 |

It can be indicated that the algal cells did not present any type of deformation to the aeration conditions raised; similar aspects were reported by (Shi et al., 2016; Sadeghizadeh et al., 2017); as observed in Table (1), the values obtained from the hydrodynamics of the FBRCs, as the aeration flows increased, all the hydrodynamic parameters also increased which indicated the close relationship in each of them, besides the possible affectation with the other cellular parameters raised in this work.

Table (2) indicates cell growth, specific growth rate (μ), and nitrogen consumption obtained at the different suggested aeration flow rates.

Table 2
*Cell growth, specific growth rate (μ), and nitrogen consumption obtained at different suggested aeration flow rates*

| Team | Aeration rate (vvm) | Max cell growth * (cell×$10^6$ $mL^{-1}$) | $\mu$ * ($h^{-1}$) | Consumption* N-$NH_4^+$ (%) |
|---|---|---|---|---|
| FBRC | 0.75 | 6.80 ± 0.03[a] | 0.0230± 0.09[a] | 65.00± 0.08[a] |
| | 1.25 | 3.10 ± 0.13[b] | 0.0170± 0.06[b] | 53.00± 0.07[b] |
| | 1.75 | 2.51 ± 0.12[b] | 0.0165± 1.70[b] | 50.00± 0.93[b] |
| | 2.25 | 2.49 ± 0.03[b] | 0.0160± 0.50[b] | 47.00± 0.05[b] |

*Note:* * Different letters in the same column indicate significant differences according to Tukey test ($p \geq 0.05$); (± Standard deviation).

On the other hand, according to the maximum cell growth values in Table (2), it can be indicated that in relation to cell growth at 0.75 vvm, cells adapted to increase their development, reaching 6.80 x $10^6$ cell $mL^{-1}$ and the highest specific growth rate μ (0.023 $h^{-1}$); however, as the aeration rate was increased, cell growth was reduced more than 50% (3.10 x $10^6$) cell $mL^{-1}$ as well as the specific growth rate in all experiments. It can be mentioned that the obtained results of growth and nitrogen removal (N-$NH_4^+$) were well below other works (Kee-Lam et al., 2016; Sadeghizadeh et al. 2017, Anto et al., 2020; Montoya, 2021).

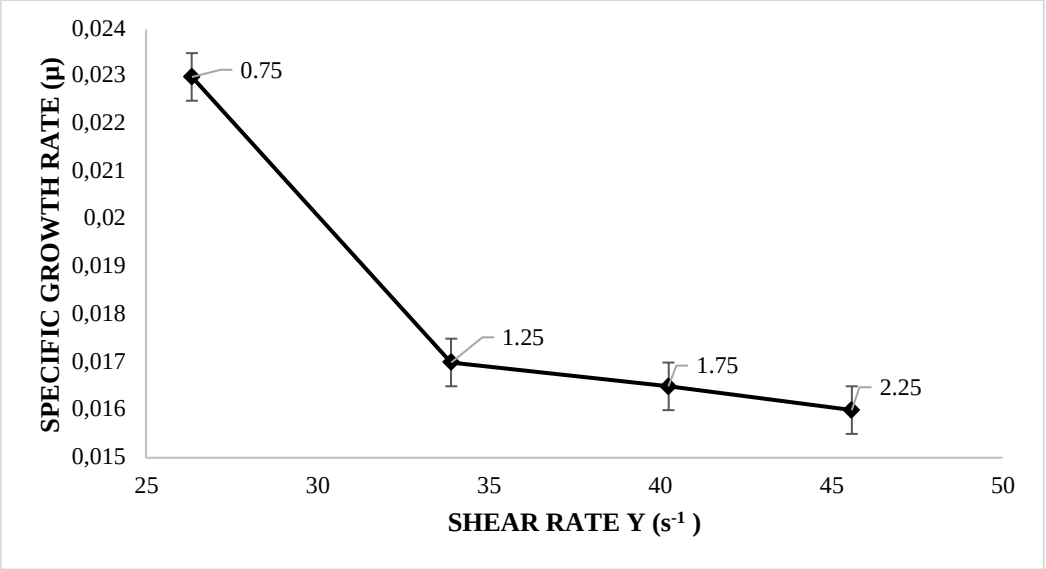


*Figure 2.* Growth rate μ *vs.* shear rate $\gamma$

According to Figure 2, it can be seen that as the aeration flow increased, the values of the shear rates increased in each experiment, which may indicate that there was a direct relationship between these two parameters; however, the opposite occurred with the specific growth rate (μ). Therefore, it can be inferred that there was an inversely proportional relationship with these two aspects, the higher the aeration, the higher the shear rate ($\gamma$), but lower the cell growth rate (μ); thus, it can be considered that the optimal air flow would be at 1.25 vvm to perform other experiments, depending on the product to be obtained, considering other factors of operation or illumination.

Table (3) shows the data of biomass dry weight, lipid content, and lipid productivity in *Chlorella vulgaris* at the proposed aeration rates.

Table 3
*Dry biomass X, lipid content w, and lipid productivity $P_L$ at the proposed aeration rates.*

| Team | Aeration rate (vvm) | $X$ ($g\ L^{-1}$) | $w_{max}$ (%$ww^{-1}$) | $P_L$ maximum ($mg\ L^{-1}\ d^{-1}$) |
|---|---|---|---|---|
| **FBRC** | 0.75 | 0.295±0.056[a] | 11.54 ± 0.03[a] | 3.99 ± 0.026[a] |
| | 1.25 | 1.395±0.010[b] | 14.28 ± 0.02[b] | 15.92 ± 0.019[b] |
| | 1.75 | 0.430±0.041[a] | 11.11 ± 0.02[a] | 7.28 ± 0.016[a] |
| | 2.25 | 0.405±0.050[a] | 10.00 ± 0.04[a] | 1.58 ± 0.016[a] |

*Note:* [*]Different letters in the same column indicate significant differences according to Tukey test ($p \geq 0.05$); (± Standard Deviation)

Generally speaking, in this work, the values achieved for productivities were low, compared to other reports (Al-Ameri and Al-Zuhair 2019; De Jesus et al., 2019). However, at 1.25 vvm, the highest lipid productivity was observed (Table 3); even though at 0.75 vvm the highest cell growth was obtained, it can be indicated then that the cells at 1.25 vvm resented the degree of agitation, probably a slight stress occurred due to shear rate, which induced them to produce higher lipid content, increasing productivity. However, as the aeration rate increased, unfavorable conditions for lipid production were presented. Other reports indicate data of 1.2 g $L^{-1}$ of biomass at different degrees of agitation, using light intensities greater than or close to 300 µmol $m^{-2}$ $s^{-1}$ (Chiu et al., 2008, Montoya, 2021), while Pham et al., (2017) report 1.35 g $L^{-1}$ of biomass at 0.3 vvm, with illumination of 300 µmol $m^{-2}$ $s^{-1}$; so, it can be inferred that the amount of light provided favors more in obtaining biomass than aeration. Similarly, other studies have reported that at illumination higher than 150 µmol $m^{-2}$ $s^{-1}$, higher biomass increases were achieved (Jiang et al., 2016; Kim et al., 2015; Alishah et al., 2019; Castellanos et al., 2020).

Table (4) shows the main fatty acids of methyl esters produced by the microalgae, taking into account the culture conditions proposed.

Table 4
*Main fatty acids of methyl esters produced by microalgae (in % of total weight of fatty acids*)*

| FAME | 0.75 vvm | 1.25 vvm | 1.75 vvm | 2.25 vvm |
|---|---|---|---|---|
| C12:0 | 23.81 (±0.38) | 22.04 (±0.71) | 23.73 (±2.02) | 20.91 (±5.08) |
| C13:0 | 0.96 (±0.27) | 0.93 (±0.14) | 0.39 (±0.02) | 0.84 (±0.69) |
| C18:0 | 1.78 (±0.07) | 1.48 (±0.038) | 1.6 (±0.13) | 1.61 (±0.10) |
| C20:0 | 0.93 (±0.04) | n.a. | 0.21(±0.11) | 0.44 (±0.005) |
| C21:0 | n.a. | n.a. | 0.27(±0.04) | 0.23(±0.07) |
| C22:0 | 5.25 (±6.95) | 13.36 (±2.25) | 8.83 (±8.73) | 10.54 (±11.32) |
| C23:0 | 0.58 (±0.006) | 1.23 (±0.079) | 1.22 (±0.061) | 1.49 (±0.25) |
| C17:1 | 0.85 (±0.11) | 0.18 (±0.05) | 0.28 (±0.45) | 0.49 (±0.073) |
| C18:1N9T | 0.06 (±1.09) | n.a. | n.a. | n.a. |
| C20:1 | 1.84 (±0.10) | 0.5 (±0.31) | 0.82 (±0.074) | 1.28 (±0.15) |
| C22:1N9 | 2.01 (±0.11) | 1.77 (±1.63) | 2.6 (±0.20) | 2.39 (±0.51) |
| C24:1 | 17.22 (±2.71) | 7.55 (±1.63) | 11.81 (±0.48) | 13.1 (±5.41) |
| C18:2N6T | n.a. | n.a. | n.a. | 0.27(±13.06) |
| C20:2 | 7.82 (±0.63) | 0.46 (±0.053) | 7.48 (±8.20) | 8.31 (±9.03) |
| C20:3N3 | n.a. | n.a. | 0.34(±10.30) | 0.74(±7.04) |
| C20:3N6 | 0.22 (±0.14) | 0.17 (±0.093) | 0.24 (±0.13) | 0.25 (±0.16) |
| C20:4N6 | 3.32 (±0.2) | 2.41 (±0.40) | 2.98 (±0.13) | 2.46 (±0.88) |
| C20:5N3 | 21.71 (±2.51) | 15.34 (±3.02) | 24.14 (±0.81) | 24.38 (±8.37) |
| C22:2 | 8.35 (±0.31) | 9.66 (±1.60) | 14.71 (±1.88) | 20.46 (±8.49) |
| C22:6N3 | 1.78 (±0.25) | 1.17 (±0.05) | 1.47 (±0.085) | 1.51 (±0.34) |
| **SFA** | 33.34(±8.57)$^{a}$ | 39.07(±8.67)$^{a}$ | 36.29(±8.73)$^{a}$ | 36.10(±7.83)$^{a}$ |
| **MUFA** | 22.00(±7.21)$^{a}$ | 10.03(±4.97)$^{b}$ | 15.53(±4.97)$^{b}$ | 17.27(±5.46)$^{b}$ |
| **PUFA** | 43.23(±7.39)$^{a}$ | 29.24(±8.75)$^{b}$ | 51.39(±8.75)$^{a}$ | 58.43(±9.74)$^{a}$ |

*Note:* *Percentages in weight, different letters in the same row indicate significant differences according to Tukey test ($p \geq 0.05$); n.d.=not detected, (± Standard deviation).

The fatty acid methyl ester compounds (Table 4) that predominated were polyunsaturated (PUFA) since they were present at 0.75 vvm, 1.75 vvm, and 2.25 vvm; however, at 1.25 vvm, a greater presence of saturated fatty acids (SFA) was reached. The elements in higher amount were C12:0; C20:5N3; C24:1; C 22:0; C22:2, similarly to those reported by: (Al-Ameri and Al-Zuhair 2019; Alishah et al., 2019).

The analysis of variance showed that there was no significant difference between SFA and PUFA; similarly, the monounsaturated (MUFA) at 1.25, 1.75, and 2.25 vvm did not reveal significant differences ($p \geq 0.05$), while at 0.75 the value of MUFA was slightly higher. From the fatty acids obtained, it can be said that the increase in aeration in FBRCs favored higher PUFA production, especially at 1.75 vvm and 2.25 vvm, contributing to the characteristics of the biofuel; it is recommended that the composition of the lipids be long-chain fatty acids, with low degree of unsaturation to avoid toxic emissions and improve fuel properties such as cetane number (CN) and oxidative stability (OS) (Knothe, 2010, Basto-Flores et al., 2022).

Considering that within the main properties of fuels is the CN (cetane number), which in a dimensionless way describes the ignition quality of a fuel and is related to the

ignition delay time experienced by a fuel (Arguelles et al., 2018); according to the FAME analysis, due to the higher amounts detected of PUFA in the experiments, it can be indicated that biodiesel would be of low quality considering the low presence of saturated fatty acids.

## Conclusions

From the above, it can be concluded that the aeration rate at 0.75 vvm was the most appropriate to obtain greater cell growth, but it should be noted that at 1.25 vvm there was a greater increase in lipid content, and therefore in lipid productivity. The working regime was in the homogeneous bubbling flow range due to the characteristics of the reactor and the surface velocity of the gas.

According to the conditions proposed in relation to cell growth, it would be convenient to manage an aeration rate of 0.75 vvm; on the other hand, in order to obtain greater lipid production, the aeration rate would be 1.25 vvm; therefore, a culture process could be considered, first growing the cells and then to conditions with agitation of 1.25 vvm to continue the culture, taking into account the concentrations of nutrients, the type of lighting, and verifying the productivity.

## Importance of the study, strengths and weaknesses

This study was carried out with the purpose of knowing and interpreting the effect of aeration and some hydrodynamic parameters such as shear rate ($\dot{\gamma}$) on the production of fatty acids of methyl esters (FAME) mainly, together with the fact of determining in the same way if these changes in aeration benefit other parameters such as cell growth, nitrogen consumption, lipid productivity, besides using correlations to determine in function of the FAME the quality of biodiesel, according to the type of components of methyl esters present at the proposed aeration flows.

In relation to the strengths, it can be indicated that there is an adequate methodological approach to calculate the hydrodynamics in the photobioreactors and other determinations, the knowledge to be able to interpret the results obtained, in addition to having the equipment to carry out the experimental part; regarding the areas of opportunity, it can be pointed out that it is necessary to continue experimenting, considering other operational factors, consider greater analysis of the determinations, and go deeper into these.

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