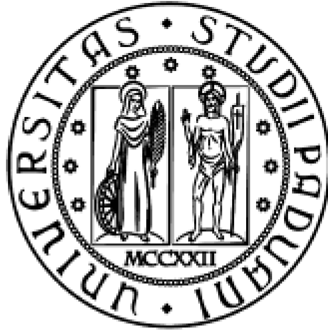


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI INGEGNERIA INDUSTRIALE

LAUREA MAGISTRALE IN INGEGNERIA CHIMICA E DEI PROCESSI INDUSTRIALI

Tesi di Laurea Magistrale in
Ingegneria Chimica e dei Processi Industriali

LABORATORY-SCALE PROCESS OPTIMIZATION OF THE ALKALINE-ENZYMATIC HYDROLYSIS OF CASTOR CAKE

Relatore: Prof.ssa Elisa Cimetta

Correlatore: Dott.ssa Clizia Franceschi

Laureando: ILARIA BUSON

ANNO ACCADEMICO 2021-2022

Riassunto

I biostimolanti sono prodotti contenenti sostanze e/o microrganismi in grado di sostenere la crescita e lo sviluppo delle piante durante tutto il ciclo di vita della coltura, dalla germinazione dei semi e dal trapianto fino alla raccolta. Hanno la capacità di stimolare quei processi naturali della pianta che migliorano l'assorbimento e l'efficienza d'uso dei nutrienti, la tolleranza agli stress abiotici e la qualità delle colture, e pertanto attualmente rappresentano un settore tecnico-scientifico in continua evoluzione. Una categoria di sostanze biostimolanti sono gli idrolizzati proteici. Essi sono delle sostanze contenenti una miscela di aminoacidi e peptidi solubili, generalmente ottenuti per idrolisi chimica o enzimatica, o mista da proteine di origine animale o vegetale. Essi contribuiscono al campo della sostenibilità e della chimica verde, per questo negli ultimi anni vi è un'attenzione e uno sviluppo notevole di questi prodotti. Il presente lavoro ha lo scopo di studiare l'ottimizzazione del processo d'idrolisi alcalino-enzimatica del pannello di ricino su scala di laboratorio per produrre un idrolizzato proteico. La materia prima è un sottoprodotto che viene riutilizzato ed è il pannello di ricino che rappresenta lo scarto di lavorazione della produzione dell'olio di ricino. Verranno studiati differenti parametri di processo, per capire quali condizioni portino ad ottenere un miglior prodotto in termini di processo produttivo. I prodotti realizzati dovranno essere poi successivamente testati ***in vivo*** al fine di verificarne l'efficacia come biostimolanti e il potenziale effetto sulle piante. Lo studio presentato nella seguente tesi rappresenta un'analisi preliminare di processo che pone le basi per la realizzazione di un futuro impianto d'idrolisi, ovvero rappresenta la prima fase del processo di scale-up. Lo scale-up di un processo consiste in tutte le considerazioni e le azioni necessarie per riprodurre i dati di laboratorio a livello industriale e rappresenta la metodologia di sviluppo di un processo chimico. La prima fase del processo presentata dimostra come gestire il processo produttivo dalla reazione, alla separazione solido-liquido e infine alla concentrazione. Si è cercato inoltre di conciliare la parte dell'ottimizzazione del processo con l'attenzione alla sostenibilità e all'ecologia. Si è cercato di sviluppare un processo che rispettasse il più possibile i dodici principi della chimica verde.

Abstract

Biostimulants are products containing substances and/or microorganisms capable of supporting the growth and development of plants throughout the life cycle of the crop, from seed germination and transplanting to harvest. They have the ability to stimulate those natural processes of the plant that improve the absorption and efficiency of use of nutrients, tolerance to abiotic stress and the quality of crops, and therefore currently represent a constantly evolving technical-scientific sector. One category of biostimulating substances are protein hydrolysates. They are substances containing a mixture of amino acids and soluble peptides, generally obtained by chemical or enzymatic hydrolysis, or mixed with proteins of animal or vegetable origin. They contribute to the field of sustainability and green chemistry, which is why in recent years there has been considerable attention and development of these products. The present work aims to study the optimization of the alkaline-enzymatic hydrolysis process of the castor panel on a laboratory scale to produce a protein hydrolysate. The raw material is a by-product that is reused and is the castor board which represents the processing waste from the production of castor oil. Different process parameters will be studied to understand which conditions lead to obtaining a better product in terms of production process. The products made will then have to be subsequently tested ***in vivo*** to verify their effectiveness as biostimulants and the potential effect on plants. The study presented in the following thesis represents a preliminary process analysis that lays the foundations for the construction of a future hydrolysis plant, that is, it represents the first phase of the scale-up process. The scale-up of a process consists of all the considerations and actions necessary to reproduce laboratory data at an industrial level and represents the methodology for developing a chemical process. The first phase of the process presented demonstrates how to manage the production process from the reaction to the solid-liquid separation and finally to the concentration. We also tried to reconcile the optimization part of the process with attention to sustainability and ecology. We tried to develop a process that respected as much as possible the twelve principles of green chemistry.

Table of Contents

Chapter 1 – Biostimulants **1**

1.1 Categories 1

1.2 Protein hydrolysates 2

1.3 Regulation of biostimulant 3

1.4 Green Chemistry 4

1.5 Circular Economy and Green Deal 6

1.5.1 Recovery of by-products 6

1.5.2 Evaluation parameters for the choice of raw material 7

1.5.3 Raw material: castor cake 8

1.6 The processes of hydrolysis 9

1.6.1 Types 9

1.6.2 Enzymes 11

1.7 Aim of the thesis 12

Chapter 2 – Materials and methods **15**

2.1 Raw material analysis 15

2.1.1 Determination of pH 15

2.1.2 Determination of electrical conductivity 16

2.1.3 Determination of total nitrogen 16

2.1.4 Determination of organic nitrogen 19

2.1.5 Determination of dry matter 19

2.1.6 Determination of ash 19

2.1.7 Determination of organic carbon 20

2.2 Hydrolysis Reaction 20

2.2.1 Equipment 20

2.2.2 Reaction 21

2.3 Solid-liquid separation 22

2.4 Concentration 24

2.5 Product Analysis 25
2.5.1 Determination of Brix degree 25
2.5.2 Determination of density 26
2.5.3 Determination of Hydrolysis Degree 26
Chapter 3 – Experimental Data **27**
3.1 Analysis data on raw material 27
3.2 Analysis data on products 28
3.2.1 Process Standardization 30
3.2.2 Process yields 35
3.2.3 Single enzyme vs combined enzymes 40
3.2.4 Centrifuge vs filter press 42
3.2.5 Optimization of alkalizing agent quantity 43
3.2.6 Batch vs Semi-Batch 45
Conclusion **47**
Bibliography **49**
Ringraziamenti **51**

Chapter 1
Biostimulants

In this Chapter some fundamental topics are introduced in order to better understand the project developed in this work of thesis. The subjects treated include a general introduction about categories of biostimulants and their regulation; a description of protein hydrolysates; Circular Economy and Green Deal; the raw material that will be used: the castor cake; lastly, an overview on types of the processes of hydrolysis.

1.1 Categories

Biostimulants are a group of substances of natural origin that contribute to boosting plant yield and nutrient uptake, while reducing the dependency on chemical fertilizers [4]. Biostimulants are not fertilizers as they do not provide nutrients directly to plants, but they can facilitate the acquisition of nutrients by supporting metabolic processes in soil and plants e.g. by mobilizing elements in the rhizosphere or by developing new routes of nutrient acquisition, like fixation of atmospheric N by the recruitment of bacterial endosymbionts. A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency abiotic stress tolerance and/or crop [8] quality traits, regardless of its nutrients content. The nature of the biostimulant is not restrictive: it can be a substance, microorganism or a mixture of both. A substance may be either a single chemical compound or a group of compounds having a well-established biological origin, e.g. plant extracts, but not necessarily a fully characterized composition. “Nutrition efficiency” may cover nutrient mobilization and uptake from the soil, root development, transport, storage and assimilation (i.e. conversion of inorganic to organic forms) of nutrients in the plant. ‘Abiotic stress’ refers to any physical or chemical stressor of non-biological origin (drought, salinity, cold, etc.). ‘Quality traits’ may be different and range from nutritional value to shelf life or flower pigmentation. Any of these effects should be distinct from those resulting from the nutrient content of the biostimulant.

There is no real categorization of biostimulants, but some major categories are widely recognized by scientists, regulators, and stakeholders, covering both substances and microorganisms and are the following:

- Humic and fulvic acids: humic substances (HS) are natural constituents of the soil organic matter, resulting from the decomposition of plant, animal and microbial residues, but also from the metabolic activity of soil microbes using these substrates. Fulvic acids are a family of organic acids, natural compounds, and components of the humus (which is a fraction of soil organic matter);

- Protein hydrolysates (PH) and other N-containing compounds: Amino-acids and peptides mixtures are obtained by chemical, thermal and enzymatic protein hydrolysis from agro industrial by-products, from both plant sources (crop residues) and animal wastes (e.g. collagen, epithelial tissues);
- Seaweed extracts and botanicals: the use of fresh seaweeds as source of organic matter and as fertiliser in agriculture dates back in ancient times, but biostimulant effects have been recorded only recently while plant extracts (botanicals), such as supercritical CO2, are more recent and are progressively becoming extremely interesting;
- Chitosan and other biopolymers: chitosan is a deacetylated form of the biopolymer chitin, produced naturally and industrially;
- Inorganic compounds: the five main beneficial elements are Al, Co, Na, Se and Si, present in soils and in plants as different inorganic salts and as insoluble forms like amorphous silica ($SiO_2.nH_2O$) in gramineous species;
- Beneficial fungi: Fungi interact with plant roots in different ways, from mutualistic symbioses (i.e. when both organisms live in direct contact with each other and establish mutually beneficial relationships) to parasitism [3].

1.2 Protein hydrolysates

PHs are a well-known group of plant biostimulants and their use in agriculture increased significantly during the last decade. Protein hydrolysates are mainly produced by thermal, chemical (with strong acids or alkalis) and/or enzymatic hydrolysis of proteins contained in agro-industrial by-products of animal (i.e., leather, viscera, feathers, blood) or plant origin (i.e., vegetable by-products) and in biomass of dedicated legume crops. [6] Chemically, PHs are a mixture of free amino acids, oligopeptides, and polypeptides. Due to different source materials and different hydrolytic processes used during production, PHs are a very complex and inhomogeneous category of products, a critical issue for both manufacturers and potential users of such products PHs obtained by agro-industrial by-products represent a sustainable solution to the problem of waste disposal, making their production interesting from environmental and economical points of view. More than 90% of the PH market in horticulture is based on products obtained through chemical hydrolysis of proteins of animal origin. PHs have been identified to improve the performance of several horticultural crops, including increased shoot, and root biomass and productivity [1][8]. PHs can be classified on the basis of protein source, and method of protein hydrolysis as shown in Figure 1.1. Production process and protein source strongly affect the chemical characteristics of PHs. Protein hydrolysates stimulate N metabolism and assimilation. In plants, all inorganic N is first reduced to ammonia before it is incorporated into organic form. The N assimilation is a key process controlling plant growth and development [2].

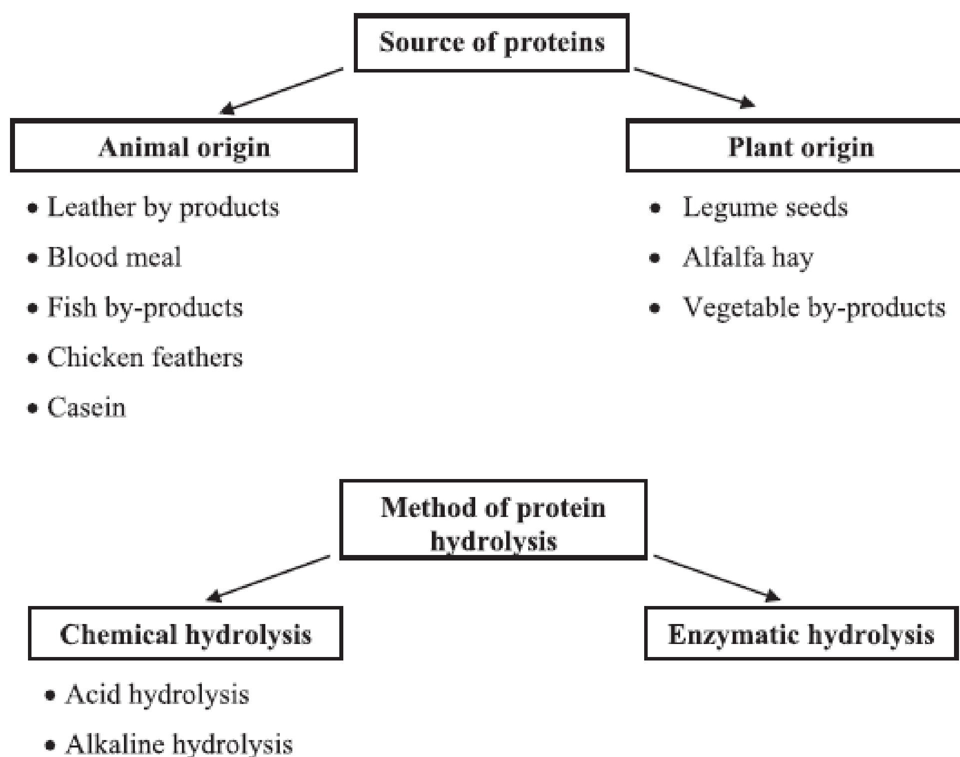


Figure 1.1 Classification criteria of protein hydrolysates on the basis of protein source and the method of protein hydrolysis used in the production process.

1.3 Regulation of biostimulants

The regulatory situation of biostimulants in general and PHs in particular is still complex today, in the absence of any harmonised and specific regulatory framework in the United States and also in the European Union (EU). The main reasons for this situation include lack of a clear definition and acceptance of the concept by regulatory bodies; thus, a supranational regulatory framework is an urgent need to promote the use of PHs as biostimulants in horticulture.

As any biostimulant is intended to influence the life processes of plants by other ways than as a nutrient, it may be regarded as a "plant protection product" from a strict regulatory viewpoint. Synthetic and natural substances (including botanicals and basic substances as mentioned before), and microorganisms, are all covered by this regulation [7]. All plant growth regulators and herbicide safeners have been registered under this PPP regulation until now and these are substances that interact with the physiology of the plant, even though they do not protect the plant against pests or diseases. Due to the lengthy and costly procedures to place a PPP on the European market, taking into consideration that many companies developing biostimulants are SMEs and that improved plant nutrition and growth are the main scope of biostimulants, an alternative route has been chosen, namely the 'fertilisers route' in which case national legislation is applied. The European law on EC fertilisers (regulation (EC) No 2003/2003) is not applied because the definition of fertilisers laid down by this

regulation is very restrictive and cannot include biostimulants. Any fertiliser must provide nutrients as its main function. This is clearly not the case of biostimulants, which by definition promote plant growth by other means than by providing nutrients. The situation in the USA is to some extent like the European situation: no approved definition of biostimulants, no harmonization between the 50 states, use of fertilisers laws for the placing on the market of certain biostimulants at the state level, and work in progress between stakeholders, representatives of regulatory bodies and federal agencies to improve the legal certainty surrounding biostimulants [3].

1.4 Green Chemistry

The chemical industry is required in particular to create products that allow the conservation of natural resources (materials and energy); to have more efficient processes (better use of raw materials, generating as little waste as possible); to use energy from renewable sources; to consider the entire life cycle of the product (LCA) and also its fate after the end of its useful life ("from cradle to grave" design approach); to operate in a safe way for health and the environment. Green chemistry was born from the demands made on chemistry for sustainable development, which has been considered the method for achieving sustainable development in that industry. The definition of Green Chemistry given by the EPA is "Green chemistry is the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances." Green chemistry applies across the life cycle of a chemical product, including its design, manufacture, use, and ultimate disposal. Green chemistry is also known as sustainable chemistry. Green chemistry:

- Prevents pollution at the molecular level;
- Is a philosophy that applies to all areas of chemistry, not a single discipline of chemistry;
- Applies innovative scientific solutions to real-world environmental problems;
- Results in source reduction because it prevents the generation of pollution;
- Reduces the negative impacts of chemical products and processes on human health and the environment;
- Lessens and sometimes eliminates hazard from existing products and processes;
- Designs chemical products and processes to reduce their intrinsic hazards.

Green chemistry is based on the "twelve principles of green chemistry":

1. **Prevent waste:** Design chemical syntheses to prevent waste. Leave no waste to treat or clean up.
2. **Maximize atom economy:** Design syntheses so that the final product contains the maximum proportion of the starting materials. Waste few or no atoms. Within this principle, the E factor

(environmental factor) can also be considered as a parameter that indicates the yield of a reaction.

3. **Design less hazardous chemical syntheses:** Design syntheses to use and generate substances with little or no toxicity to either humans or the environment.
4. **Design safer chemicals and products:** Design chemical products that are fully effective yet have little or no toxicity.
5. **Use safer solvents and reaction conditions:** Avoid using solvents, separation agents, or other auxiliary chemicals. If you must use these chemicals, use safer ones.
6. **Increase energy efficiency:** Run chemical reactions at room temperature and pressure whenever possible.
7. **Use renewable feedstocks:** Use starting materials (also known as feedstocks) that are renewable rather than depletable. The source of renewable feedstocks is often agricultural products or the wastes of other processes; the source of depletable feedstocks is often fossil fuels (petroleum, natural gas, or coal) or mining operations.
8. **Avoid chemical derivatives:** Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.
9. **Use catalysts, not stoichiometric reagents:** Minimize waste by using catalytic reactions. Catalysts are effective in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and carry out a reaction only once.
10. **Design chemicals and products to degrade after use:** Design chemical products to break down into innocuous substances after use so that they do not accumulate in the environment.
11. **Analyse in real time to prevent pollution:** Include in-process, real-time monitoring and control during syntheses to minimize or eliminate the formation of by-products.
12. **Minimize the potential for accidents:** Design chemicals and their physical forms (solid, liquid, or gas) to minimize the potential for chemical accidents including explosions, fires, and releases to the environment.

Sustainable processes try to comply as much as possible with the 12 principles. They can impact the production process differently and can have different effects at different stages. The application of these principles has the main purpose of improving sustainability, but this is only possible if the process is also economically sustainable. Often their application leads to a series of advantages that have important repercussions also at an economic level and this represents a strong push for the application of the principles themselves. For example, if we consider the first principle that provides for the minimization of waste: when they are produced, they must be treated, and the more they are generated, the more costs increase; if, on the other hand, waste is minimized, costs are consequently reduced and an economic benefit is therefore obtained [15].

1.5 Circular Economy and Green Deal

The circular economy is a model of production and consumption, which involves sharing, leasing, reusing, repairing, refurbishing and recycling existing materials and products as long as possible. In this way, the life cycle of products is extended. In practice, it implies reducing waste to a minimum. When a product reaches the end of its life, its materials are kept within the economy wherever possible. These can be productively used again and again, thereby creating further value. This is a departure from the traditional, linear economic model, which is based on a take-make-consume-throw away pattern. This model relies on large quantities of cheap, easily accessible materials and energy. Also, part of this model is planned obsolescence, when a product has been designed to have a limited lifespan to encourage consumers to buy it again [13].

The European Green Deal is a set of policy initiatives by the European Commission with the overarching aim of making Europe climate neutral in 2050. An impact assessed plan will also be presented to increase the EU's greenhouse gas emission reductions target for 2030 to at least 50% and towards 55% compared with 1990 levels. The plan is to review each existing law on its climate merits, and also introduce new legislation on the circular economy, building renovation, biodiversity, farming and innovation. The European Commission's climate change strategy, launched in 2020, is focused on the promise to make Europe a net-zero emitter of greenhouse gases by 2050 and to demonstrate that economies will develop without increasing resource usage. Among the various topics dealt with in the Green Deal, one point in particular refers to the field of agriculture. Some objectives are: reduce by 50% the use of pesticides by the year 2030, and reduce the use of Fertilizers by 20% by the year 2030 [14].

1.5.1 Recovery of by-products

Valorisation is the process of converting by-products to high value substances. The development of novel tools to secure crop yield are objectives that are of high importance on the agenda of the research funding bodies across Europe and worldwide. On the other hand, increasing price of oil directly affects the cost of fertilizers, which urges farmers to look for novel ways to reduce the input cost. The concept of circular economy creates opportunities for the agricultural and food industry aiming at valorising by-products. Indeed, recycling raw organic material from waste is a first step toward reducing energy and material input costs in the production process. Waste streams from food and agricultural industries are equally important sources for biostimulant development. The biostimulants generated from waste streams are extracts from food waste [7], composts, manures, vermicompost, aquaculture waste streams and sewage treatments. Recycling of organic waste as biofertilizers is historically a common practice. Manure has been and is applied to the field as soil fertilizer across the world.

The concept of circular economy emphasizes the process of converting waste materials and products that have reached the end of their life cycle into new sources. A new EU regulation that entered into force on January 1st, 2018 aims at boosting the use of bio-base wastes as new types of fertilizers. This waste originated fertilizers usually contain biostimulatory substances. When a waste turns into raw material for industrial or agricultural application, then it is no longer waste. As the definition of "waste" describes the material to be discarded, it is sometimes more appropriate to label the material as by-product. A by-product is lawfully used, not deliberately produced, of certain use, ready for use without further processing and produced as an integral part of the production process (European Commission, 2007). Through research and development of using industrial waste for re-manufacturing, reuse and recycling, one can make fundamental steps toward biomass optimization and resources use efficiency and sustainability (European Commission, 2017). Therefore, developing biostimulants from by-products provides the innovative methods to prevent inadvertent disposal and results in environmental-friendly solutions for waste re-use [4].

1.5.2 Evaluation parameters for the choice of raw material

As the first step toward developing biostimulants from organic waste, the choice of biomass resource is critical. Different active ingredients found in industrial waste streams and by-products of biological origins pose the perfect opportunity to extract molecules for better growth and pathogen resistance of valuable crop plants. However, some understanding of the intrinsic biochemical characteristics of raw materials is needed. The environmental and economic evaluation must be carried out as well, to assess whether a new biostimulant product has good prospect to become successful. To assess if the raw material is suitable for the development of biostimulants, several factors must be taken into consideration:

- Absence of Pesticides: By-product derived from plant species that has been treated with pesticides could potentially cause problems for biostimulants production, as it no longer will be seen as a "natural" product and will be considered as an alternative preparation of the regulated agrochemical. Even the smallest amount of contamination with pesticides may cause problems with the legislation. To this end, the source of plant biostimulants should not contain pesticides that otherwise would lead to problems with the registration and permission to use in sustainable agriculture;
- Low cost of collection and storage: The initial economic value of the waste material should be low, and it preferably requires additional processing to dispose of at a financial cost. The reason for this is that the yield increase attributed to biostimulant application is typically in the range of 5–10% and this limits the profitability of biostimulant sales. The economic burden of a by-product resource can be broken down into three main components: collection, conservation, storage and transport. Despite the complexity of organizing the logistic of storage for perishable biomass, agricultural production is typically linked with seasonal production activities and poor conservation methods may in addition result in deterioration of

the material that could affect the stability of bioactive ingredients. Many natural products are unstable and undergo chemical modification when exposed to heat, light or oxygen, which would result in loss of bioactivity;

- Sufficient Availability: An effective agrochemical product is preferably available in sufficient quantities to accommodate the market demand. It is therefore critical that the biomass from which a biostimulant product is derived is available in large quantities and readily available for processing. It should also be avoided to develop biostimulants derived from by-products which are prone to perturbation, i.e., the material that strongly varies in composition or for which the supply is uncertain. An abundant product has, in addition, the advantage of attracting further valorisation and development for other usage;
- Positive and Negative Impact of Competing Use: If different valorisation strategies applied to the same waste stream are in competition with each other, this may turn out to have a negative impact on the development of a biostimulant product. This is because biostimulants are typically less valuable than animal feed and much less than valorisation through the isolation of fine chemicals as pharmaceutical agents. As a result, to be suitable as biostimulant source, the waste source is preferably not intensively used for feed or other higher value uses. There are therefore both positive and negative aspects to consider when a given biomass is used in diversified sectors. Researchers and entrepreneurs are suggested to carry out a SWOT analysis, identifying the pros and cons when developing biostimulants from material that is being exploited for different purposes already.

The European Commission has proposed to revise EU legislation on waste to set clear targets to establish a long-term goal for waste management (European Commission, 2017). It is expected the prospect of a circular economy to require more research and technology development, focusing on bringing up sustainable products onto the market and that, with the trend of replacing chemical fertilizers, biostimulant derived from by-products will be more commonly used if valorisation chain is well established and mode of action is further investigated [4].

1.5.3 Raw material: Castor cake

The raw material that will be used to produce protein hydrolysates is castor cake which is the waste obtained from the castor oil production process.

The castor oil plant, *Ricinus communis*, is a species of flowering plant in the spurge family, Euphorbiaceae. Castor is indigenous to the south-eastern Mediterranean Basin, Eastern Africa, and India, but is widespread throughout tropical regions. Castor seed is the source of castor oil, which has a wide variety of uses. The seeds contain between 40% and 60% oil that is rich in triglycerides, mainly ricinolein. The seed contains ricin, a toxin, which is also present in lower concentrations throughout the plant and therefore castor oil is inedible. Castor oil has many industrial applications as it is widely used in manufacturing of soaps, cosmetics, paint, varnishes, adhesives, lubricants etc. Being a mild

laxative and smoothing agent, it is widely used in many medicinal preparations as well. Castor oil seed contains about 30%–50% oil (m/m) depending on variety. Castor oil can be extracted from castor beans by mechanical pressing, solvent extraction, or a combination of pressing and extraction. After harvesting, the seeds are allowed to dry so that the seed hull will split open, releasing the seed inside. The extraction process begins with the removal of the hull from the seeds. This can be accomplished mechanically with the aid of a castor bean de-huller or manually with the hands. When economically feasible, the use of a machine to aid in the de-hulling process is more preferable. After the hull is removed from the seed, the seeds are then cleaned to remove any foreign materials. These materials can usually be removed using a series of revolving screens or reels. Magnets used above the conveyer belts can remove iron. The seeds can then be heated to harden the interior of the seeds for extraction. In this process, the seeds are warmed in a steam-jacketed press to remove moisture, and this hardening process will aid in extraction. The cooked seeds are then dried before the extraction process begins. A continuous screw or hydraulic press is used to crush the castor oil seeds to facilitate removal of the oil. The first part of this extraction phase is called pre-pressing and usually involves using a screw press called an oil expeller. The oil expeller is a high-pressure continuous screw press to extract the oil.

The waste at the end of this production process is the castor cake which from the analysis has good titles as will be presented later and respects the parameters described above so it can be used as a raw material.

1.6 The process of Hydrolysis

Various chemical reactions fall under the generic name of hydrolysis in which the molecules are split into two or more parts by the effect of water and can sometimes be considered as the inverse of the condensation reaction. We speak of hydrolysis, for those reactions that see the interaction between an ion and water.

1.6.1 Types

There are mainly three types of hydrolysis:

- **Acid hydrolysis**: It is a very aggressive process carried out at high temperature (>121◦C) and pressure (>220.6 kPa). In acid hydrolysis, hydrochloric and sulphuric acid are mainly used to hydrolyse proteins, the most common being hydrochloric acid;
- **Alkaline Hydrolysis:** it is a simple and straightforward process where proteins are solubilised by heating followed by the addition of alkaline agents, such as Ca, Na or potassium hydroxide, and maintaining the temperature to a desired set point;
- **Enzymatic Hydrolysis:** The protein hydrolysis is carried out by proteolytic enzymes originating from animal organs (e.g., pepsin), plants (e.g., papain) or microorganisms (e.g.,

alcalase). Proteolytic enzymes hydrolyse proteins more gently than acids/alkalis and do not require high temperature (<60◦C), and they usually target specific peptide bonds.

Acid and alkaline hydrolysis are chemical hydrolysis. These types of reactions can also be combined with each other.

Chemical hydrolysis [5] is usually adopted for producing animal--based PHs. This type of reaction attacks all peptide bonds of proteins, leading to a high degree of protein hydrolysis (high content of free amino acids in total) and destruction of several amino acids. Moreover, other useful thermolabile compounds (e.g., vitamins) are also mostly destroyed during chemical hydrolysis. One other critical aspect of chemical hydrolysis is the conversion of free amino acids from the L-form to D-form (racemisation). Since the amino acids in proteins of living organisms are only in the L-form, plants cannot directly use D-amino acids in their metabolism, making the PHs less effective or even potentially toxic for plants. Finally, the large use of acids/alkalis during chemical hydrolysis leads to an increase in salinity of PHs.

Enzymatic hydrolysis [5] is usually adopted for producing plant--based PHs. PHs resulting from enzymatic hydrolysis are a mixture of amino acids and peptides of varying length with a low salinity and a constant composition over time and other bioactive molecules. PH production processes based on enzymatic hydrolysis are also more environmentally friendly than those based on chemical hydrolysis due to the low energy requirement and the carbon dioxide emissions. Combined chemical and enzymatic hydrolytic processes have also been proposed to save energy and to preserve the structure of amino acids. Animal--derived PHs usually contain a higher amount of total amino acids than plant derived PHs. The major amino acids in PHs differ according to the source of proteins. Peptide concentration and molecular weight distribution can be extremely variable depending on the production process. Peptide concentrations are usually higher in enzymatically derived PHs than in chemically--derived ones. Enzymatic hydrolysis, being more selective compared to chemical hydrolysis, enables industry to maximise yield of these bioactive peptides in PHs. Besides amino acids and peptides, PHs contain other compounds that can contribute to the biostimulant action. These compounds include fats, carbohydrates, phenols, mineral elements, plant growth regulators, and other organic compounds. Moreover, plant--based PHs contain soluble carbohydrates and phenols, which play an important role in energy metabolism and oxidative stress defences. Animal--derived PHs, conversely, lack carbohydrates, phenols and phytohormones. Mineral content is also affected by protein source, usually being higher in the products from plant--derived PHs [1]. Hydrolysis of peptide bonds therefore increases the number of free amino groups (-NH2), released after the cleavage of peptide bonds. The ratio between the number of cleaved peptide bonds and the total number of peptide bonds defines the degree of hydrolysis. The DH of a product is determined by calculating the ratio between free amino groups and total organic nitrogen. This indicator is very important, since it is a quantitative expression of the hydrolytic efficiency of the process. [5]

1.6.2 Enzymes

Enzymes are the catalyst of enzymatic hydrolysis. The conditions of this reaction, atmospheric pressure and low temperatures, allow the use of enzymes and avoid their denaturation. This hydrolysis can be incorporated into the green chemistry thanks to its process conditions. The choice of enzymes must be very thoughtful, as there are several specific and selective enzymes. In this thesis, preliminary bibliographic research was carried out to understand which enzyme was most suitable for the raw material under examination.

The enzymes needed to perform enzymatic hydrolysis on castor are proteases because they need to enzymes that catalyse proteolysis, breaking down proteins into smaller polypeptides or single amino acids, and spurring the formation of new protein products. Proteases belong to the class of enzymes known as hydrolases catalysing the reaction of hydrolysis of various bonds with the participation of a water molecule. The reaction of proteolysis of a peptide bond is:

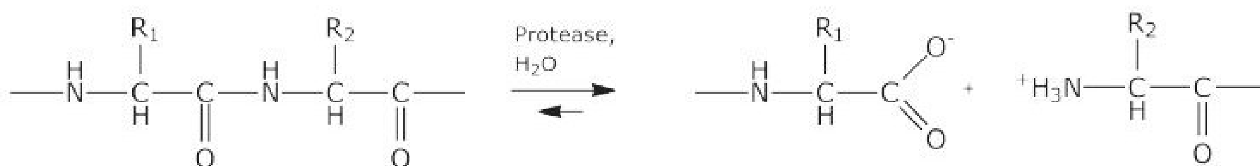


Figure 1.2: Reaction of proteolysis of a peptide bond

Proteases can be divided into two large groupings based on the structural properties of the substrate:

- Exopeptidases: they shorten the polypeptide chain by removing one residue at a time. If this occurs from the N-terminus it is called aminopeptidase, if instead the removal occurs at the C-terminus, it is called of carboxypeptidase;
- Endopeptidases: act by fragmenting the polypeptide chain internally and not at the ends.

Moreover, they can also be classified according to the catalytic amino acid and the pH. After comparing different enzymes also from different companies, it was decided to use two enzymes presented in the table 1.1:

Enzyme	**Type**	**Form**	**Optimum usage conditions**
Protease 1	Serine endopeptidase	Liquid	T = 30-65 °C; pH = 7-9
Protease 2	Serine endopeptidase	Liquid	T = 30-70 °C; pH = 8-10

Table 1.1: Enzymes chosen for the process

Both enzymes chosen are serine endo peptidases, according to the classification based on the catalytic amino acid and the type of substrate. Serine proteases contain a serine group in their active site which is essential for substrate binding and cleavage. Serine proteases are characterized by their broad substrate specificity and their activity extends beyond purely peptidase to include esterase and amidase activities. The common reaction mechanism is in the form of a catalytic centre containing serine as a nucleophile, aspartate as an electrophile and histidine as a base. The reaction mechanism involves the formation of covalently linked enzyme substrate intermediate through acylation resulting in loss of the corresponding amino acid or peptide fragment. Nucleophilic attack on the intermediate by water results in diacylation thereby completing hydrolysis of the peptide. The first enzyme acts as an esterase, enabling it to catalyse stereoselective hydrolysis of some esters. It also efficiently hydrolyses amino esters which include heterocyclic amino esters. The second enzyme is an endopeptidase with a broad specificity which performs well in alkaline conditions and at elevated temperatures as compared to other microbial serine proteases. The main advantages of using enzymes are:

- Cost saving: reduction in raw material input, avoids use of costly chiral resolving agents or costly metal-based catalysts as well as lower equipment, labour and energy costs required;
- Improved productivity: shortened synthesis routes, higher yields;
- Improved quality of intermediate: fewer or no by-products leading to reduced impurities in the final products, high stereo-, regio-, and chemo selectivity, less residual solvent carry over from reduced solvent use;
- Environmental friendliness: reduction of waste products produced and solvent usage, higher energy savings.

1.7 Aim of the thesis

The main objective of the thesis is the study and optimization of the alkaline-enzymatic hydrolysis process of the castor panel on a laboratory scale. The first phase of the study was carried out with bibliographic research regarding the hydrolysis process, the enzymes to be selected and the analytical methods to characterize the raw material and the final product. After re-elaborating the information acquired, the experimental part was performed. First, the raw material was analitycally characterized and evaluated with the previously described parameters to understand if it could be a suitable raw material for obtaining biostimulants. Subsequently various finished products were created by studying some key process parameters, that is:

- Single enzyme vs combined enzymes;
- Centrifuge vs filter press;
- Optimization of basifying agent quantity;
- Batch process vs Semi-Batch process.

The finished products obtained are also subjected to analytical characterization and compared using a set of yield parameters to understand which are the best process conditions in order to obtain a biostimulant. This study on process optimization on a laboratory scale sets the foundations for the future development of an industrial hydrolysis plant.

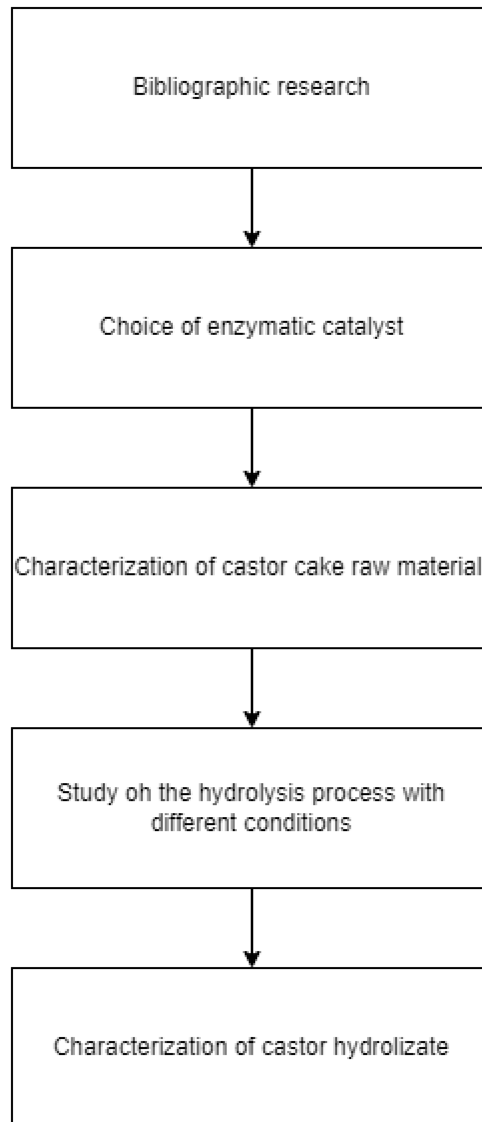


Figure 1.2 Workflow of thesis

Chapter 2
Materials and methods

In this chapter we will explain the steps of the process which are as follows:

- Raw material analysis;
- Hydrolysis reaction;
- Solid/liquid separation;
- Concentration;
- Product analysis.

2.1 Raw material analysis

The analyses carried out on the raw material, and derive from the specific legislation of the sector or from an internal development of the methods, will be illustrated below.

2.1.1 Determination of pH

The pH is determined with a pH meter with a measuring and a reference electrode. The measuring electrode is a glass electrode that measures the difference in electric potential between the outside and the inside of a glass membrane. This potential difference is related to the difference between the concentrations of hydrogen ions inside and outside the membrane. It is also equipped with a temperature probe which corrects the pH value based on the temperature of the sample. Before being used, the pH meter must be calibrated with the buffer solutions. To perform the pH of the protein hydrolysate the procedure is the following: weigh 3g of product with an analytical balance, add 50 ml of distilled water and stir. While the solution is stirring, the measurement is continued until it stabilizes.

Figure 2.1: pH meter

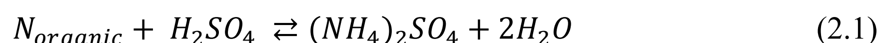
2.1.2 Determination of electrical conductivity

The determination of the specific electrical conductivity is performed by measuring the specific electrical resistance of an aliquot of the solution using a Kohlrausch bridge. The instrument used for the measurement is a conductivity meter. To measure the hydrolysate, the procedure is the following: 2 g of sample weighted with an analytical balance, are added of distilled water up to a total volume of 200 ml. The solution is stirred and the measurement is performed until it stabilizes.

2.1.3 Determination of Total Nitrogen

The Kjeldahl method is an analytical method that allows to determine the nitrogen content of organic and inorganic substances. The method consists of two operational phases:

- Mineralization of the sample to transform the organic nitrogen into ammonia form with concentrated boiling sulfuric acid:



$$N_{organic} + H_2SO_4 \rightleftarrows (NH_4)_2SO_4 + 2H_2O \quad (2.1)$$

- Dosage of the ammonium ion, by distillation in an alkaline environment and absorption on sulfuric acid, the excess of which is titrated with Sodium Hydroxide.
  Reactions:

$NH_4^+ + OH^- \rightleftarrows NH_3 + H_2O$ Distillation (2.2)

$NH_3 + H^+ \rightleftarrows NH_4^+$ Fixation on known volume of acid (2.3)

$H^+ + OH^- \rightleftarrows H_2O$ Titration of excess acid (2.4)

The procedure consists of three phases:

1. Mineralization: Weigh about 1-2 g of sample and insert inside the special glass test tube. Add a tablet of KjTabs catalyst and 15 ml of 96% sulfuric acid. Place the test tube inside the mineralizer, carefully positioning the aspiration system with the water tap open to aspirate. Start the work cycle program which is composed of a first set of 15 minutes at 200°C, a second set of 15 minutes at 310°C and a last set at 70 minutes at 420°C. When the cycle is completed, the test tube is allowed to cool, turning off the suction system or closing the water from the tap.
2. Distillation: The walls of the test tube are rinsed with distilled water. Add 50 ml of 4% boric acid inside a 300ml graduated flask with indicator and stir bar. Insert the test tube and the graduated flask into the appropriate spaces in the Kjedahl distiller by setting 60 ml of NaOH at 30-33% with a duration of 7 minutes and the distillation is started.
3. Titration: The flask is placed to shake and titrated with 0.25N sulfuric acid. When the color changes from green to pink, the titration stops and the volume used is noted.

To calculate the total nitrogen content in% N, the following formula is used:

$$N\,\% = \frac{V * N_{NaOH} * 0.1 * R * ME_N}{P} * 100 \quad (2.5)$$

Where:

V = ml di H_2SO_4 0.25N used to titrate the sample

R = dilution factor which in this case is equal to 1

P = sample weight in g

ME_N = 14 g/eq

N_{NaOH} = sulfuric acid normality

Figure 2.2: Digester of Kjeldahl

Figure 2.3: Distiller of Kjeldahl

2.1.4 Determination of Organic Nitrogen

Organic nitrogen is obtained from the difference between total nitrogen and ammoniacal nitrogen. The process for the determination of ammoniacal nitrogen is partly that of total nitrogen. The first phase is no longer mineralization but consists in the preparation of the solution to be distilled. The procedure is therefore divided into:

1. Preparation of the solution to be distilled: Weigh about 4.5-5 g of sample and place inside a 250 ml flask with a stir bar inside. The flask is about half filled with demineralized water and shaken for 30 minutes. After the time has elapsed, the stir bar is removed and brought up to volume. The solution is homogenized and filtered through a white banded paper filter into another 250 ml flask.
2. Distillation: 50 ml of filtered solution are taken and transferred to the test tube.

The remaining procedure is the same as that of total nitrogen. To calculate the ammonia nitrogen content in% N, the formula n.2.5 is used with an R = 5.

2.1.5 Determination of dry matter

The dry matter is the part of the sample that remains after the total evaporation of the water. It is determined by placing a weighed quantity of sample in an oven at 105 ° C and evaluating the weight loss after 12 hours.

$$dry\ matter\ (\%) = \frac{P'}{P} * 100 \tag{2.6}$$

where P 'is the weight in grams after drying and P the weight in grams of the wet sample.

2.1.6 Determination of ash

Ash is the portion of the sample that does not burn turns into volatile compounds during combustion. The sample is placed in the muffle at 550 ° C until constant weight. It is weighed again, and the percentage of ashes present is determined with the formula:

$$ash\ (\%) = \frac{P'}{P} * 100 \tag{2.7}$$

where P 'is the weight in grams after incineration and P the weight in grams before incineration.

2.1.7 Determination of Organic Carbon

Organic carbon is calculated with the following formula:

$$C\ \% = \frac{(100 - Humidity - Ash)}{1{,}724} \tag{2.8}$$

Where the denominator is the conversion factor to transform the g/kg of organic substance into the corresponding organic carbon content.

2.2 Hydrolysis Reaction

2.2.1 Equipment

Figure 2.4: Bioreactor

A glass bioreactor is used to carry out the hydrolysis reaction. A constant temperature is guaranteed by a jacket placed on the glass vessel in which water flows at a controlled temperature heated by a thermal bath. The temperature inside the reactor is monitored with a thermometer. The mixing system is double 4PBT (pitched blade turbine) connected to a motor placed at the head of the bioreactor. The shape, size, distance and speed of rotation of the blades can be changed according to needs. Through an outlet valve, located under the reactor, it is possible to empty the contents of the reactor.

To monitor the progress of the reaction, the pH and Brix degree are checked every hour on samples from different points of the reactor. It is not possible to detect other parameters, as the values (eg N, C, degree of hydrolysis etc.) are below the detection threshold of the methods applied internally with the tools available. A bench pH-meter is used to monitor the pH while the sample is stirred. A bench refractometer is used to monitor the brix degree after centrifuging the sample.

Figure 2.5: Complete apparatus for carrying out the hydrolysis reaction

2.2.2 Reaction

The reaction can be divided into three stages

1. Reagent insertion and maceration: To carry out the reaction, 5 litres of water are added to the reactor with the castor cake while the reactor is kept under agitation. The amount of water and castor powder were obtained from previous studies. The reactor is heated through the jacket maintaining a constant temperature of 65°C and atmospheric pressure. After adding the reagents, the castor powder maceration begins and ends when the temperature designated for the reaction is reached.

2. Alkaline Hydrolysis: The basifying agent, which is calcium hydroxide, is added and alkaline hydrolysis begins. It is necessary to add a basifying agent to create the suitable working environment in terms of pH and temperature for the enzyme that will be inserted later to carry out the enzymatic hydrolysis.
3. Enzymatic Hydrolysis: After the desired pH and temperature have been reached, it is possible to proceed with the insertion of the chosen enzyme and enzymatic hydrolysis begins. The amount of enzyme to be add was previously obtained from bibliographic searches and previous studies. The reaction is continuously monitored and ends after the pH and degree of Brix are constant, usually after 3 hours. Calcium hydroxide can be added during enzymatic hydrolysis to raise the pH to ensure an optimal environment for the enzyme.

When the reaction ends, the reactor is discharged, keeping it stirred to avoid packing, through the outlet valve located at the bottom of the reactor and proceeding with the next phase.

2.3 Solid-liquid separation

Two different methods were used to conduct the solid-liquid separation.

The first method is to use the centrifuge. Centrifugation is a mechanical process which involves the use of the centrifugal force to separate particles from a solution according to their size, shape, density, viscosity, and rotor speed. The denser components of the mixture migrate away from the axis of the centrifuge, while the less dense components of the mixture migrate towards the axis. The remaining liquid that lies above the precipitate is called a supernatant. There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity. The larger the size and the larger the density of the particles, the faster they separate from the mixture. By applying a larger effective gravitational force to the mixture, like a centrifuge does, the separation of the particles is accelerated. The particles' settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity. The product obtained from the reaction is placed inside the centrifuge and the program was set at 1500 rpm for 7 minutes.

Figure 2.6: Liquid extracted with centrifuge

The second method is to use the filter press. An industrial filter press is a tool used in separation processes, specifically to separate solids and liquids. The machine stacks many filter elements and allows the filter to be easily opened to remove the filtered solids and allows easy cleaning or replacement of the filter media. Filter presses cannot be operated in a continuous process but can offer very high performance, particularly when low residual liquid in the solid is desired.

Figure 2.7: Liquid extracted with filter press

After both methods, the solid obtained is placed in an oven to dry at 105°C, while the liquid is ready to be concentrated.

2.4 Concentration

A rotavapor is used to concentrate the liquid. A rotary evaporator is a device used for the efficient and gentle removal of solvents from samples by evaporation.

The main components of a rotary evaporator are:

- A motor unit that rotates the evaporation flask;
- A vapor duct that is the axis for sample rotation and is a vacuum-tight conduit for the vapor being drawn off the sample;
- A vacuum system, to substantially reduce the pressure within the evaporator system.
- A heated fluid bath to heat the sample;
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures;
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses;
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

Figure 2.8: Rotavapor

The operating principle of this machine is based on the ability of the vacuum to lower the boiling temperature of a given solvent. The vacuum is generated by depression values, and the pump is very important because it avoids using too high temperatures. It allows the temperature of the thermostatic bath to be kept at a value that is not excessively high, so as not to damage the heat-sensitive products. Under these conditions, all solvents evaporate at lower temperatures than those which should be used in atmospheric pressure processing. Evaporation is further facilitated by the presence of the evaporating flask and the thermostatic bath, which, by further heating the temperature, increase the speed of the process. During these events, the solvent vapours move away and, once they reach the surface of the condenser, they liquify due to the colder temperature. They are then collected in the collecting flask. The first phase of the procedure consists in introducing the solution to be evaporated inside the evaporation flask. This is then connected to the rotation mechanism, which in turn is connected to the capacitor. The evaporating flask is introduced into the thermostatic bath, after which the rotation is started. At this point, the vacuum is applied. At the end of this, a tap in the condenser allows the entry of air, necessary to interrupt the vacuum. The process can be considered concluded when the solute is completely separated from the solvent. In particular, the first will be in the evaporating flask, while the second will be contained in the collecting flask.

The bath temperature is kept at 50 ° C, the rotation speed is 80 rpm and the pressure is approx. 0.095 MPa. Evaporation continues until the hydrolysate is sufficiently concentrated, which means that it has a brix degree greater than 53° Bx.

2.5 Product analysis

The same analyses are performed on the products already done for the raw material and the analyses explained below are added.

2.5.1 Determination of Brix Degree

Brix is a measure of solid-state substances dissolved in a liquid. One degree Brix (symbol ° Bx) corresponds to 1 part of solid substance (dry weight) in 99 parts of solution. The measurement is carried out either with the saccharimeter, which measures the density of the liquid or more easily with the refractometer which calculates the Brix degrees based on the refractive index. Place a few drops of the sample on the refractometer and read the value expressed in% corresponding to ° Bx.

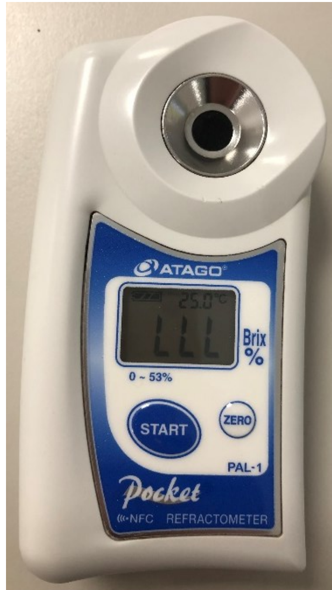


Figure 2.9: Bench refractometer

2.5.2 Determination of density

Density is measured by weighing a known volume of sample.

$$density\left(\frac{kg}{dm^3}\right) = \frac{mass}{volume} \tag{2.9}$$

2.5.3 Determination of Hydrolysis Degree

The method proposed by Frank C. Church, Harold E. Swaisgood, David H. Porter, And George L. Catignani in 1983 was used and adapted as an internal method for measuring the degree of hydrolysis.

Chapter 3
Experimental Data

In this chapter the results obtained from the hydrolysis tests will be presented.

3.1 Analysis data on raw material

In order to characterize the raw material and understand if it could be used to make hydrolysates, the analyses explained above were conducted which gave the following results:

Parameter	**Value**
pH	6,33
Electric conductibility [mS/cm]	1,88
N total [%]	8,30
N organic [%]	8,06
Moisture [%]	8,25
Ash [%]	9,29
Organic carbon [%]	47,83

Table 3.1: Raw material analysis

The most interesting data for the production of a hydrolysate is the quantity of total and organic nitrogen because it is an essential nutritional element for plants. From the analyses presented above it can be seen that castor powder has a lot of total nitrogen so it is suitable as a raw material to produce biostimulants.

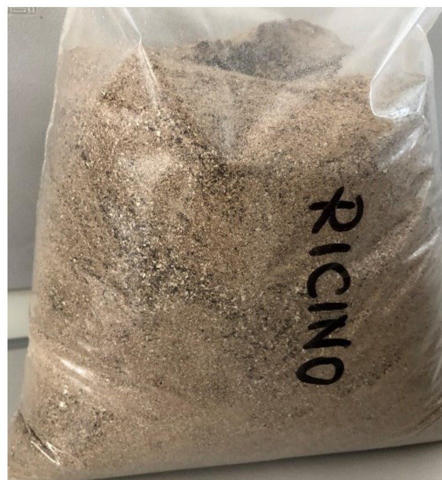


Figure 3.1: Castor cake

3.2 Analysis data on products

Six different products were made in duplicate. Each product is characterized by a different process or recipe conditions. The following table shows the various combinations used to make the products that are named according to the first 6 letters of the alphabet with the sequential numbering below:

	A	B	C	D	E	F
1 enzyme	●			●		●
2 enzymes (Protease 1+ Protease 2)		●				
2 enzymes (Protease 2+ Protease 1)			●		●	
Less $Ca(OH)_2$	●	●	●		●	●
More $Ca(OH)_2$				●		
Centrifuge	●	●	●	●		
Filter Press					●	●
Batch	●	●	●	●	●	
Semi-Batch						●

Table 3.2: Product denomination

The results of the analyses described above for all liquid hydrolysed products and for the liquid solid part are presented below:

	pH	**Electric Cond. [mS/cm]**	**N total [%]**	**N organic [%]**	**Density [kg/dm^3]**	**Moisture [%]**	**Ash [%]**	**C organic [%]**	**Brix Degree [°Bx]**
A.1	7,01	0,88	6,42	5,44	1,151	50,79	4,08	26,18	53
A.2	6,96	0,75	6,52	5,62	1,156	52,25	4,03	25,36	53
B.1	6,82	1,73	6,84	5,13	1,167	49,31	5,78	26,05	61
B.2	7,27	0,87	6,46	5,49	1,150	52,13	4,73	25,02	56
C.1	7,40	0,98	6,95	6,15	1,170	46,80	5,60	27,61	56
C.2	7,20	0,71	6,32	5,55	1,173	53,09	4,62	24,53	53
D.1	5,43	1,63	6,43	4,66	1,157	47,50	5,54	27,24	60
D.2	6,38	1,45	6,38	4,68	1,150	47,60	4,94	27,53	59
E.1	7,28	0,82	6,18	5,30	1,162	52,20	4,73	24,98	61
E.2	7,11	1,01	6,35	5,24	1,144	52,10	4,85	24,97	55
F.1	6,27	0,94	5,74	4,99	1,185	49,32	7,17	25,24	54
F.2	6,23	0,88	5,68	4,99	1,182	50,37	6,95	24,76	54

Table 3.3: Liquid product analysis

From the analyses it can be seen that the various samples have good repeatability. It can be noted that in the products we find a good percentage of total and organic nitrogen, a fundamental element for the composition of biostimulants.

Residue	N total [%]	N organic [%]	Moisture [%]	Ash [%]	C organic [%]
A.1	5,53	5,13	0,65	12,59	50,32
A.2	6,02	5,63	5,15	12,56	47,73
B.1	4,90	4,55	6,82	17,44	43,93
B.2	6,88	6,38	3,4	12,80	48,61
C.1	5,43	5,01	5,96	15,07	45,81
C.2	5,77	5,40	5,31	16,65	45,27
D.1	4,50	4,24	5,11	10,17	49,14
D.2	4,11	3,82	5,96	14,63	46,06
E.1	3,12	3,02	4,64	12,49	48,07
E.2	2,65	2,50	1,32	13,20	49,58
F.1	6,54	6,44	62,5	13,65	48,14
F.2	5,77	5,68	58,4	12,7	46,56

Table 3.4: Residues analysis

Observing the data, the hydrolysates have a good repeatability. In the solid residues, currently considered as waste, it is noted that there is a good percentage of total and organic nitrogen. For this reason, they can be reused, after grinding treatment, to produce, for example, fertilizers or other solid biostimulants, thus implementing the policy of the circular economy. In this case the process would have null rejects.

Figure 3.2: Castor hydrolysate

3.2.1 Process Standardization

To demonstrate process standardization, all products with the same recipe are considered. The graphs of pH and Brix degree monitored during the reaction with the same time intervals are shown below:

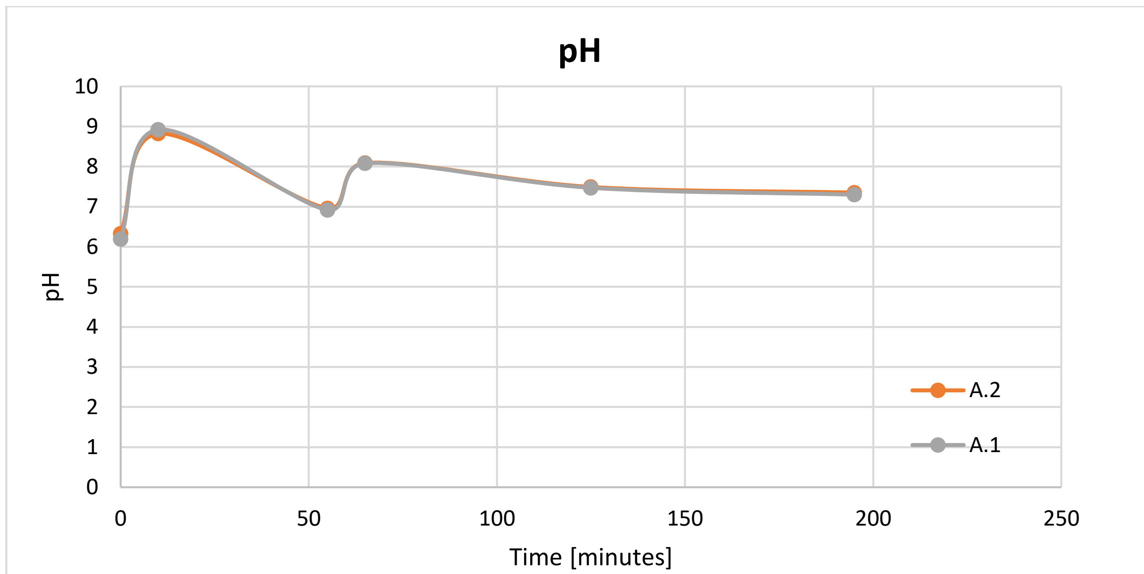


Figure 3.2: pH of products A

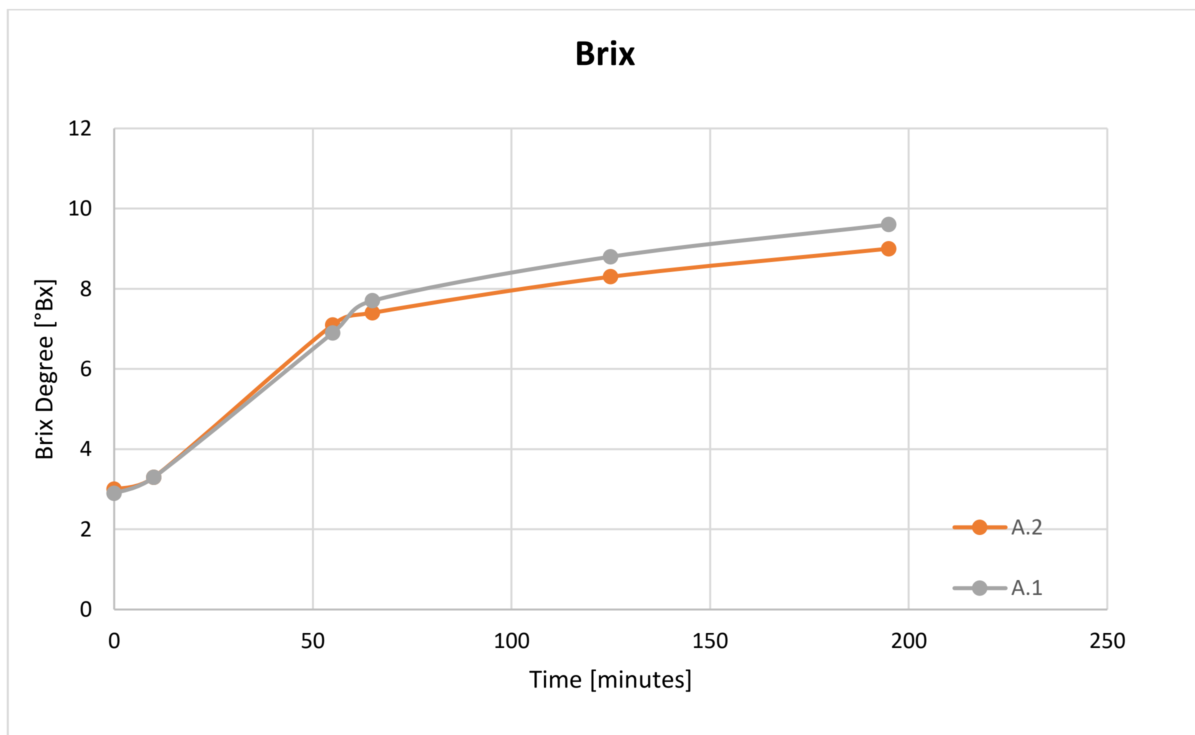


Figure 3.3: Brix Degree pf products A

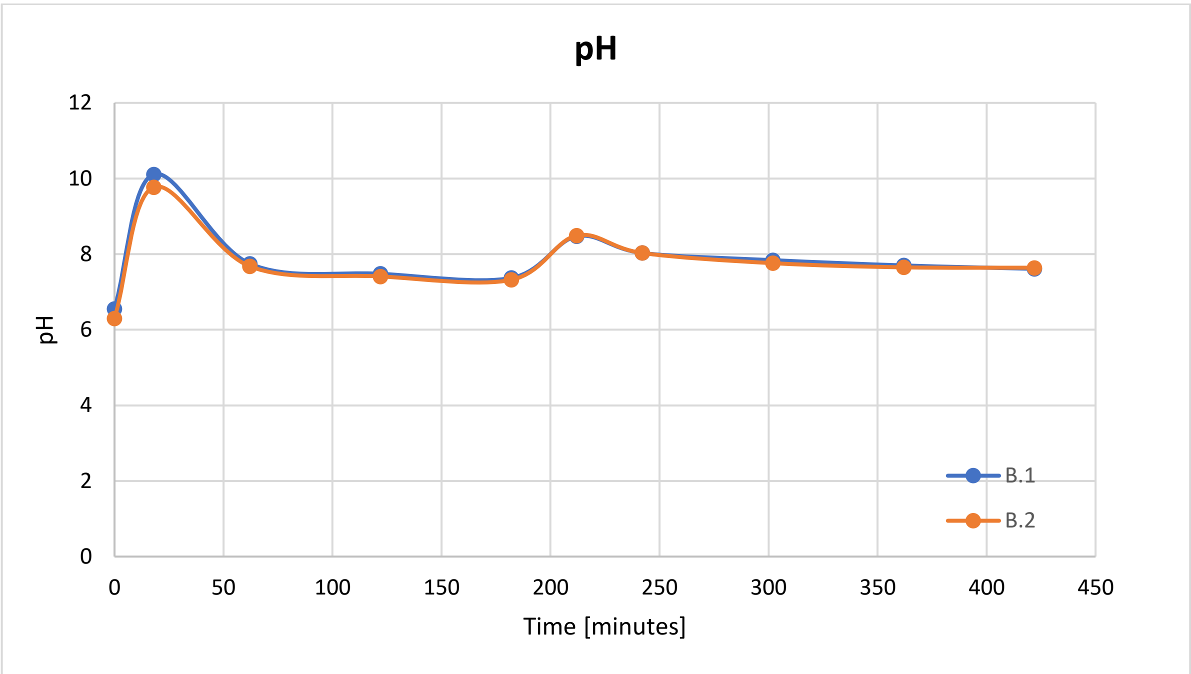


Figure 3.4: pH of products B

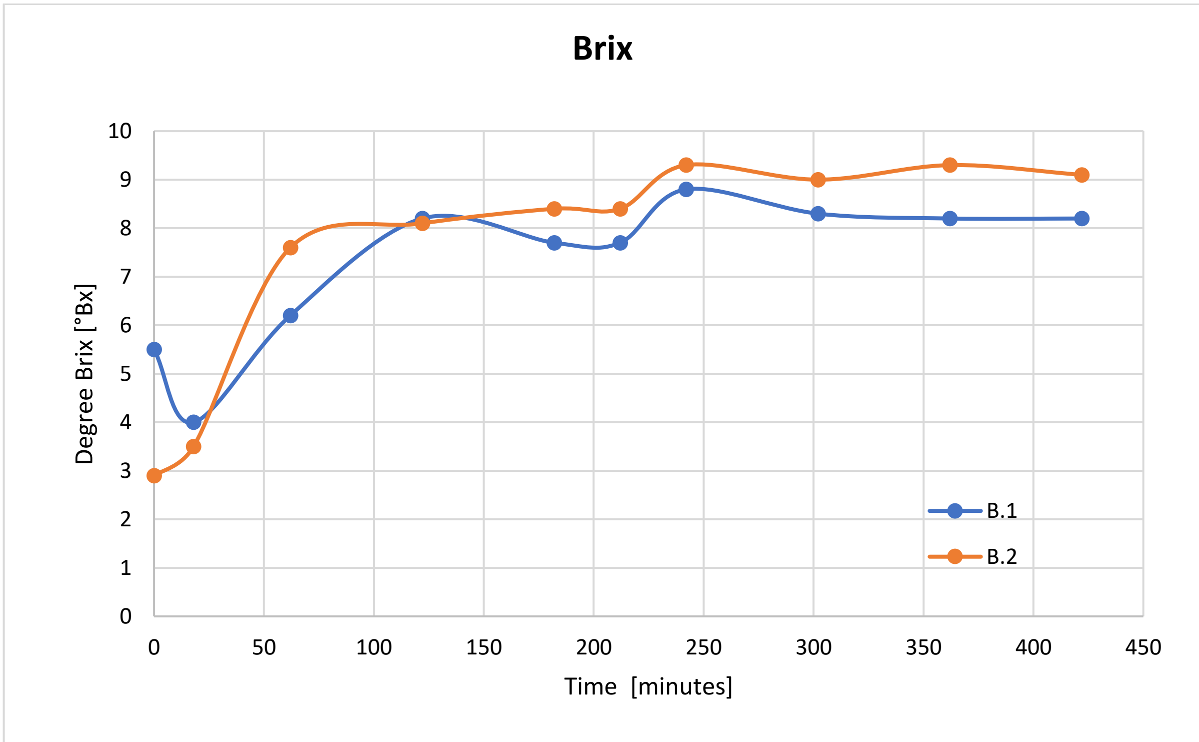


Figure 3.5: Brix Degree pf products B

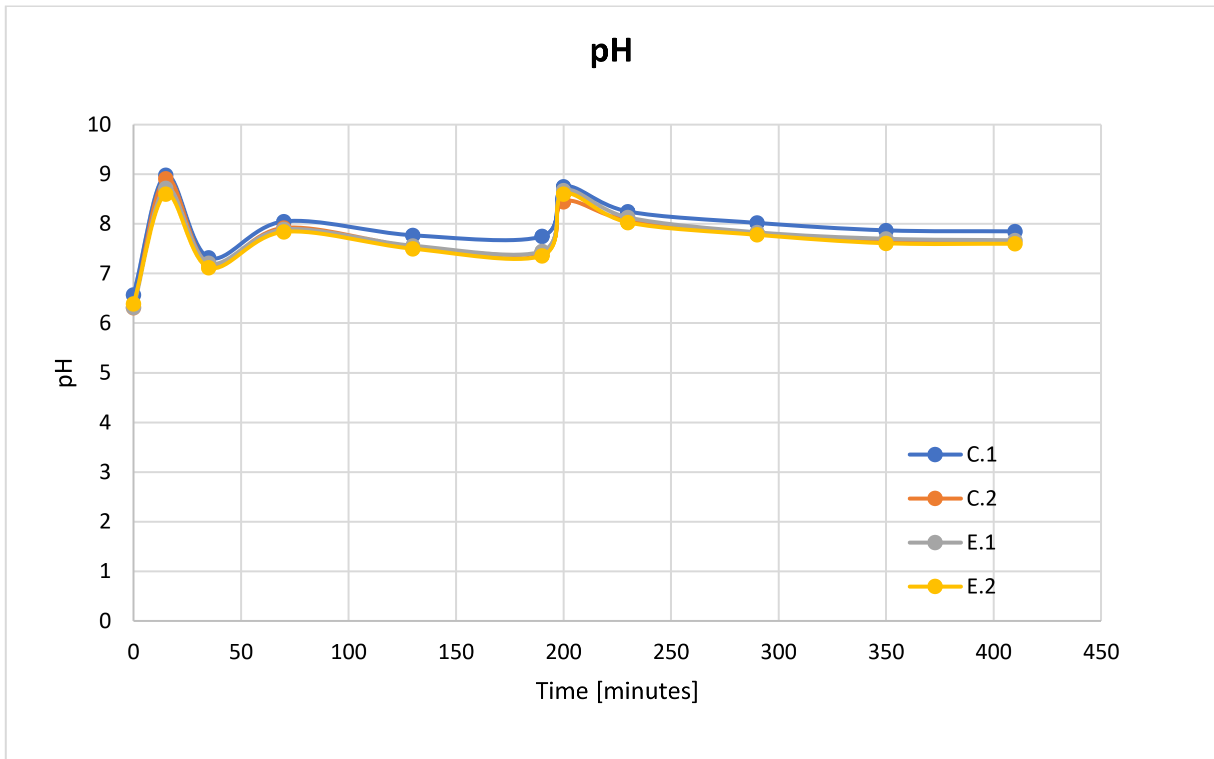


Figure 3.6: pH of products C and E

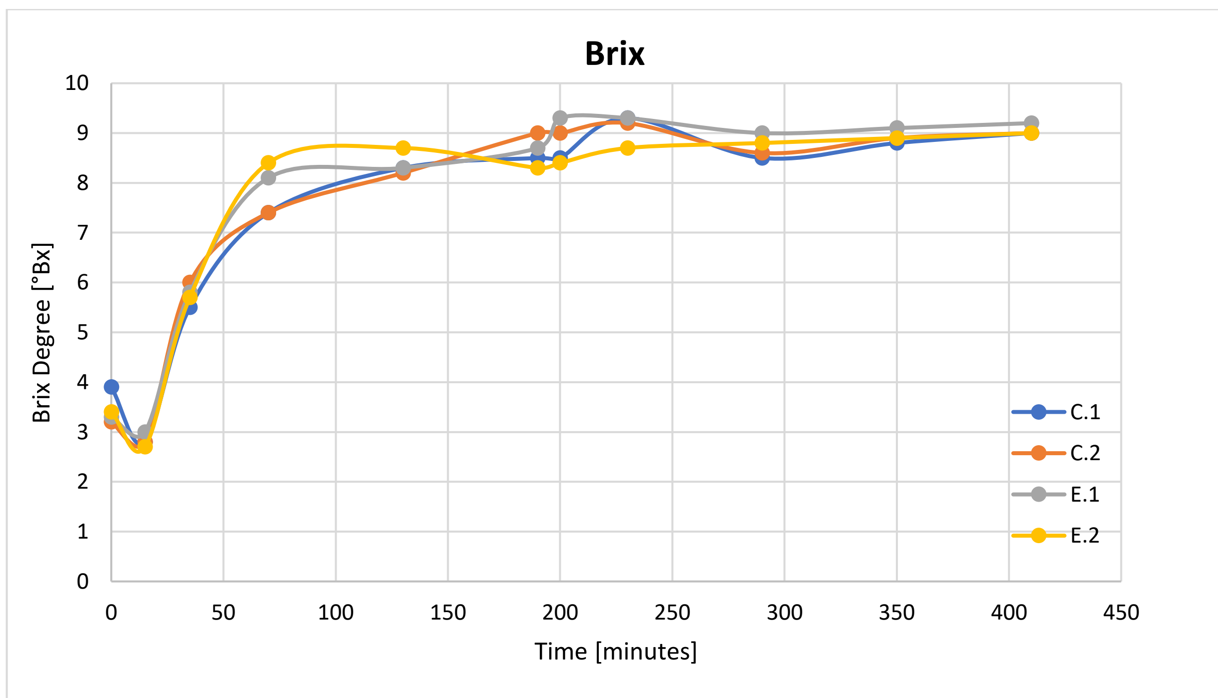


Figure 3.7: Brix Degree of products C and E

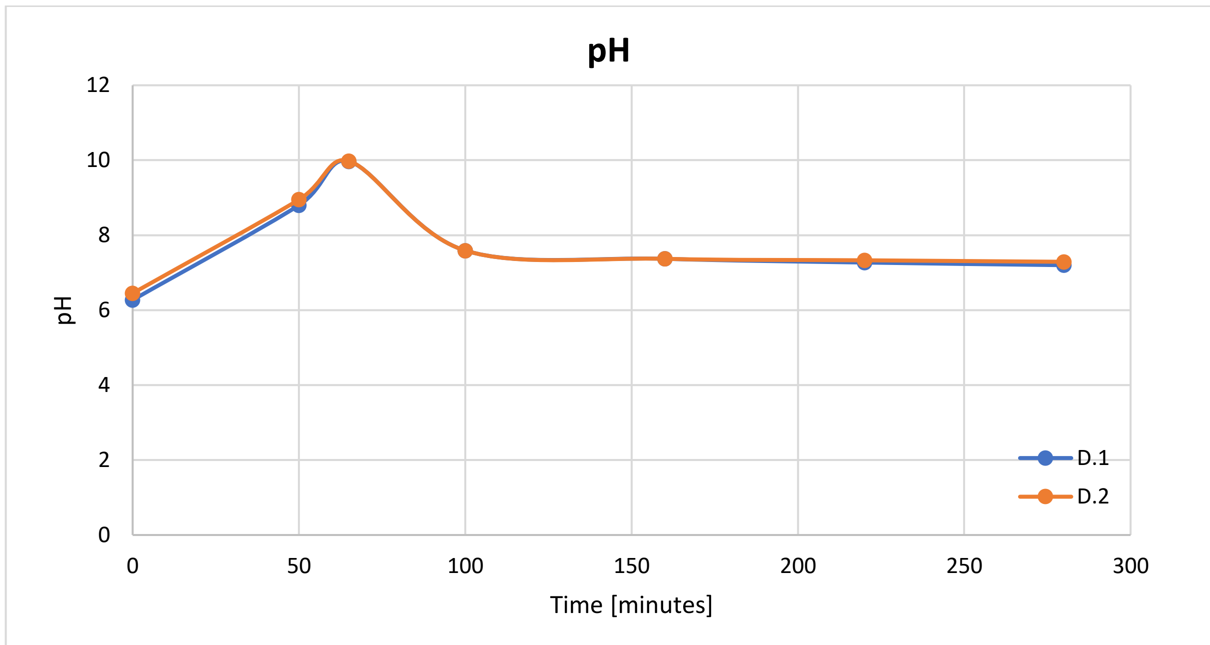


Figure 3.8: pH of products D

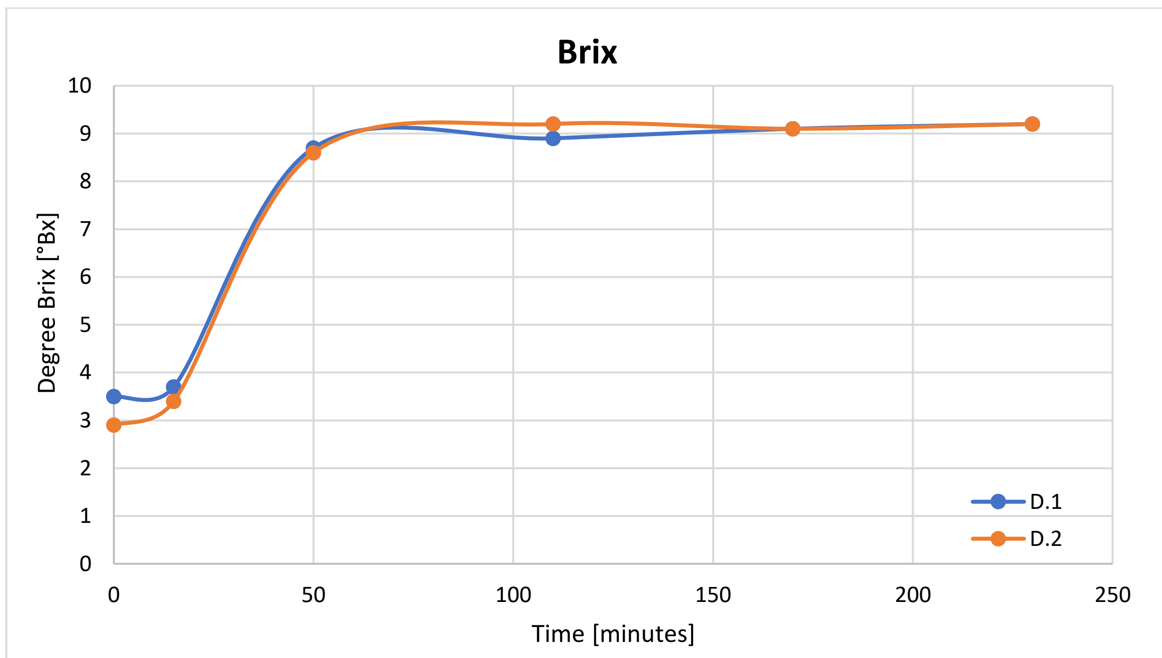


Figure 3.9: Brix Degree of products D

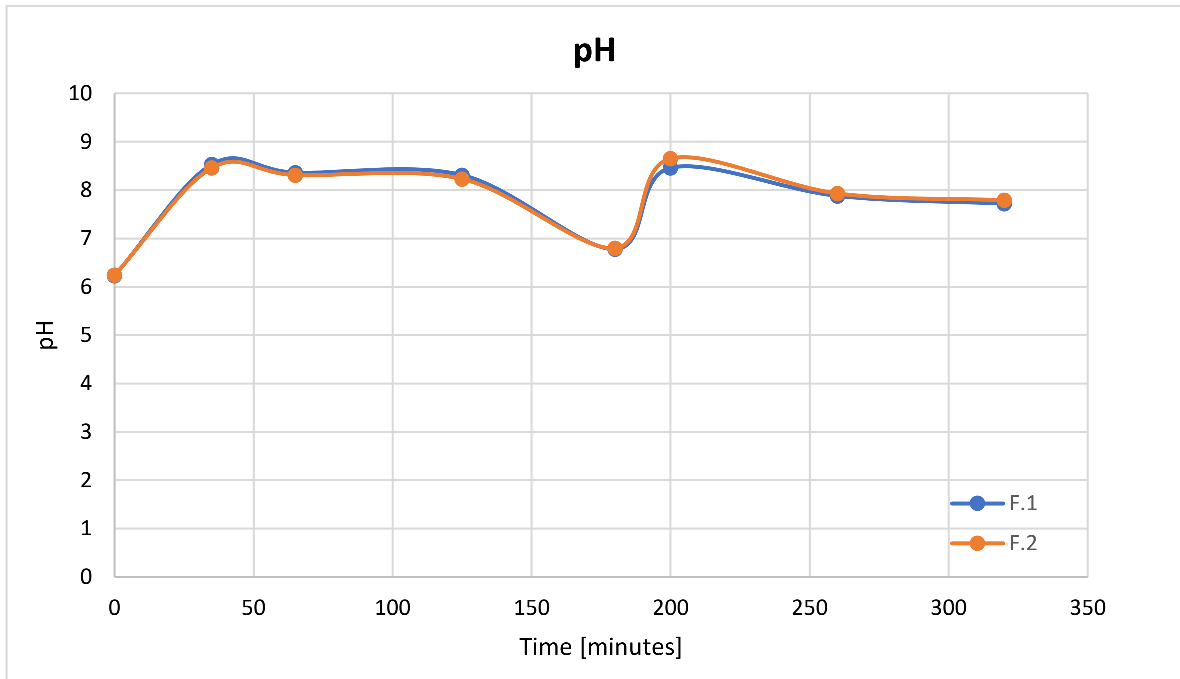


Figure 3.10: pH of products F

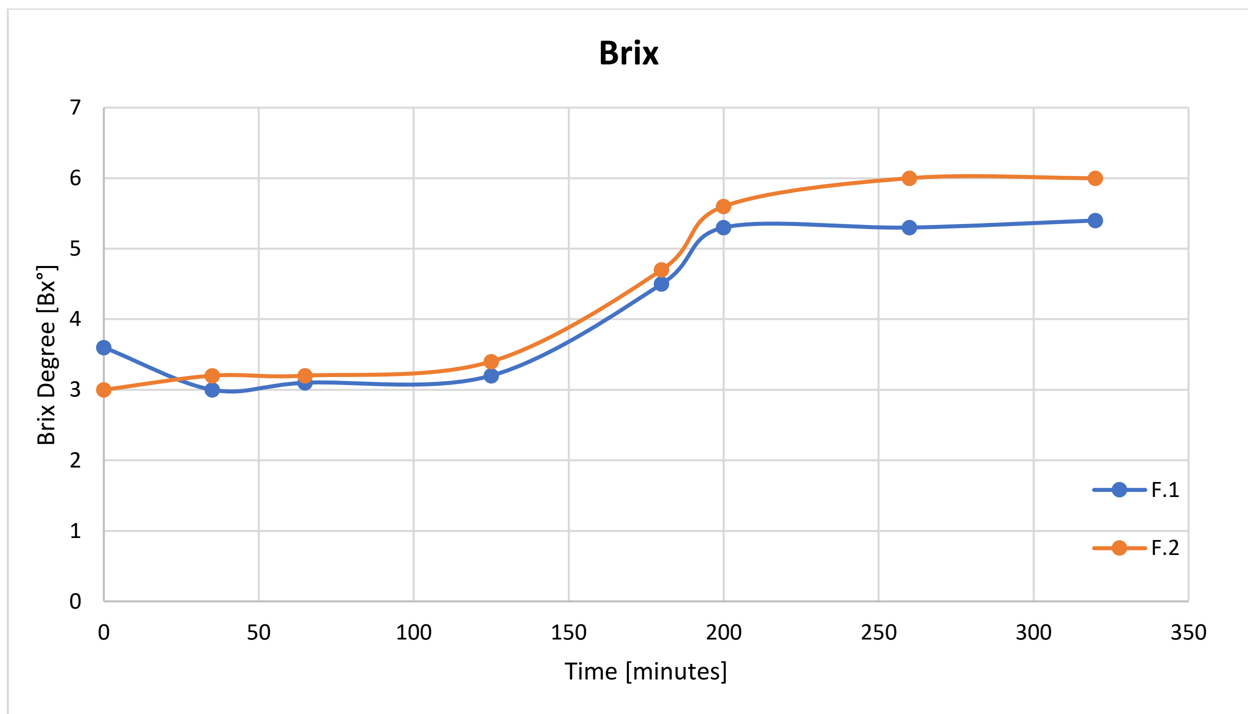


Figure 3.11: Brix Degree of products F

From the graphs presented it can be seen that the pH trend is almost the same for the samples, while the Brix degree has some slight variations, but the overall trend is the same. These variations could be due to non-homogeneity of the sample and from the sampling operation during the reaction.

As a first result it can be said that there is a good standardization of the process.

3.2.2 Process yields

To compare the performance of the different hydrolysates obtained, three yield indicators were used defined as follows:

- Yield of saleable product: it represents the quantity of product that can be sold compared to the quantity of raw material used and the formula is:

$$\frac{Liquid\ out\ [g]}{Castor\ powder\ mass\ in\ [g]} * 100 \tag{3.1}$$

- Weight Yield: it represents the quantity of product obtained by weight and the formula is:

$$100 - \left(\frac{Dry\ solid\ residue\ out}{Dry\ castor\ powder\ mass\ in} * 100\right) \tag{3.2}$$

- Nitrogen Yield: it represents the amount of total nitrogen present in the product compared to the total nitrogen present in the raw material and the formula is:

$$\frac{N\ tot\ Liquid\ out}{N\ tot\ castor\ powder\ mass\ in} * 100 \tag{3.3}$$

The following results were obtained:

Products	Yield of saleable product [%]	Weight Yield [%]	Nitrogen Yield [%]
A	71,78	47,92	57,05
B	67,67	43,51	54,11
C	75,06	46,02	59,72
D	58,01	46,87	44,75
E	89,37	58,96	67,87
F	51,33	24,80	35,79

Table 3.5: Liquid product yield indicators

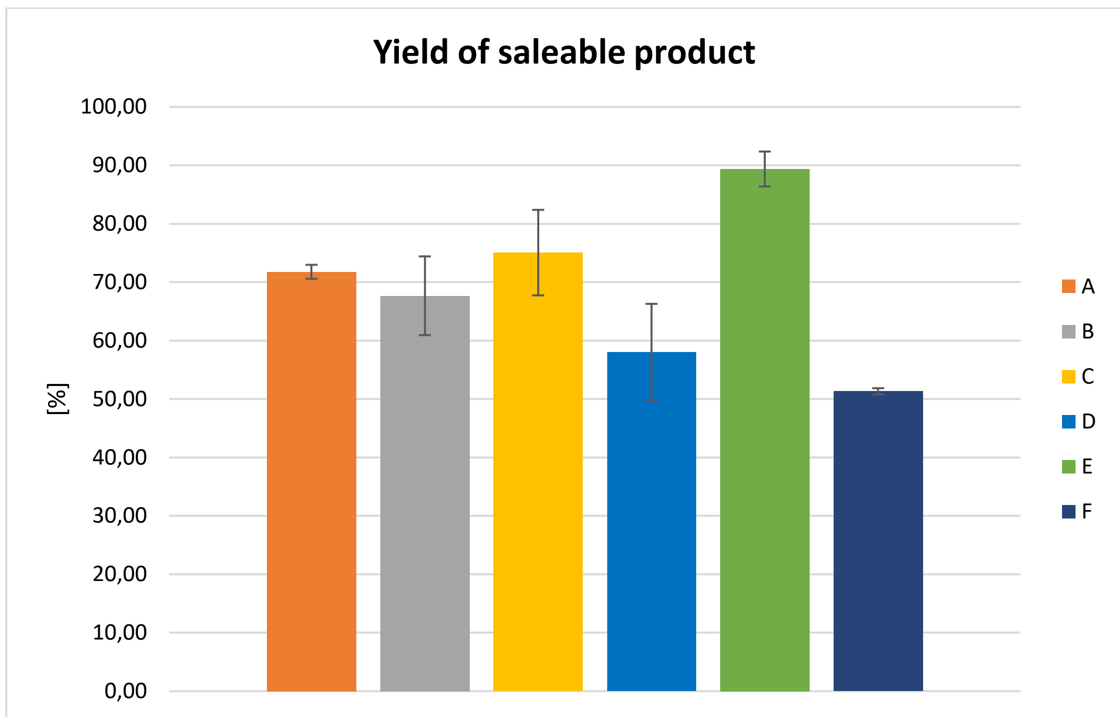


Figure 3.12: Yields of saleable liquid products

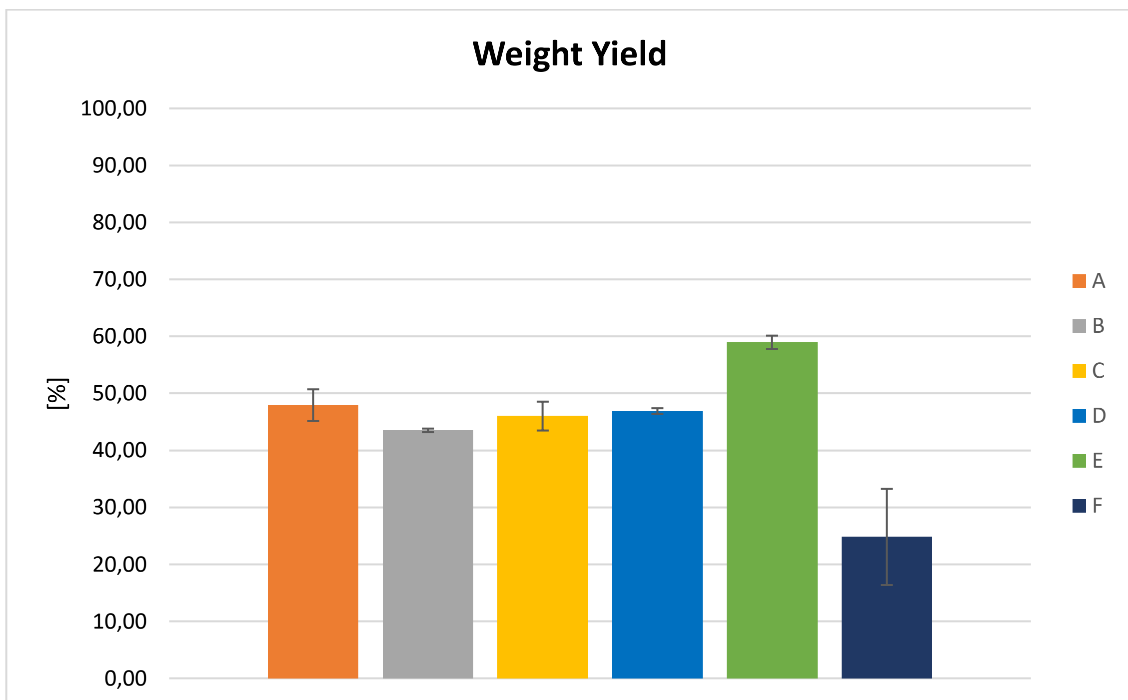


Figure 3.13: Weight yields of liquid products

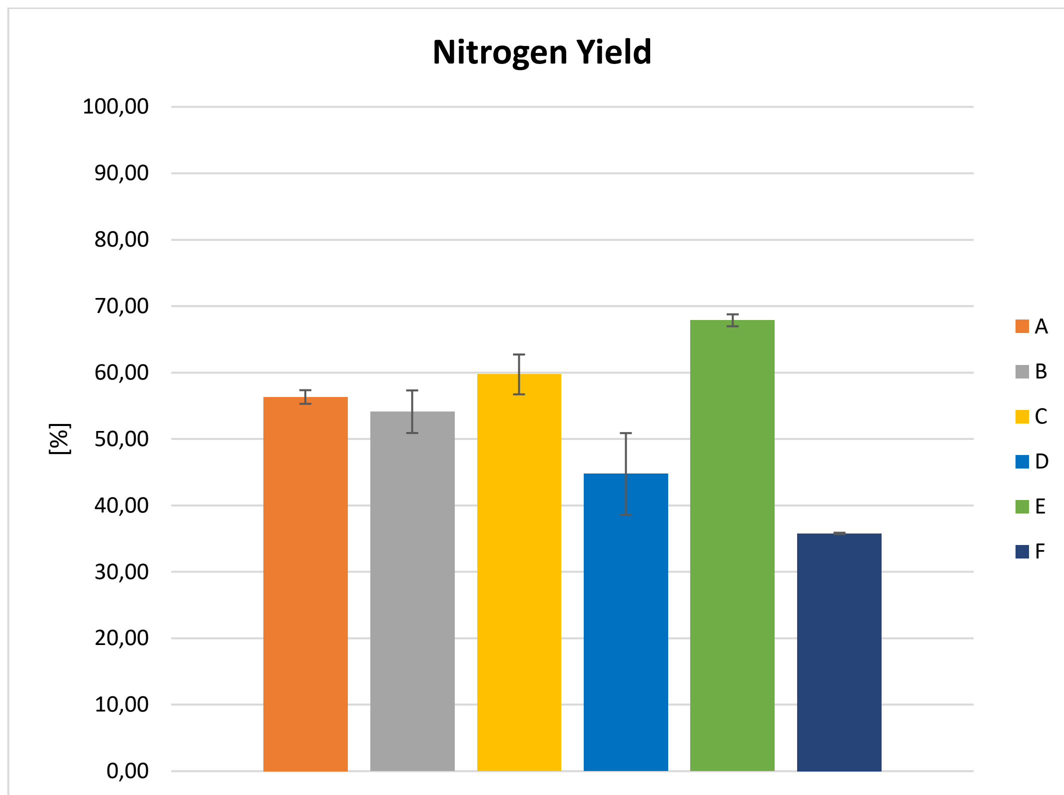


Figure 3.14: Nitrogen yields of liquid products

The best product turns out to be product E, this is because with the use of the filter press for the liquid-solid separation we are able to obtain more liquid to be concentrated and therefore more product. The worst product is product F, made with the semi-batch process.

Another parameter is used to compare the products with each other: the degree of hydrolysis. For the calculation of the degree of hydrolysis with the method presented above, three different measurements were collected:

Products	Measure 1 [%]	Measure 2 [%]	Measure 3 [%]	Average [%]	Standard deviation
A.1	31,65	33,57	33,13	32,78	1,00
A.2	49,23	48,77	49,57	49,19	0,40
B.1	41,37	40,67	39,31	40,45	1,05
B.2	43,24	41,15	42,29	42,23	1,04
C.1	44,35	45,98	45,01	45,12	0,82
C.2	30,84	31,28	30,74	30,95	0,28
D.1	21,24	22,69	23,66	22,53	1,21
D.2	30,78	28,58	30,72	30,03	1,25
E.1	30,54	29,59	31,26	30,46	0,84
E.2	33,29	25,15	30,11	29,52	4,10
F.1	21,32	19,68	20,37	20,46	0,83
F.2	25,76	24,03	23,58	24,46	1,15

Table 3.6: Hydrolysis degree of products

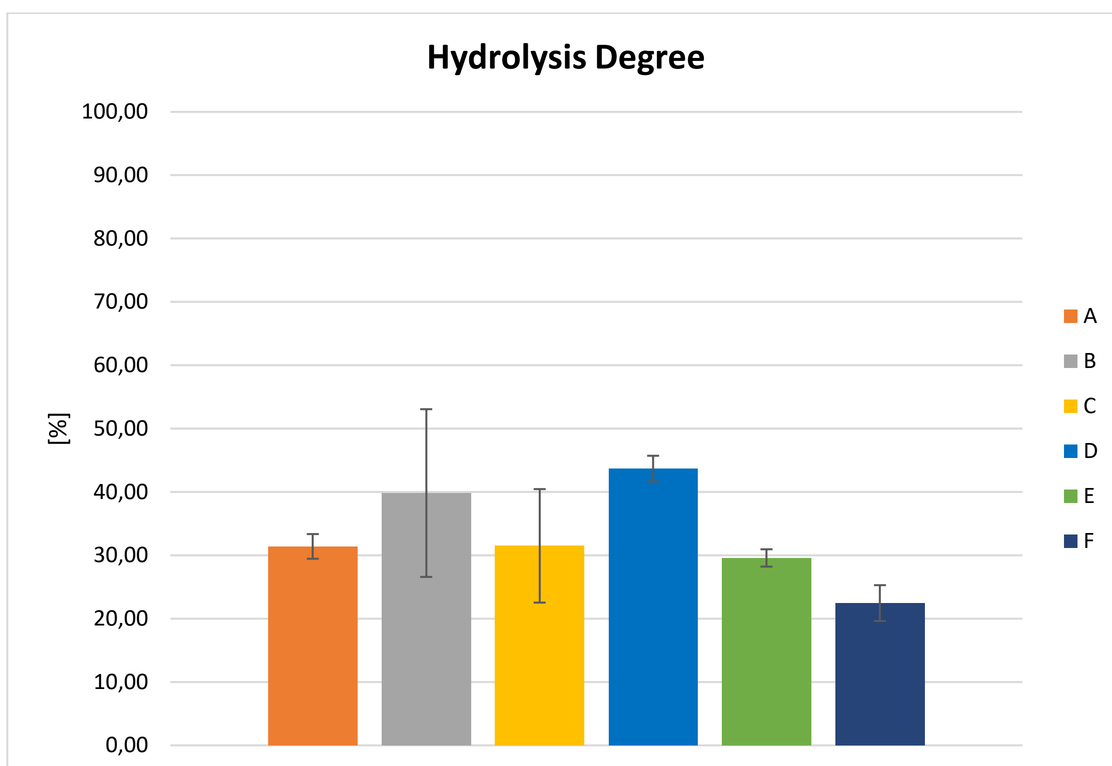


Figure 3.15: Hydrolysis degree of products

Product D has the highest degree of hydrolysis; this means that the peptide bonds have been cut more and it has a lower molecular weight than the others. A lower molecular weight means it is easier to assimilate nutrients for plant metabolism. Product F has the lowest degree of hydrolysis, which means that the peptide chains have been cut less and therefore it will generally have a higher molecular weight. The measurement of the degree of hydrolysis is correlated through inverse proportionality to the molecular weight, which is why it indirectly provides us with information about it.

The second principle of green chemistry presents some chemical metrics for the evaluation of the process and one of this is the E factor (environmental factor), which is simple metric of how "green" a reaction is. It is defined as the ratio of the mass of waste per mass of product:

$$E\ factor = \frac{mass\ of\ total\ waste}{mass\ of\ product} \tag{3.4}$$

The wastes considered in the calculation are only those of the process as there are no compounds necessary for the abatement or treatment. The lower the number of this factor, the closer the process is to the objective of having a deviation equal to 0.

It was calculated to understand which product was the "greenest" and the following results were obtained:

Product	**E factor**
A	0,67
B	0,75
C	0,65
D	0,80
E	0,41
F	1,34

Table 3.7: Liquid product E factor's

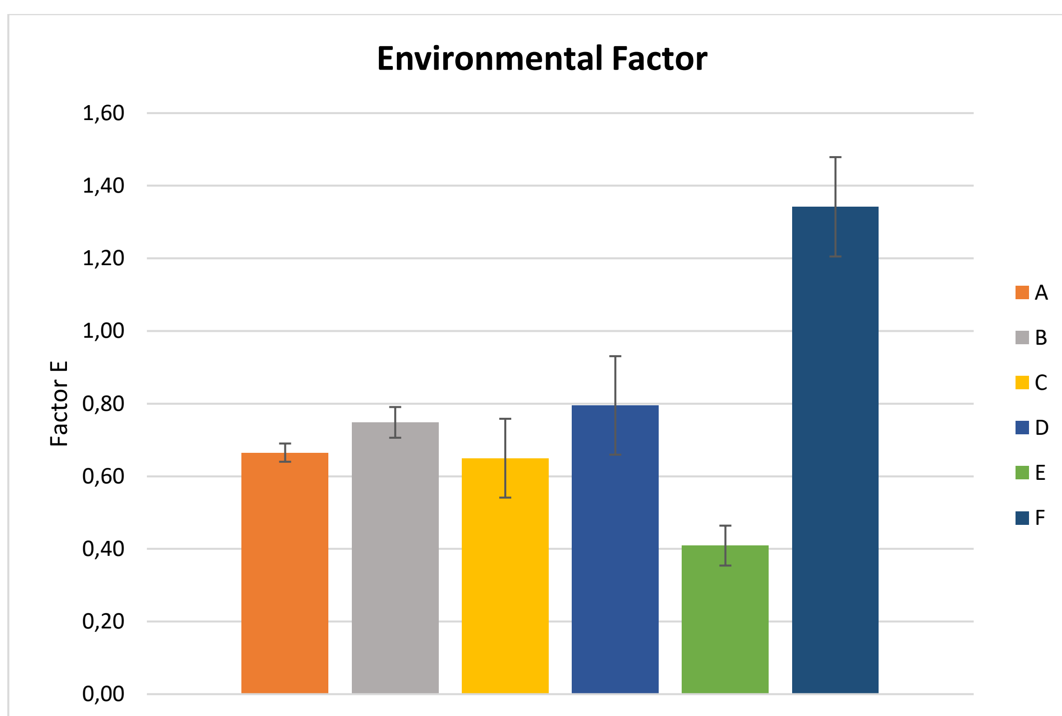


Figure 3.16: E Factor of products

From the calculations it emerges that the best product is E and therefore the most sustainable.

The E factor does not replace the considerations previously seen on the yield indicators of the product of the yield but it is a further consideration that it has been widely implemented by the chemical industry with great impacts on sustainability and also on the economy.

3.2.3 Single enzyme vs combined enzymes

The first target conducted was the comparison between two different recipes, one with the use of a single protease and the other with the use of two different proteases.

In the recipe with the single enzyme after introducing the basifying agent, the enzyme is introduced. After about 1 hour it is necessary to further basify in order to create the right environment for the enzyme to work. It is noted that the enzyme is able to work for about 3 hours after being inserted. The overall process takes about 4 hours.

In the recipe with the combination of two different enzymes, after introducing the basifying agent the first enzyme is introduced. After about 3 hours, the basic environment is recreated and the second enzyme is introduced and will work for the next 3 hours. The overall process takes about 7 hours.

To evaluate which of the recipes is better, the yields were considered and the following results were obtained:

Products	Yield of saleable product [%]	Weight Yield [%]	Nitrogen Yield [%]
A.1	70,93	49,89	55,6
A.2	72,62	45,95	57,05
B.1	62,91	43,73	51,84
B.2	72,44	43,28	56,38
C.1	67,74	43,49	56,72
C.2	82,37	48,55	62,72
D.1	52,16	46,51	40,41
D.2	63,87	47,24	49,09
E.1	92,36	60,14	68,77
E.2	86,38	57,78	66,97

Table 3.8: Liquid product yield indicators

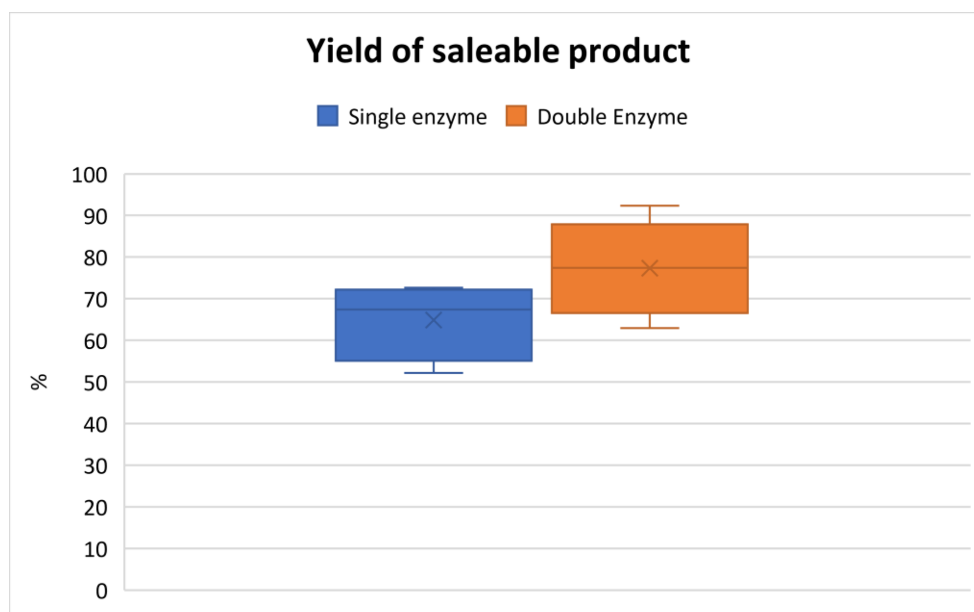


Figure 3.17: Yield of saleable product of single enzyme vs combined enzymes

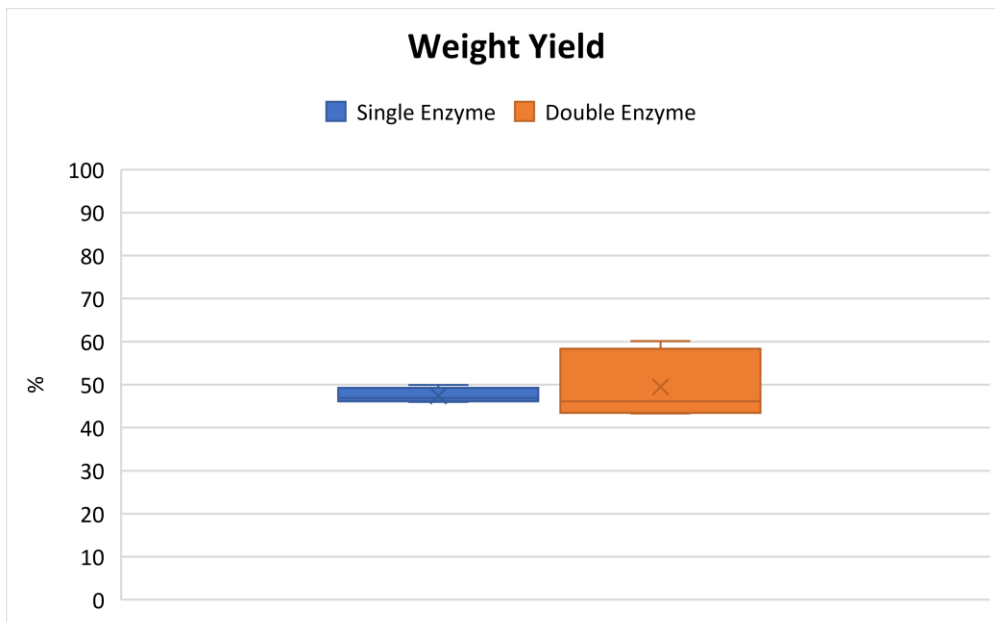


Figure 3.18: Weight yield of single enzyme vs combined enzymes

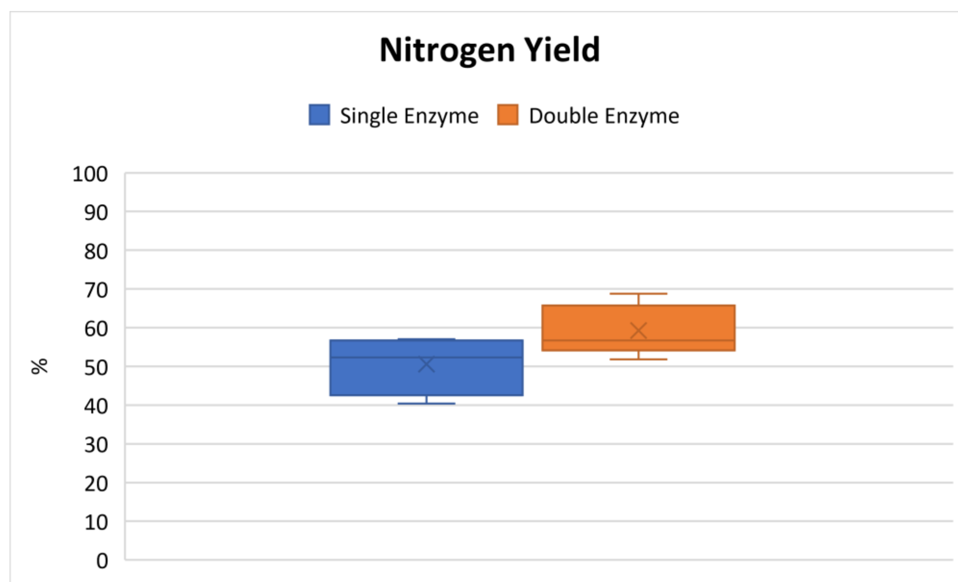


Figure 3.19: Nitrogen yield of single enzyme vs combined enzymes

From the box plots obtained the recipe using the combination of several proteases is better than using the single enzyme. The combination of enzymes is better because the endoproteases used fragment the polypeptide chain of the castor in a different and combined way, managing to fragment much more than using the single enzyme.

3.2.4 Centrifuge vs filter press

The second target was carried out maintaining the same recipe but changing a process parameter, the method for separating the liquid from the solid.

The first test was conducted with the use of the centrifuge while the second with the use of the filter press as described in chapter 2.

To evaluate which test is better, the yields were considered and the following results were obtained:

Products	**Yield of saleable product [%]**	**Weight Yield [%]**	**Nitrogen Yield [%]**
C.1	67,74	43,39	56,72
C.2	82,37	48,55	62,72
E.1	92,36	60,14	68,77
E.2	86,38	57,78	66,97

Table 3.9: Yield indicators of product C and E

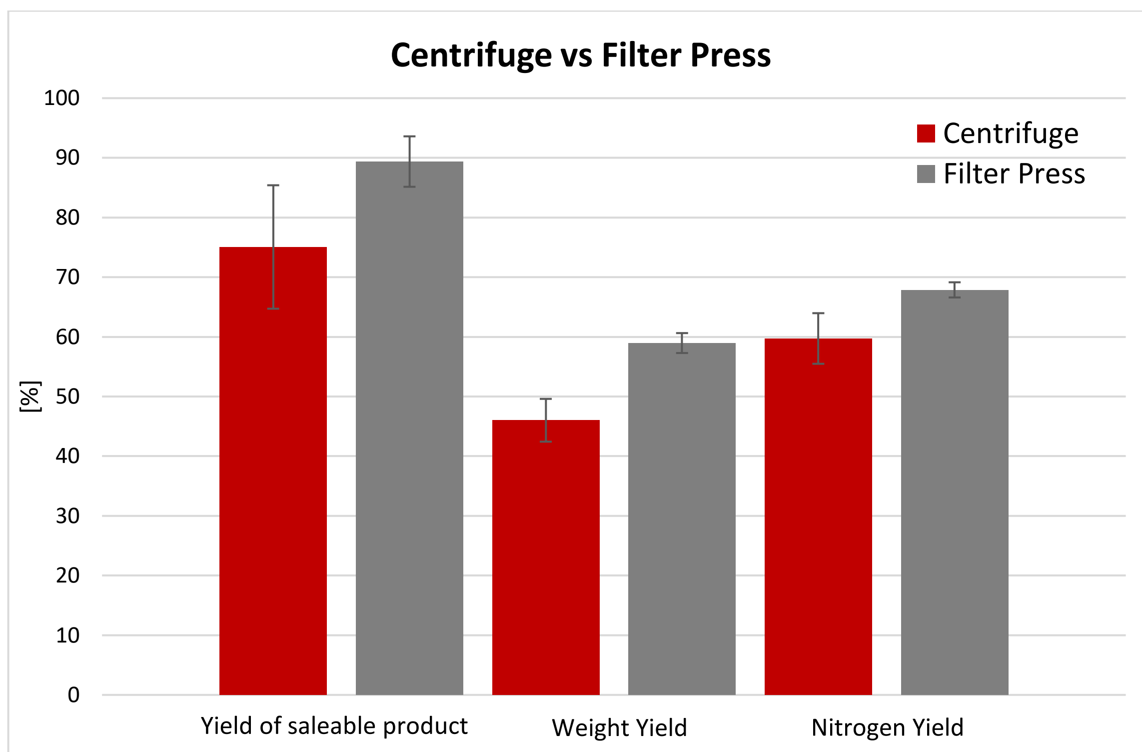


Figure 3.20: Comparison of yield indicators relating to the use of centrifuge vs filter press

All three yields show that the use of the filter press is much better than the use of the centrifuge, this is because with the use of the filter press it is possible to obtain a less humid solid and therefore it is possible to extract more liquid to concentrate.

3.2.5 Optimization of alkalizing agent quantity

The third target was carried out by modifying the quantity used to carry out the alkaline hydrolysis. 10% more calcium hydroxide was used in D products than in A products.

To evaluate which recipe is better, both the process parameters pH and Brix degree and the yields were considered, and the following results were obtained:

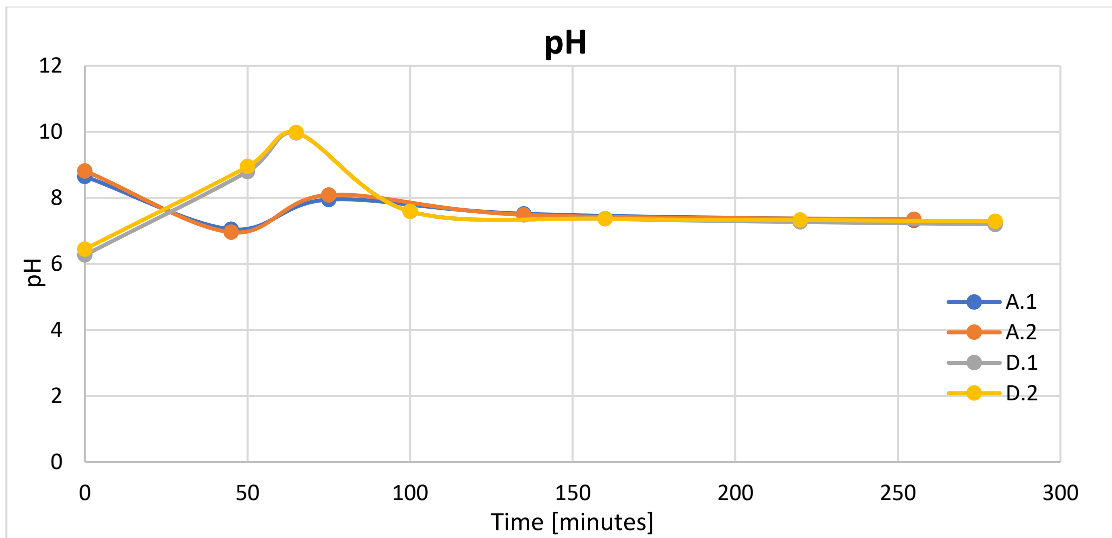


Figure 3.21: pH of products A and D

By using a greater quantity of basifying agent, it is noted that a peak is obtained where the reaction environment is more basic which then stabilizes. This represents a disadvantage for the protease used because it exceeds the upper limit of its optimal pH range.

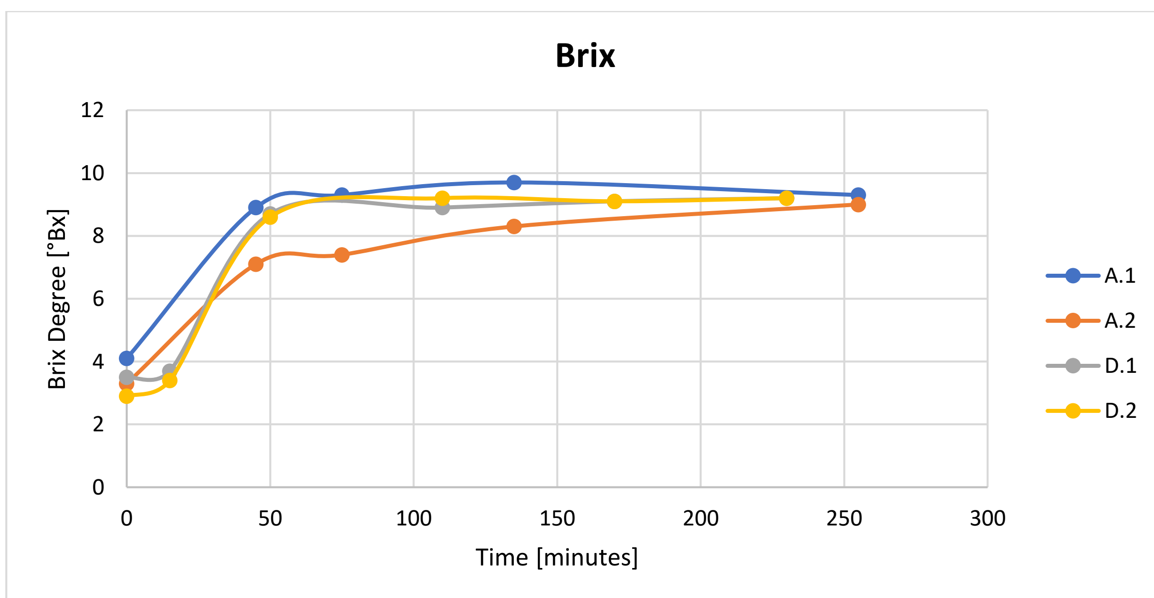


Figure 3.22: Brix Degree of products A and D

The trend of Brix, on the other hand, is almost the same despite the variation in the quantity of the alkalizing agent.

Products	Yield of saleable product [%]	Weight Yield [%]	Nitrogen Yield [%]
A.1	70,93	49,95	55,6
A.2	72,62	45,95	57,05
D.1	52,16	46,51	40,41
D.2	63,87	57,24	49,09

Table 3.10: Yield indicators of product A and D

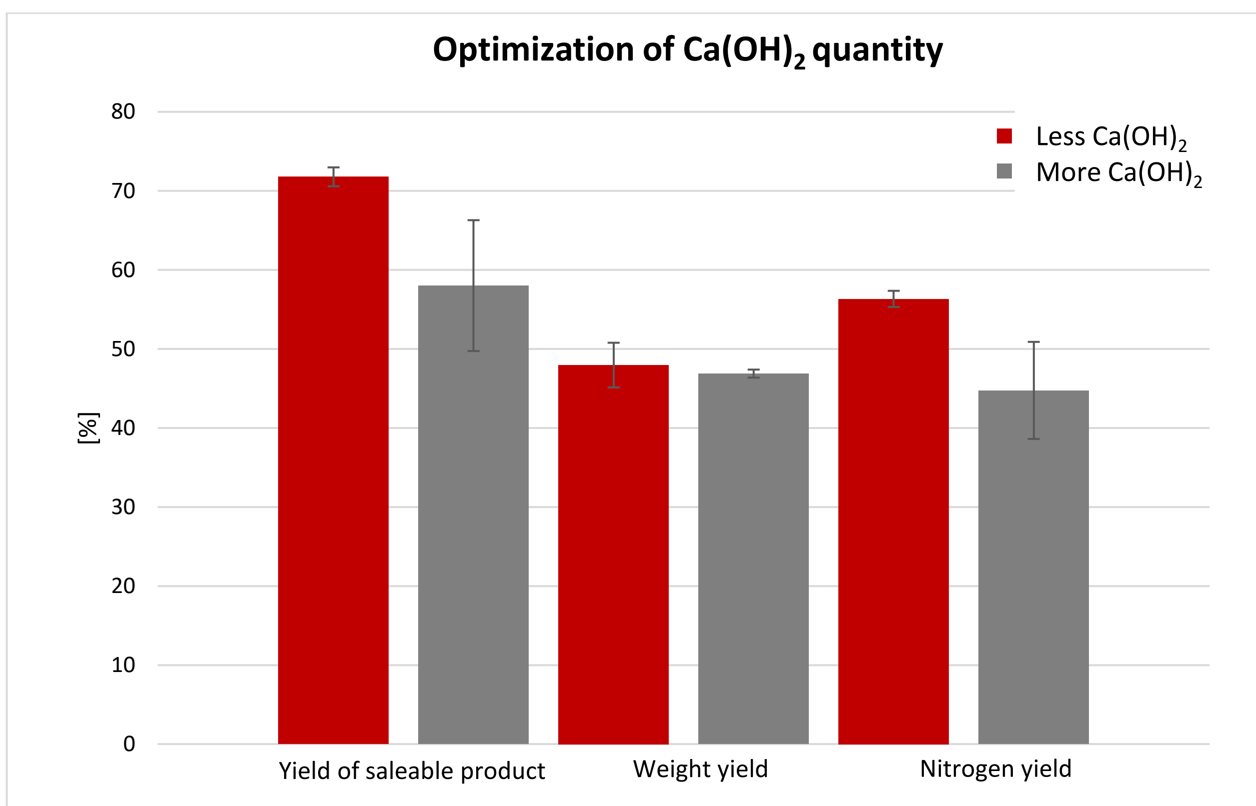


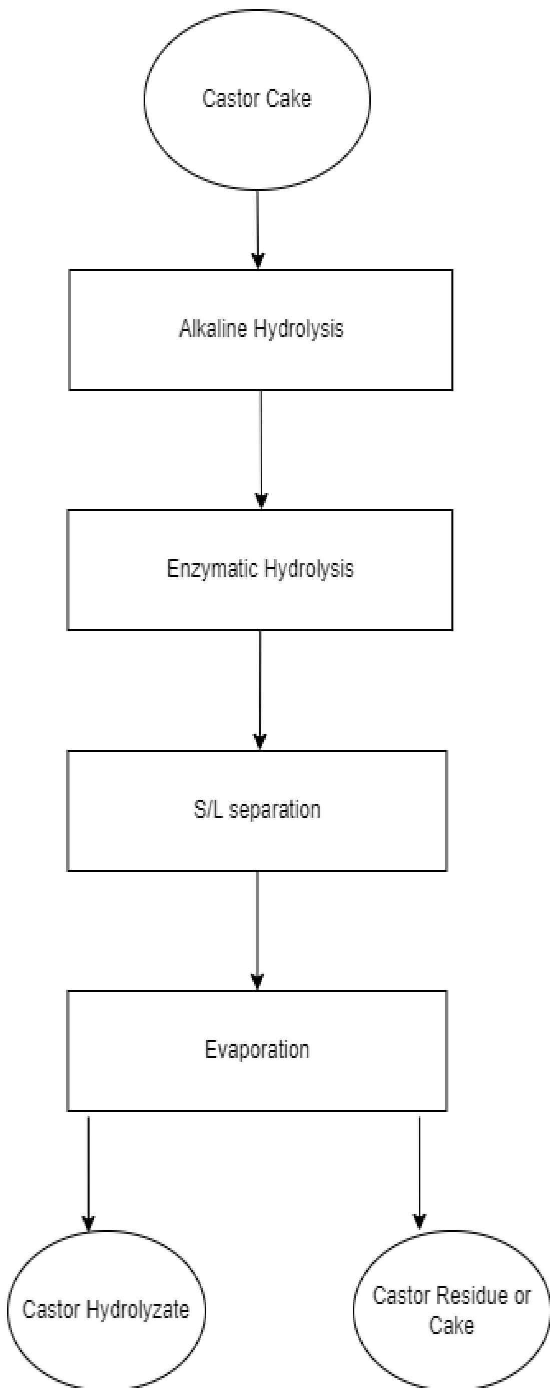
Figure 3.23: Comparison of yield indicators relating to the optimization of $Ca(OH)_2$ quantity

From the graph it is concluded that by using less calcium hydroxide, a product with better yields is obtained. This is true because increasing the amount of basifying agent raises the pH above the optimal range established for the enzyme used and probably other reactions occur during the hydrolysis that subsequently hinder the subsequent L / S separation phase. By increasing the amount of calcium hydroxide from an agronomic point of view it has also been noted that more ash is produced.

3.2.6 Batch vs Semi-Batch

The fourth target consists in modifying the execution of the process by conducting in a batch or semi-batch way.

The two processes are illustrated below with the help of two flow charts:



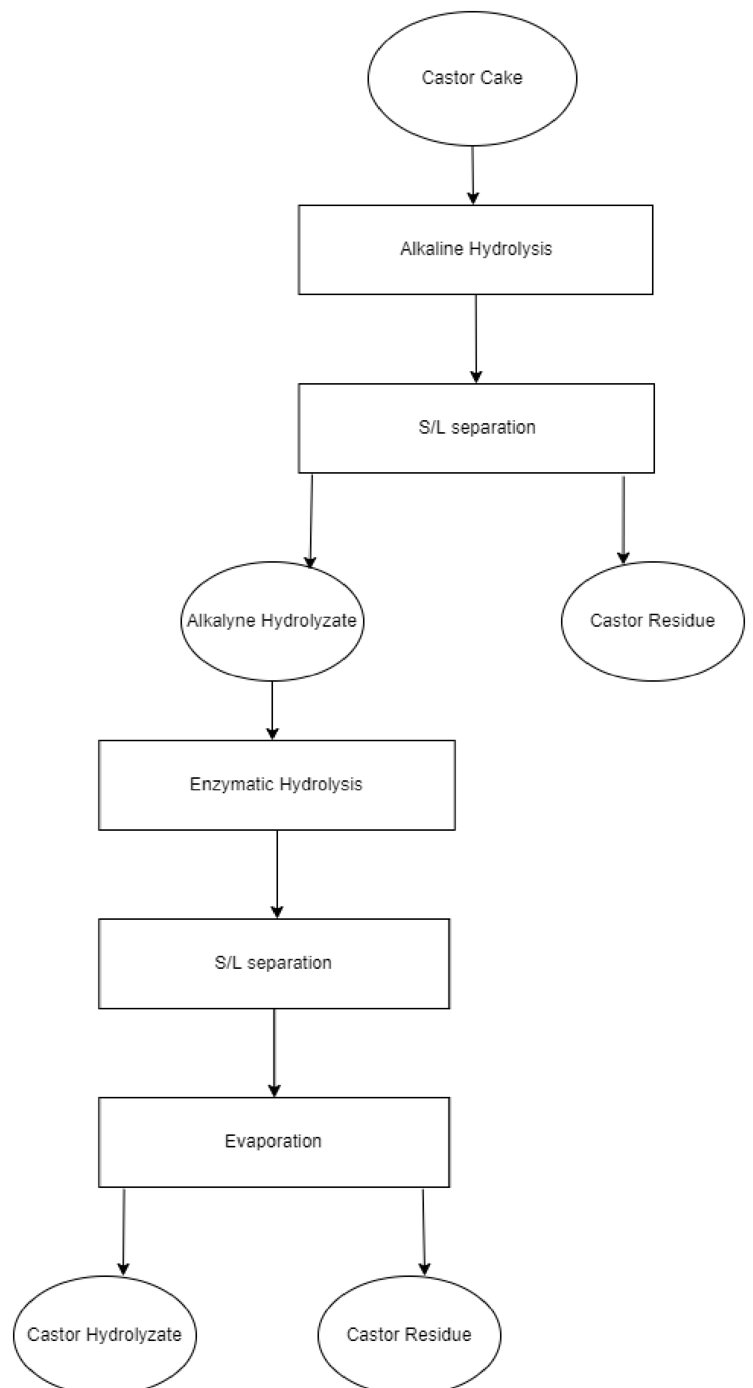


Figure 3.24: Flow chart of Batch Process

Figure 3.25: Flow chart of Semi-Batch process

The substantial difference between batch and semi-batch process lies in the fact that at the end of the alkaline hydrolysis there is the S/L separation process and the enzymatic hydrolysis is subsequently carried out only on the liquid.

To evaluate which of the two processes is the best, the yields and the degree of hydrolysis are taken into consideration:

Products	Yield of saleable product [%]	Weight Yield [%]	Nitrogen Yield [%]	Hydrolysis Degree [%]
A.1	70,93	49,95	55,60	32,78
A.2	72,62	45,95	57,05	30,03
F.1	50,95	30,77	35,71	20,46
F.2	51,70	18,83	35,86	24,46

Table 3.11: Yield indicators and hydrolysis degree of product A and F

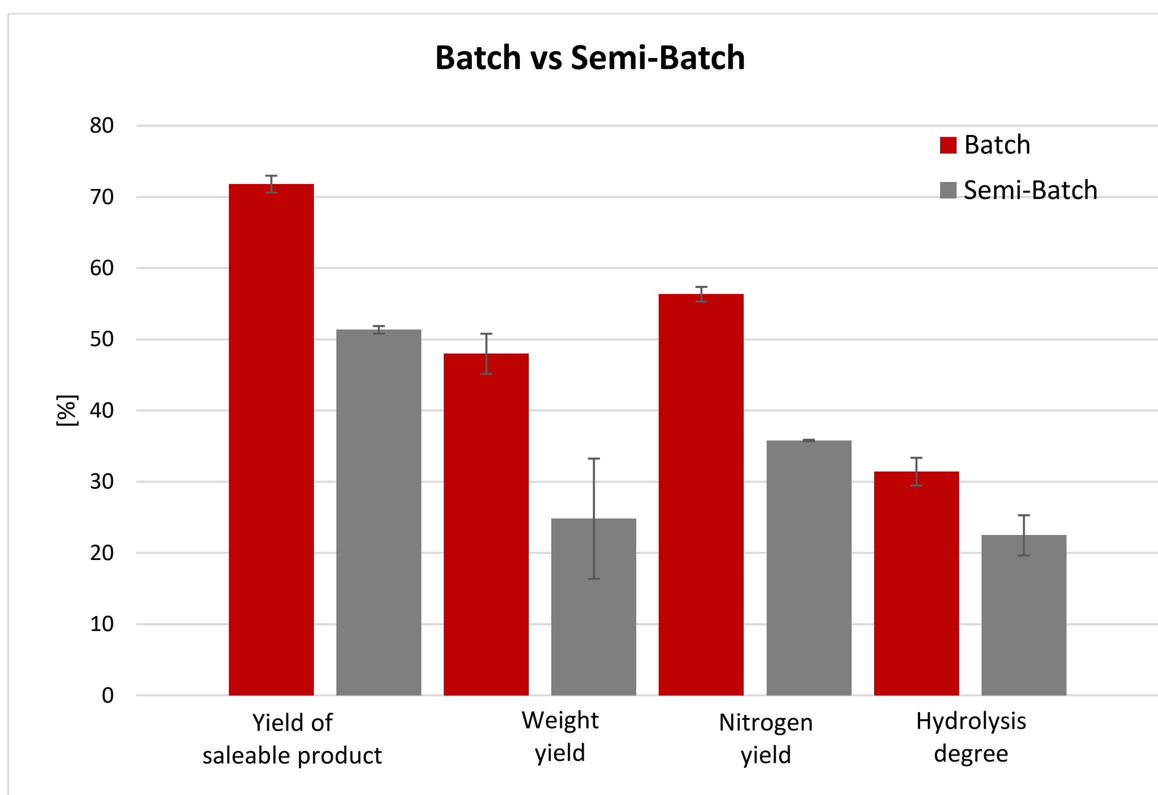


Figure 3.26: Comparison of yield indicators and hydrolysis degree relating to batch and semi-batch process

The yields show that the batch process is more efficient than the batch process. The degree of hydrolysis is greater in the batch process than in the semi-batch process, meaning that the enzymes have been able to cut the chains more. The semi-batch process is disadvantaged because in the S / L separation phase after alkaline hydrolysis part of the raw material is lost and consequently less product will be obtained.

Conclusion

Worldwide in recent years increasing attention has been paid to environmental issues. At the European level, as previously mentioned within the Green Deal, attention is paid to green chemistry which aims to direct the approach to the chemical industry towards sustainability and ecology. Within this discussion there is also the field of agriculture where better efficiency is constantly sought with the lowest possible environmental impact. New studies on innovative products can represent ecological alternatives to fertilizers, and biostimulants are a representative category of such products. They are used in low dosages allowing assimilation in the metabolic pathways of the plant helping it in its own growth and development. In this context, the development of the process for producing a protein hydrolysate deriving from a by-product of agriculture, the castor cake, is placed. In this context, the study of the process to produce a protein hydrolysate deriving from a by-product of agriculture, the castor cake, is an innovation that needs to be developed. The following parameters were studied:

- Single enzyme vs combined enzymes;
- Centrifuge vs filter press;
- Optimization of basifying agent quantity;
- Batch process vs Semi-Batch process.

Some key process parameters were set before starting the practical tests while others were tested and matured thanks to the observations conducted during the tests. 6 different products were created and analysed in duplicate for a total of 12 protein hydrolysates. To complete all the stages of the process and obtain the finished product, it takes an average of 1 week per product. The duplicate test was used to verify the repeatability of the process both for the process and for the physical and chemical properties analysed. A good process standardization was obtained from the tests carried out. It has been seen that the use of the combination of several enzymes, the use of the filter press, the use of a smaller quantity of alkalizing agent combined with a batch process are the best process and product parameters. The focus was not only on the part of process optimization but also on the part of sustainability and ecology. In particular, an attempt was made to develop a process that respected as much as possible the twelve principles of green chemistry.

After carrying out the chemical and physical analyses on the solid residue, it was found that it has good potential for use indeed. Thanks to the quantity of nitrogen present, albeit in a smaller quantity than the respective liquid, it is possible to note that even the solid could have a use as a fertilizer. In this way, the first and second principles of green chemistry would be fully respected because the process would have E factor equal to zero and would have no waste.

The third and fourth principle is respected as the castor panel contains ricin which is a toxic substance but after being treated with enzymatic alkaline hydrolysis it has been verified that in the liquid and in the solid there is no ricin.

The fifth principle has been respected as no solvents or auxiliaries are used to conduct the reaction.

The sixth principle is partially respected as the reaction is carried out at ambient pressure but not at ambient temperature, even thought if not at high temperatures are not necessary.

The seventh principle is respected as castor cake is a by-product of castor oil processing and has been defined as renewable biomass.

Principle eight is respected because there are no derivatives.

Principle nine is respected as a biocatalyst with enzymes is used.

The tenth principle is respected as the hydrolysates, respecting their main function, will be absorbed by the plants in a harmless way and will not persist in the environment.

The products produced will then be studied ***in vivo*** on plants, in order to verify their effectiveness as biostimulants and the potential effect on plants. If the results are efficient, the study presented in this thesis represents the first step for the scale-up of a hydrolysis plant for the production of castor--based biostimulants in an ecological and sustainable way as demonstrated by the respect of most of the principles of green chemistry.

Bibliography

1. Luciano Cavani, Alja Margon, Luigi Sciubba, Claudio Ciavatta, and Claudio Marzadori. (2017). What we talk about when we talk about protein hydrolyzate-based Biostimulants. AIMS Agriculture and Food, doi: 10.3934/agrfood.2017.3.221

2. Giuseppe Colla, Serenella Nardib, Mariateresa Cardarellic, Andrea Ertanib, Luigi Lucinid, Renaud Canaguiere, Youssef Rouphaelfa (2015). Protein hydrolysates as biostimulants in horticulture. Scientia Horticulturae, http://dx.doi.org/10.1016/j.scienta.2015.08.037

3. Patrick du Jardin. (2015). Plant biostimulants: Definition, concept, main categories and regulation. Scientia Horticulturae, http://dx.doi.org/10.1016/j.scienta.2015.09.021

4. Lin Xu, Danny Geelen. (2018). Developing Biostimulants From Agro-Food and Industrial By-Products. Front. Plant Sci. 9:1567. doi: 10.3389/fpls.2018.01567

5. Andrea Colantoni, Lucia Recchia, Guido Bernabei, Mariateresa Cardarelli, Youssef Rouphael, Giuseppe Colla. (2017). Analyzing the Environmental Impact of Chemically-Produced Protein Hydrolysate from Leather Waste vs. Enzymatically-Produced Protein Hydrolysate from Legume Grains. Agriculture, 7, 62; doi:10.3390/agriculture7080062

6. Cecilia Anzani, Barbara Prandi, Tullia Tedeschi, Chiara Baldinelli, Giovanni Sorlini, Arnaldo Dossena, Stefano Sforza. (2019). Enzymatic hydrolysis as a means of recovering bovine hides: laboratory and medium scale trials, characterization of hydrolysates and scale-up to semi-industrial scale. Detritus / Volume 05 - 2019 / pages 57-65 https://doi.org/10.31025/2611-4135/2019.13782

7. Moses Madende, Maria Hayes. (2020). Fish By-Product Use as Biostimulants: An Overview of the Current State of the Art, Including Relevant Legislation and Regulations within the EU and USA. Molecules, doi:10.3390/molecules25051122

8. Gianluca Caruso, Stefania De Pascale, Eugenio Cozzolino, Maria Giordano, Christophe El-Nakhel, Antonio Cuciniello, Vincenzo Cenvinzo, Giuseppe Colla, Youssef Rouphael. (2019). Protein Hydrolysate or Plant Extract-based Biostimulants Enhanced Yield and Quality Performances of Greenhouse Perennial Wall Rocket Grown in Different Seasons. Plants, 8, 208; doi:10.3390/plants8070208

9. Frank C. Church, Harold E. Swaisgood, David H. Porter, George L. Catignani. (1983). Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins. Dairy Sci 66:1219-1227

Sitography

10. https://www.europarl.europa.eu/news/en/headlines/economy/20151201STO05603/circular-economy-definition-importance-and-benefits
11. https://www.europarl.europa.eu/news/en/headlines/priorities/climate-change/20200618STO81513/green-deal-key-to-a-climate-neutral-and-sustainable-eu
12. https://www.epa.gov/greenchemistry/basics-green-chemistry

Ringraziamenti

Mi sento in dovere di dedicare questa pagina del presente elaborato alle persone che mi hanno supportato nella redazione dello stesso.

Innanzitutto, ringrazio la mia relatrice Cimetta Elisa, sempre pronta a darmi le giuste indicazioni in ogni fase della realizzazione dell'elaborato.

Un ringraziamento speciale va alla mia Tutor aziendale Franceschi Clizia che mi ha seguito, con la sua infinita disponibilità, in ogni passo dello sviluppo della tesi con molta cura e attenzione.

Ringrazio tutto lo staff dell'azienda Fomet S.p.a in cui ho svolto un tirocinio formativo della durata di 6 mesi e complementare alla redazione della tesi, per l'ospitalità e per le skills acquisite sul campo. Grazie a voi ho accresciuto le mie conoscenze e le mie competenze.

Ringrazio i miei genitori e le mie sorelle che mi hanno aiutato a superare i momenti più difficili e non hanno mai smesso di credere in me nonostante le difficoltà. Senza di loro non avrei mai potuto intraprendere questo percorso di studi.

Un ringraziamento speciale è per Filippo per avermi sopportato, supportato, spronato e incoraggiato. Grazie per esserci sempre stato e per avermi fatto sentire quanto credessi in me ogni giorno.

Ringrazio i miei compagni di corso che hanno condiviso con me gioie e fatiche di questi due anni trascorsi insieme. Grazie per aver ascoltato i miei sfoghi, grazie per tutti i momenti di spensieratezza.

Alle mie amiche di sempre, il mio porto sicuro dove rifugiarmi quando il mare è in tempesta: grazie per volermi bene per quella che sono e per essere sempre al mio fianco.

Una dedica speciale a tutti i miei amici, che ogni giorno hanno condiviso con me gioie, sacrifici e successi, senza voltarmi mai le spalle. L'affetto e il sostegno che mi hanno dimostrato rendono questo traguardo ancora più prezioso

Ringrazio anche chi c'è stato e non c'è più perché è anche grazie a loro che sono diventata la persona che sono.

Infine, dedico questa tesi a me stesso, ai miei sacrifici e alla mia tenacia che mi hanno permesso di arrivare fin qui.

Grazie a Tutti, senza di Voi non ce l'avrei fatta!