PROJECT
DEVELOPMENT OF SE-YEAST FOR NUTRITIONAL THERAPIES IN NEURODEGENERATIVE DISEASE [SE-YEAST]

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Development of Se-yeast for nutritional therapies in neurodegenerative disease [Se-Yeast]

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CHAP 1: POTENTIALITY OF MILK SERUM -WHEY PRODUCTION

1.1.Introduction

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The first yeast originated hundreds of millions of years ago, and 1,500 species are currently identified. They are estimated to constitute 1% of all described fungal species. Yeasts are unicellular organisms which evolved from multicellular ancestors, with some species having the ability to develop multicellular characteristics by forming strings of connected budding cells known as pseudo-hyphae or false hyphae Yeast sizes vary greatly, depending on species and environment, typically measuring 3–4 µm in diameter, although some yeasts can grow to 40 µm in size. Most yeasts reproduce asexually by mitosis, and many do so by the asymmetric division process known as budding. Yeasts are unicellular, eukaryotic and polyphyletic organisms classified in the kingdom fungi. Fermentation of sugars by yeast is the oldest application in the making of bread, beer and wine. Apart from the production of bread and beverages, ethanol production is vital for different applications. Yeasts are also involved in single cell protein production. The growth of Baker’s yeast market is directly linked to the increasing trend of processed and fast food consumption, especially bakery items. The principal use of baker’s yeast is as an essential bakery ingredient for causing fermentation in the dough used in making bakery items.

The global yeast market is dominated by baker's yeast which accounted for over 31% of the total market. Growing market for bakery products, beer, wine, animal feed, and bio-ethanol is supposed to be the major driver for global yeast market. Yeast is one of the mostly and commonly used microorganisms in the manufacturing of food and beverages. Yeast is used in the process of fermentation for its abilities in aiding carbon - release, imparting better aroma, taste, texture, and flavor to food. Yeast is considered as the most consistent fermenting agent in spite of various other chemical fermentation agents available in the market. The global market for yeast is expected to grow at CAGR of more than 8% from 2014 to2018. The global yeast market by types can be segmented as global specialty yeast market, yeast extract market, autolyzed yeast market, supplement yeast market, and yeast derivatives market. Yeasts areeukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The first yeast originated hundreds of millions of years ago, and 1,500 species are currently identified. They are estimated to constitute 1% of all described fungal species.
Yeast sizes vary greatly, depending on species and environment, typically measuring 3–4 µm in diameter, although some yeast can grow to 40 µm in size. Most yeasts reproduce asexually by mitosis, and many doso by the asymmetric division process known as budding. Yeasts are unicellular, eukaryotic and polyphyletic organisms classified in the kingdom fungi.
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USD 7.88 billion at a continuous rate of 9.1% during the forecasted period 2017 to 2027. The global market for yeast products is expected to grow to $9.2 billion by 2019, with a compound annual growth rate (CAGR) of 7.9% from 2013 to 2019.

1.2.The importance of yeast for humans

The by-products of fermentation—carbon dioxide and alcohol—have been used by humans for centuries in the production of breads and alcoholic beverages. Before the mid-nineteenth century, however, bakers and brewers knew very little about the nature of the organisms that helped make their products. The experiments of French microbiologist Louis Pasteur (1822–1895) showed that fermentation could only take place in the presence of living yeast cells. He also deduced that anaerobic conditions were necessary for proper fermentation of wine and beer (in the presence of oxygen, yeast convert alcohol to acetic acid. Brewer's yeast is added to liquids derived from grains and fruits to brew beer and wine. The natural starches and sugars in the liquids provide food for the yeast. Deprived of oxygen during the fermentation process, yeasts produce alcohol as a by-product of incomplete sugar breakdown. Yeasts that occur naturally on the skins of grapes also play a vital role in fermentation, converting the sugars of grapes into alcohol for wine production.
Baker's yeast, another variety of yeast, is added to dough made from the starchy portion of ground grains (such as wheat or rye flour). The yeast breaks down some of the starch and sugar present in the mixture, producing carbon dioxide. The carbon dioxide bubbles through the dough, forming many air holes and causing the bread to rise. Since oxygen is present, no alcohol is produced when the bread is rising. When the bread is baked, the air holes give the bread a lighter texture.
In recent times, yeasts have been used to aid in the production of alternative energy sources that do not produce toxic chemicals as byproducts. Yeasts are placed in huge vats of corn or other organic material. When fermentation takes place, the yeasts convert the organic material into ethanol fuel. Present-day geneticists are working on developing yeast strains that will convert even larger organic biomasses (living material) into ethanol more efficiently.

1.3.Brief history of yeast

Yeasts can be considered man’s oldest industrial microorganism. It’s likely that man used yeast even before the development of a written language. Hieroglyphics suggest that that ancient Egyptians were using yeast and the process of fermentation to produce alcoholic beverages and to leaven bread over5,000 years ago. The biochemical process of fermentation that is responsible for these actions was not understood and undoubtedly looked upon by early man as a mysterious and even magical phenomenon.

It is believed that these early fermentation systems for alcohol production and bread making were formed by natural microbial contaminants of flour, other milled grains and from fruit or other juices containing sugar. Such microbial flora would have included wild yeasts and lactic acid bacteria that are found associated with cultivated grains and fruits. Leaven, referred to in the Bible, was a soft dough-like medium. A small portion of this dough was used to start or leaven new bread dough. Over the course of time, the use of these starter cultures helped to select for improved yeasts by saving a “good” batch of wine, beer or dough for inoculating the next batch. For hundreds of years, it was traditional for bakers to obtain the yeast to leaven their bread as by-products of brewing and wine making. As a result, these early bakers have also contributed to the selection of these important industrial microorganisms.

It was not until the invention of the microscope followed by the pioneering scientific work of Louis Pasteur in the late 1860's that yeast was identified as a living organism and the agent responsible for alcoholic fermentation and dough leavening. Shortly following these discoveries, it became possible to isolate yeast in pure culture form. With this new found knowledge that yeast was a living organism and

the ability to isolate yeast strains in pure culture form, the stage was set for commercial production of baker's yeast that began around the turn of the 20th century. Since that time, bakers, scientists and yeast manufacturers have been working to find and produce pure strains of yeast that meet the exacting and specialized needs of the baking industry.

Yeasts are single-celled fungi. As fungi, they are related to the other fungi that people are more familiar with. These include edible mushrooms available at the supermarket, common baker's yeast used to leavenbread, molds that ripen blue cheese and the molds that produce antibiotics for medical and veterinary use. Many consider edible yeast and fungi to be as natural as fruits and vegetables.

1.4.Yeast Cells

Over 600 different species of yeast are known and they are widely distributed in nature. They are found in association with other microorganisms as part of the normal inhabitants of soil, vegetation, marine and other aqueous environments. Some yeast species are also natural inhabitants of man and animals. While some species are highly specialized and found only in certain habitats at certain times of the year, other species are generalists and can be isolated from many different sources.

Baker's yeast is used to leaven bread throughout the world and it is the type of yeast that people are most familiar with. Baker's yeast is produced from the genus and species of yeast called *Saccharomyces cerevisiae*. The scientific name of the genus of baker's yeast, *Saccharomyces*, refers to "*saccharo*" meaning sugar and "*myces*" meaning fungus. The species name, *cerevisiae*, is derived from the name Ceres, the Roman goddess of agriculture. Baker's yeast products are made from strains of this yeast selected for their special qualities relating to the needs of the baking industry.

The typical yeast cell is approximately equal in size to a human red blood cell and is spherical to ellipsoidal in shape. Yeasts reproduce vegetatively by budding, a process during which a new bud grows from the side of the existing cell wall. This bud eventually breaks away from the mother cell to form a separate daughter cell. Each yeast cell, on average, undergoes this budding process 12 to 15 times beforeit is no longer capable of reproducing. During commercial production, yeast is grown under carefully controlled conditions on a sugar containing media typically composed of beet and cane molasses. Under ideal growth conditions a yeast cell reproduces every two to three hours.

Yeast is the essential ingredient in many bakery products. It is responsible for leavening the dough and imparting a delicious yeast fermentation flavor to the product. It is used in rather small amounts in most bakery products, but having good yeast and using the yeast properly often makes the difference between success and something less than success in a bakery operation.

Kluyveromyces marxianus is an *ascomycetous* yeast and member of the genus *Kluyveromyces*. It is the sexual stage of *Telosaccharomyces pseudotropicalis* also known as *Candida pseudotropicalis* (*Candida kefyr* today). This species has a homothallic mating system and is often isolated from dairy products (Rocha *et al* 2011).

This species was first described in the genus *Saccharomyces* as *S. marxianus* by the Danish mycologist, Emil

Christian Hansen from beer wort (Fonseca *et al* 2008). He named the species for the zymologist, Louis Marx of Marseille who first isolated it from grape (Fonseca *et al* 2008). The species was transferred to the genus *Kluyveromyces* by van der Walt in 1956. Since then, 45 species have been recognized in this genus. The closest relative of *Kluyveromyces marxianus* is the yeast *Kluyveromyces lactis*, often used in the dairy industry (Lane et al 2011). Both Kluyveromyces and Saccharomyces are considered a part of the "Sacchromyces complex", subclade of the Saccharomycetes Kluyveromyces marxianus is an aerobic yeast capable of respiro-fermentative metabolism that consists of simultaneously generating energy from both respiration via the TCA cycle and ethanol fermentation (Fonseca et al 2008).[2] K. marxianus is widely used in industry because of its ability to use lactose. K. marxianus is highly thermotolerant and able to withstand temperatures up to 45°C (Rocha et al 2011). K. marxianus is also able to use multiple carbon substrata at the same time making it highly suited to industrial use. When glucose concentration falls down to 6 g/L, the lactose co-transport initiates (Fonseca et al 2013).

Industrial use of K. marxianus is mainly in the conversion of lactose to ethanol as a precursor for the production of biofuel (Lane et al 2011).[4] The ability for K. marxianus to reduce lactose is useful because of the potential to transform industrial whey waste, a problematic waste product for disposal, into useful biomass for animal feed, food additives or fuel (Lane et al 2010). Certain strains of the fungus can also be used to convert whey to ethyl acetate, an alternative fuel source. K. marxianus is also used to produce the industrial enzymes: inulinase, β-galactosidase, and pectinase (Lane et al 2010). Due to the heat tolerance of K. marxianus, high heat fermentations are feasible, reducing the costs normally expended for cooling as well as the potential for contamination by other fungi or bacteria. In addition, fermentations at higher temperatures occur more rapidly, making production much more efficient (Yang et al 2015). Due to the ability of K. marxianus to simultaneously utilize lactose and glucose, the prevalence of K. marxianus in industrial settings is high as it decreases production time and increases productivity (Fonseca et al 2013).

1.5.Whey as a co-product from cheese making

Whey is the liquid fraction that remains following manufacture of cheese. The world production of whey is estimated at about 165 million tones. Of which cheese whey contributes about 95 %. The utilization of whey was 75% in Europe and probably less than 50% in the rest of the world and as a result a very large amount of material with potential value as a food or feed is wasted (Aneja *et al* 2002). Whey is a serious pollutant as it imposes a very high BOD of 30,000- 50,000 mg/lit and chemical oxygen demand of 60,000-80,000 mg/lit. Discarding of whey also constitutes a significant loss of potential nutrients and energy and has been looked upon seriously by the environmentalists and technologists due to its potent polluting strength. In addition the dairy industry suffers from an economic blow due to several treatment costs that are incurred in proper disposal of whey.

Whey is a by-product of the dairy industry, which for years was thought to be insignificant and was either used as an animal feed or it was disposed of as waste. Considering that over 145 million tons of whey is produced worldwide annually, the desire for new methods to utilize whey can be appreciated. Over thelast years several studies were carried out concerning the importance of whey is nutritional value and the properties of its ingredients. It is now accepted that main content, whey proteins, have antimicrobial, antiviral and anti-oxidant properties. Due to the substantial difficulties encountered in the treatment of whey as a biological waste and its high potential to be valuable raw material for added value food and bioactive substances production the later tend to be the only accepted and popular trend of dealing with this dairy industry by-product. Productions of whey proteins by ultrafiltration, lactose hydrolysis products, and the use of whole whey or whey permeate as a fermentation feedstock are possible options.A large number of workers have carried out studies on the composition and processing of whey for its usein foods and animal feeds besides studying the nutritive, therapeutic and functional properties of whey. Fig 1 shows the typical scheme for cheese making

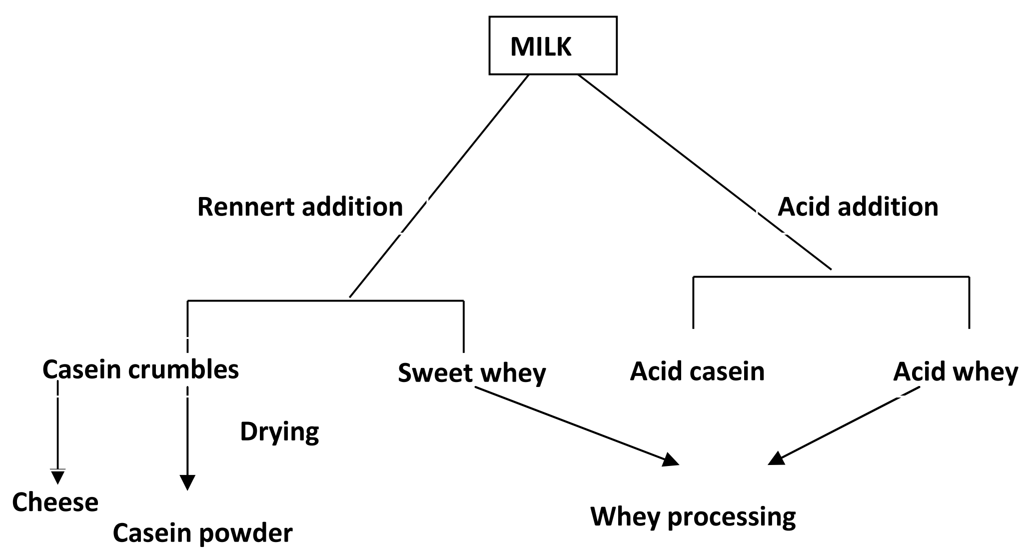


Fig 1: Scheme for cheese making

1.6. Yeast from cane molasses

Molasses is a co-product resulting from refining sugarcane into sugar. Molasses varies by amount of sugar, method of extraction, and age of plant. Sugarcane molasses is primarily used for sweetening and flavoring foods everywhere. Molasses is a defining component of fine commercial brown sugar.

As a final product, molasses still contains large amounts of sugars, namely sucrose, glucose and fructose that can be used for microorganism to propagate or to produce several important products nowadays.

The production of fodder yeast by cane molasses is widely documented. It has been produced in Cuba in several commercial plants with a yield of about 4 tons of molasses per ton of yeast. This yield affects the use of molasses for this purpose since the substrate cost is high as compared to other substrates

However, molasses as a substrate for biomass growth is a complete medium that only needs some inorganic salts of nitrogen and phosphorus to sustain microbial propagation. Table 2 shows the average composition of both kind of molasses. although these vales can vary from one country to other or even in the same country at different places and time of production

Component, % (dry matter basis)	**Sugarcane**	**Beet**
Water	15-20	16-20
Organic matter	74	72
Total reducing sugars	46-52	45-50
Free reducing sugars	15-20	0.2-1.2
Sucrose	30-40	45-49
Glucose	14	1
Fructose	16	1
Rafinose	Non detected	0.5-2.0
Non fermentable substances	2-4	None reported
Kjeldahl nitrogen	0.51	1.7
Ashes	8-11	8-11

Table 2. Composition of cane and beet molasses* (Otero 1997)

Among ashes the most abundant metals are potassium, calcium and magnesium (Otero *et al* 1993)

1.7. Use of industrial wastes to produce yeast biomass

Several industrial wastes have been tested for the production of yeast biomass as glycerol (Kurcz *et al* 2018*)*, paper mill wastes (Bengtsson *et al* 2007), fruit wastes (Mondal *et al* 2012) and distillery slops (Otero *et al* 2003, Martinez *et al* 2004), among other. With the only exception of distillery slops that was scaled up to commercial installations with low yields, the rest of industrial wastes never has gone farther than lab or pilot plant scale.

1.8. Cheese whey as substrate for yeast growth

1.8.1 Cheese whey composition

The lactose present as a major component in cheese whey can be used for the production of single cell protein (SCP) using yeasts capable of consume this sugar like those of the genus *Kluyveromyces* highly productive and safe for human consumption. There are also other yeasts belonging to the genus *Candida*, especially *Candida kefyr* (formerly *Candida pseudotropicalis*). The latter, however, is not advisable for large-scale use due to potential toxicity events (Dufresne *et al* 2014). This is a fundamental step in the production process, all the work will be carried out by the inoculated microorganism, and therefore, its selection must be very careful. This must meet several requirements, namely:

• High biomass / substrate yields.

• High cell duplication rates, and

• High protein content.

The most convenient is to select the strains to study from the natural flora of the carbon source and energy to be used. These populations already bring advanced adaptation to the substrate. The use of genetically modified microorganisms is not recommended as there is the possibility of mutation reversal during the process. A method used in the selection of microorganisms is the use of runs in continuous culture for prolonged times and select the populations that prevail over time and show the best production conditions (Arai *et al* 2003).

1.8.2 Microorganism screening for whey consumption

It has been reported previously the consumption of lactose and whey proteins for *Kluyveromyces* genus (Koushki *et al* 2012, Zoppellari & Bardi 2013). These authors observed lactose consumption higher than 95% of initial content while protein consumption reached about 70%.

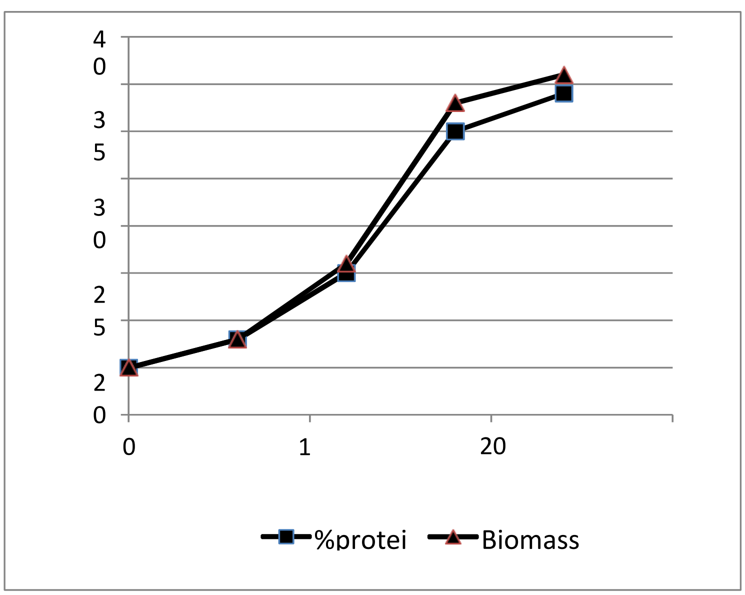


Fig 2 Protein and biomass production of yeast from whey in batch culture

Data obtained from the amount of biomass yield corresponds with that of protein produced during fermentation. About 82% of total protein was produced in the first 18 h of propagation, which can be an indication of the exponential phase of the yeast growth.

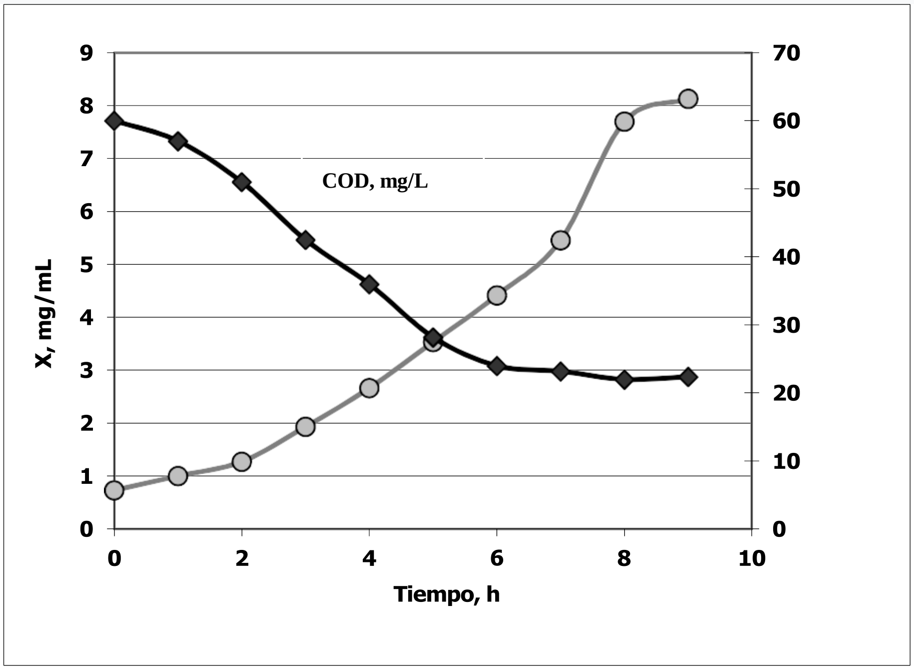


Fig 3 Kinetics for cell growth of *K. marxianus* in mixtures of whey and cane molasses (80:20 based on COD). Process is based on an initial COD = 50 000 mg/L

1.8.3 Basic medium for Kluyveromyces marxianus

The yeast *K. marxianus* can grow in a medium rich in sugars, including glucose and lactose. It will be used for growth in lyophilized lacto-whey Petri dishes stored at a temperature of -40 °C. Commercial house Chr. Hansen. Yeast lactose positive.

Residual serum was used for the preparation fresh cheese. This type of cheese corresponds to the categoryof fresh cheeses that are made from curd without maturation. The Latin American tradition is based on white cheeses made from cow milk.

Preparation of whey for yeast propagation at lab scale

The preparation of all of the cheese whey for lab scale culture was done by means of coagulation by thermoacid treatment, in which adjusting pH to 4.5 (H_2SO_4) followed by heating at 115 °C for 20 minutesin an autoclave.

he serum cooled and proteins were separated by decantation and filtration with Whatman paper Nº41. The final protein content of the serum used in fermentations should be around 0.38%.

Inoculum preparation

Kluyveromyces marxianus were incubated for 24 hours. Cellular concentration should be measured by means of a Neubauer chamber (Counting Chamber, Hausser Scientific Company).

Yeast is then transferred to a 1 L erlenmeyer flask where several slants of yeast were poured into and taketo a rotary shaker for 8-10 hours with a medium prepared as follows: cheese whey (50 000 mg/L COD) 1L, 1 g/L $(NH_4)_2SO_4$, 1 g/L $(NH_4)_2PO_4$ and pH adjusted to 5.0 with H_2SO_4. Temperature was set on 32°C and 300

min^{-1}.

Second inoculum step

Once the first stage of inoculum at shaker level the whole volume is transferred to a 10 L stainless steel loaded with same medium as before and an air flow of 1 volume of air per medium volume per minute (VVM) for an additional 8-10 hours.

Cultivator

An 800 L jacketed culture tank equipped with mechanical stirring is used as inoculum third stage with similar medium a medium composition as in previous steps for 12 hours and then taken to a 15 000 L prefermenter tank with same condition as before.

Prefermenter

Once pre-fermenting stage was over the commercial fermenter (200 000 L working volume) and its fully taken to this volume in consecutive 70 000 L volume of fresh medium in 18-24 hours. Once the full volume is reached the process is swift to continuous culture feeding a fresh medium flow 50 000L/hour in continuous feeding ($\mu = 0.25h^{-1}$)

The whole process is repeated again for second and third fermenter until the three commercial fermenter are under operation. During operation at commercial scale a temperature of 32 ºC, aeration flow 0.6-0.8 VVM and agitation 300 rpm.

Liquid whey is defined as liquid waste from cheese manufacturing. These residues contain fermentable carbon material in the form of sugars - basically lactose-, proteins, lipids and minerals. About 85-90% of the milk components are transferred to the whey so the production of this co-product is 9 L of whey per10 l of milk. When the whey is directly discharged on the farmland adjacent to the cheese factories, this causes a serious contamination problem, which has led to numerous studies for its treatment. One of the several available processes is the production of Single Cell Protein (SCP), which meets the double objective of using lactose for the production of fodder yeast and concomitantly reduces itspolluting power.

1.8.4 Yeast Propagation

It is the most important stage of the production process because the quality of the final product and the main economic indicators of the process (industrial yield and productivity) will depend on the successful course of this operation. The empirical reaction for the formation of yeast biomass can be represented as follows

$$200g\ C_{12}H_{22}O_{11} + 10.4\ g\ NH_3 + 102.5\ g\ O_2 \longrightarrow 100\ g\ \text{Dry biomass} + 142.5\ g\ CO_2 + 77.2\ g\ H_2O$$

$$\Delta H = -353\ \text{Kcal}$$

As a balanced result it will yield

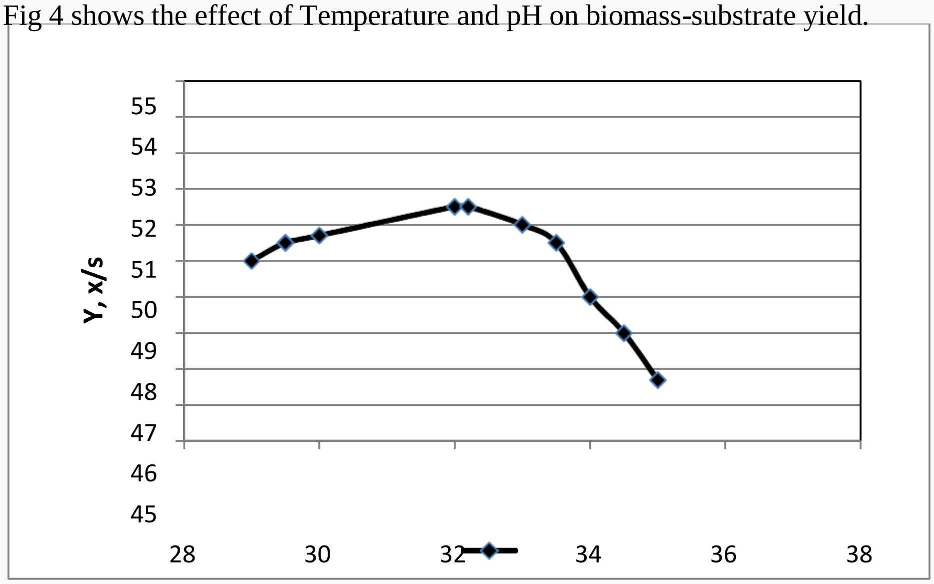
$$0.585\ C_{12}H_{22}O_{11} + 0.612\ g\ NH_3 + 3.203\ g\ O_2 \longrightarrow C_{3.72}O_{1.952}N_{0.612} + 3.3\ g\ CO_2 + 4.289\ g\ H_2O$$

$\Delta H = -\ 0.11$ Kcal/mmol O_2

However, this balance is subject to the conditions under which the propagation of yeast occurs, which will depend on the transformation of sugar into biomass.

Biomass-substrate yield

Fig 4 shows the effect of Temperature and pH on biomass-substrate yield.



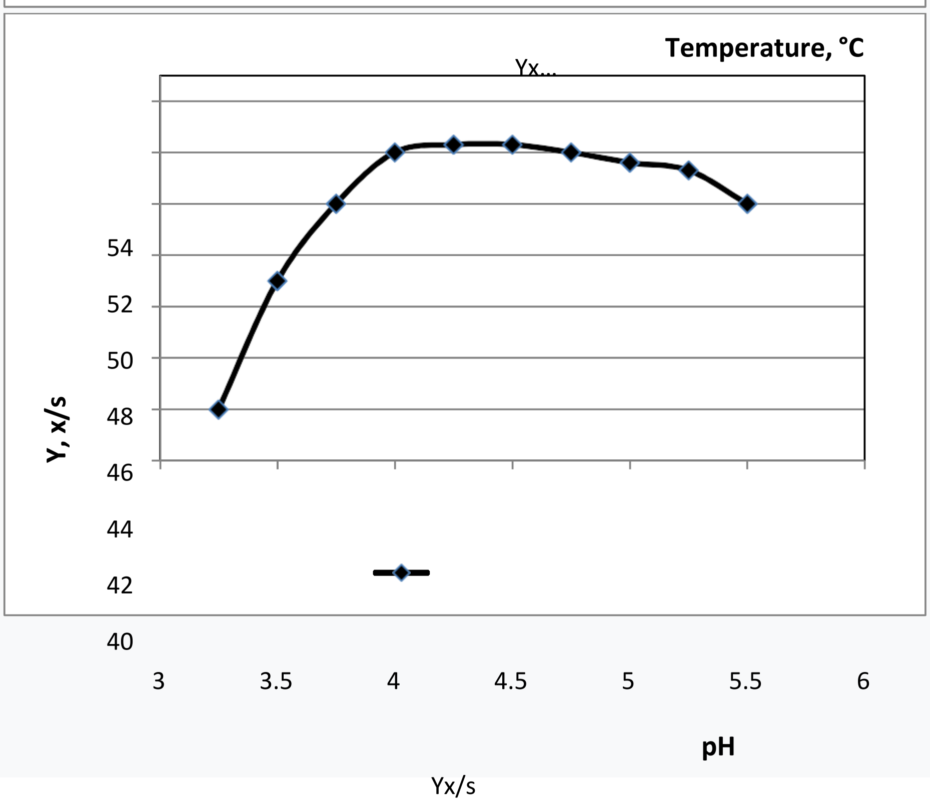


Fig 4. Biomass-substrate yield of *Kluyveromyces marxianus* grown on lactose medium at different temperature and pH values

Yields greater than 50% are obtained at temperature values in the range of 29-33 °C and pH between 3.75 and 4.5. Values in the environment of pH 5 are not recommended due to the increased danger of contamination by bacteria. Higher temperatures also reduce the capabilities of cells to growth.
Other parameters of vital importance to control are the dilution rate (μ) during commercial propagation. In general, under the conditions of continuous cultivation in which the greatest productivity is obtained, work is done at this scale at suboptimal dilution rates to avoid system wash out.

Propagation indexes

Cell propagation is carried out in a 200 000 L capacity industrial fermenter equipped with agitation, aeration, level control, pH, foam, cooling system and fresh medium dosing systems. Commercially there are two types of fermenter, namely: mechanically agitated Vogelbush type and air agitated Lefrancois type.

Nutrient salts

Although the whey contains nitrogen and phosphorus, these are not sufficient to support the growth of the yeast and therefore must be supplemented to the propagation medium in the quantities required for an efficient consumption of the carbon source (lactose). Nitrogen supply is essential for amino acid and subsequent protein synthesis while phosphate is an essential component in nucleic acids and ATP.
The supply of these nutrients is carried out by the addition of inorganic salts that are preferred to organic nutrients for economic reasons. If desired, the pH can be controlled using a suitable ratio from different sources such as ammonium sulfate and urea.
The composition of yeast cells concerning to these nutrients is the following

Table 3. Nitrogen and phosphorus content in yeast cells assuming biomass-substrate yield 45%

Component	**% in yeast biomass**
Nitrogen	8.0-8.5 (as Kjeldahl nitrogen)
Phosphorus	3.0-3.5 as P_4O_{10}

Even though whey contains some amount of both nutrients those should be neglected during nutrient calculation since concentration can vary in function of whey source.

Whey pre-treatment

Whey contains important amounts of microorganisms typical from milk and process of cheese making.This micro flora comprises bacteria, yeast and fungi in about 1000 CFU/mL of whey. Thesemicroorganisms have to be eliminated prior to propagation to avoid further culture contamination. Theprocess of pasteurization should be carried out using plate heat-exchangers for several seconds at 95 °C. Once whey is pasteurized and cooled to working temperature nutrient salts are added in an appropriateinstallation and the whole medium pumped to feed the industrial fermenter.

Yeast cell harvesting

Lactose exhaust medium containing produced yeast that is overflow from fermenter is then fed to the separation section which harvests the yeast biomass previous de-aeration of yeast cream –usually build-upin centrifuge system. This procedure is carried out mainly by centrifugation where a yeast suspension is obtained. The process comprises two batteries of centrifuges with an intermediate washing.

Once the cream is washed (this second cream shows a concentration of about 15-17% of biomass) it is pumped to a evaporation system.

It has to be taken into account that the effluents from centrifugation step still contain suspended solids and therefore should be treated to reduce pollution capacity before be disposed in some water recipient. The average composition of effluents, referred as oxygen demands, are shown in Table 4.

pH	4-5
BOD	3000-5000 mg/L
COD	10000-15000 mg/L
Total solids	13000-14000 mg/L
Total reducing sugars	1000-1500 mg/L

Table 4: Typical composition of centrifugation effluents

Evaporation and drying

The most commonly used form is vacuum evaporation at a temperature of 80 °C, which significantly reduces the viability of the cells and the water content in them. The evaporation system comprises pre-heater, thermolizer and evaporator. The evaporated cream leaves the system with a concentration around 200-220 g/L and then transferred to a drying section which it is usually a spray dryer working at input temperatures of 300 °C, but the drying process is carried out at as low as 80 °C.

Final product characteristics

The harvested yeast is packed in 25 kg multilayer paper bags and stored in a dry and ventilated place in 1 ton pallets until used.

Dry product shows the characteristic appearing in Table 5.

Index	**%**
Moisture	5-8
Protein (Kjeldahl Nx6.25)	45-50
Phosphorus (as P_4O_{10})	3-3.5
Total carbohydrates	25-30
Fat	1.0-1.5
[illegible]	[illegible]
Bulk density	~420 Kg/m^3
Granulometry	200-400 mesh

Table 5. Composition and properties of dry fodder yeast

1.9. Plant design

The capacity of the plant to be installed will depend on the following factors:

a) Availability of raw material

b) Market for the products

c) Typical capacity of the required equipment

a) Regarding the availability of the raw material the following observations can be made. It is unlikely that a single cheese factory could supply the volume of whey needed for the continuous production of yeast 330 days/year. However, several producers can join their effluents to feed the plant. In every case a reception section has to be implemented to receive and pre-treat the raw material. The retention of whey should be minimized to no more than a few hours since induced cost for conservation at low temperature and transportation, is expensive.

b) Regarding the size of the market and the level of competition existing in it, the following should be taken into account: local, national potential market size and export potential. The strength of the companyto introduce a product in the market and the fight with the competition. In the case of the latest products, with little background for the consumer, it will be necessary to create the need for the product. This means a more or less slow process depending on the specific market dynamics. As a general rule, products for individual or domestic consumption more easily allow the entry of a new product and a new manufacturer even of small size than the products that constitute raw material for the food industry.

c) It is known that most equipment is normally produced with a working capacity within a typical range. Very small capacity equipment is more expensive per unit of product handled or processed. This is the case of centrifuges, evaporators and dryers. On the other hand, having oversized equipment makes investment more expensive and raises operating costs due to energy consumption and other factors. Additionally, the following should be taken into account:

- Weekly work hours of the installation and number of shifts.
- Convenience of performing some operations in a short time to give more space to others more complicated or longer because of having smaller capacity equipment.
- Relative cost of labor with respect to the cost of equipment according to its capacity.
- Possibility of buying second-hand equipment at low cost.
- Based on the three previous arguments, the following capacities and production structure have been selected as an example of calculation

In Table 6, the characteristics of the plant and the cost of equipment are described

Capacity 30 ton/day			
Production cost	Ton/ton of yeast	U$D/ton	Total U$D
Cheese whey	27	3.19	86.00
Other consumables			
Diammonium phosphate	0.13	350.00	44.10
Diammonium sulfate	0.46	35.00	56.9
Cane molasses	0.03	35.00	1.10
Auxiliary services			
Cooling water (m^3/ton)	206	0.06	12.40
Process water (m^3/ton)	151	0.32	48.30
Electricity (Kw-h/t)	714.29	0.064	45.70
Sub-total			280.10
Equipment	**Qty**	**U$D**	**Total U$D**
Fermenters	3	250 000	750 000
Centrifuge 1st Step	1	120 000	120 000
Centrifuge 2nd Step	1	120 000	120 000
Stand by	1	120 000	120 000
Evaporator	1	250 000	250 000
Spray dryer	1	300 000	300 000
Other	1		1 660 000
Total			3 320 000

Table 6. Plant design

CHAP II. POTENTIALITY OF MILK SERUM -FOOD INDUSTRY

2.1. General Aspects of Cheese Industry

In both Argentina and Ecuador, the dairy industry plays a crucial role in their economies. Cheese production is particularly significant, ranging from large corporations to small and medium-sized enterprises with diverse technological and financial capabilities. While larger companies tend to effectively reintegrate whey into the production process, smaller ones still face challenges in managing this by-product.

In Ecuador, it is estimated that in 2019 dairy companies generated around 900,000 liters of whey daily, but only 10% was reintegrated into the production system. This poses a significant challenge in terms of environmental management and resource utilization. Additionally, many farmers produce whey in small quantities informally, lacking options to add value to the effluent generated.

On the other hand, in Argentina, between 2016 and 2018, more than 600 cheese-making companies were registered throughout the country, with the majority processing volumes of milk below 50,000 liters per day. This situation reflects the predominant presence of small and medium-sized cheese producers, who face similar challenges regarding effluent management and environmental sustainability.

The contaminating potential of whey, alongside its significant nutritional value, has prompted extensive research into its large-scale industrial utilization (Monsalve and González, 2005). In the context of ongoing globalization and technological progress, there has been a growing emphasis on adopting environmental management practices. However, addressing the complexities of whey treatment depends on various factors, including company size, type, and effluent composition. This poses significant challenges, particularly for small and medium-sized enterprises (SMEs) within the sector (Cuellas and Wagner, 2008). Despite advancements in membrane separation technology facilitating protein recovery, its implementation necessitates substantial investments in equipment and processes, often restricting its economic viability to plants processing over 300,000 liters of milk per day (Grasselli, 1997; Jelen, 2003; Gonzáles Cáseres, 2012). Consequently, smaller companies have encountered obstacles in their progress due to the high costs associated with effluent treatment machinery.

Small and medium-sized cheese producers frequently lack the resources or industrial equipment required for effective whey treatment and wastewater management systems. While some have established whey collection facilities for pig feed, the volume of effluent produced often surpasses such capacities, demanding the adoption of more sophisticated treatments and solutions (Sánchez et al., 2012). Therefore, scientific and technological research on the recovery and revaluation of this effluent is of vital importance, both for its comprehensive utilization and for the development of simple and economical methods applicable in medium and small-scale industries (Miranda, 2007).

Given this situation, it is imperative to devise alternatives to make use of cheese whey. In this context, it is crucial to leverage the nutritional properties of the effluent and explore various options for its application in the food industry. Within the framework of this project, two alternatives of functional dairy beverages were evaluated, which allow for the incorporation of Se-yeasts with potential use in nutritional treatments for neurodegenerative diseases.

2.2. Types and Composition of Whey

Whey may be defined broadly as the serum or watery part of milk remaining after separation of the curd; which results from the coagulation of milk proteins by acid or proteolytic enzymes. The type and composition of whey at dairy plants mainly depends upon the processing techniques used for casein removal from liquid milk. The most often encountered type of whey originates from manufacture of cheese or certain casein products, where processing is based on coagulating the casein by rennet, an industrial casein-clotting preparation containing chymosin or other casein coagulating enzymes (Fox *et al* 2000). Rennet-induced coagulation of casein occurs at approximately pH 6.5; this type of whey is referredto as sweet whey (Table 7). The second type of whey, acid whey (pH < 5), results from processes using fermentation or addition of organic or mineral acids to coagulate the casein, as in the manufacture of freshcheese or most industrial casein (Jelen 2003).

Components	Sweet whey	Acid whey
Total solids (%)	7.0	7.0
Fat (%)	0.3	0.1
Protein (%)	0.9	1.0
Lactose (%)	4.9	5.1
Ash (%)	0.6	0.7

Table 7. Typical composition of whey (Gupta 2000)

In the production of cheese or casein, about 80 to 90 % of original milk used yields whey as a by-product, which contains about 50 % of the milk constituents. Whey retains about 45-55 % of the milk nutrients comprising serum proteins, lactose, minerals and vitamins. The composition of whey varies depending onthe type of coagulation. The main components of both sweet and acid whey, after water, are lactose (approximately 70–72% of the total solids), whey proteins (approximately 8–10%) and minerals (approximately 12–15%) (Gupta2000). The main differences between the two whey types are in the mineral content, acidity and composition of the whey protein fraction.

Whey is a rich source of nutritious components. Whey protein has potential as a functional food component to contribute to the regulation of body weight by providing satiety signals that affect both short-term and long-term food intake regulation. Because whey is an inexpensive source of high nutritional quality protein, the utilization of whey as a physiologically functional food ingredient for weight management is of current interest. Table 8 shows the major constituents of whey protein fraction

Proteins	**% of Whey**
α-lactalbumin	20-25
Seroalbumin	5-10
Lactoferrin	~1-2
Glycomacropeptide (casein peptide)	10-15
β-lactoglobulin	50-55
Inmunoglobulins	10
Proteosa peptona	12
Lactoperoxidasa	~0.5

Table 8. Major Constituents of Whey Protein Fraction

2.3. Whey proteins

Acid and thermal denaturation of whey proteins

Protein is denatured when its native structure deforms due to the breakage of some of the hydrogen bonds. These weak bonds break when a lot of heat is applied or when they are exposed to the action of acids. When deformed, part of the structure is dismantled and some sections of it that were hidden are exposed promoting the formation of bonds with other protein molecules and coagulation (Wijayanti *et al* 2014). The microenterprises of the dairy sector in Ecuador manufacture cheese mainly of the unripened fresh type, that is, the one that must be consumed immediately after manufacturing, using raw milk or pasteurized milk as the raw material (Mejía-López *et al* 2017).

Studies carried out in different studies show that heat treatments cause the denaturation of proteins, that is, they produce a change in their physical structure (secondary, tertiary and quaternary structures), but in general they do not affect the composition of amino acids (primary structure much more stable) and therefore to the nutritional properties of milk (Rynne *et al* 2004). Milk pasteurization in microenterprises is carried out discontinuously by applying direct fire, or steam, at different temperatures. The most used temperatures are between 63 to 65 ° C for a time of 30 minutes and 72 ° C for 15 to 20 seconds. Fig 5 illustrates the process for protein denaturation

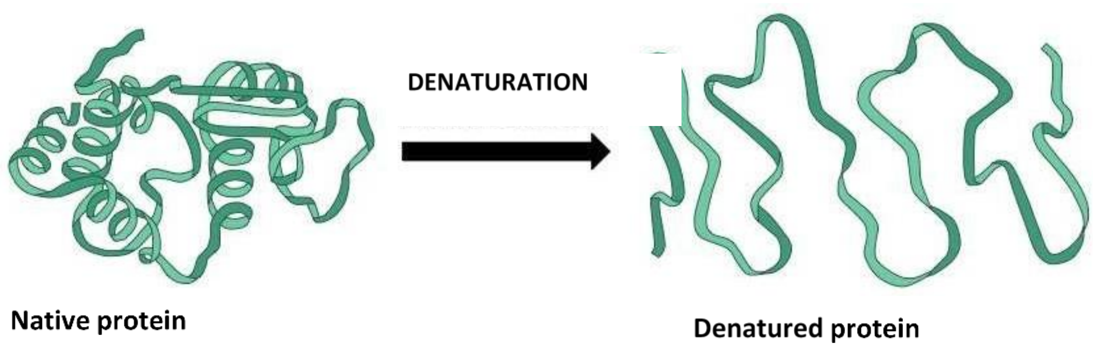


Fig 5 Protein denaturation

Whey protein concentrates and isolates

UF membranes have a molecular weight cut (cut-off) between 3000 and 100,000 Da. The most common in this industry is the standard 10,000 Da. This is the traditional cut to eliminate lactose and retain proteins. In this way, both concentrates (WPC) between 35% and 85% protein are produced, as well as isolates (WPI) with 90% or more protein content. The direct serum drying alternative does not compete in quality with products processed by membrane technologies due to its granulometry and low solubility. The main problem of membrane use lies in the formation of surface artifacts, especially when the serum has relatively high calcium phosphate contents. The control of these depositions can be carried out if constant volume diafiltration is used to keep the salt dissolved. The disadvantage is that it generates high volumes of permeate free of highly contaminating proteins (Mollea et al 2013).

2.4. Environmental impact of whey

The main problem in the management of whey is its environmental disposition. Due to its biodegradable components, BOD rises to 40,000-60,000 mg/L. The COD is even greater 50,000-80,000 mg/L (Chatzipaschali & Stamatis 2012). This high polluting potential makes its environmental disposition prohibitive, even after treatment of degradation of organic matter. Lactose, the majority component, is primarily responsible for these, BOD and COD values (Patel & Murthy 2011). The production of wheyon a global scale is almost equal to the volumes of processed milk in the manufacture of cheeses and is estimated at around 190 million tons per year (Baldasso *et al* 2011). The ratio of cheese to whey is 9 L of whey/kg of cheese and its volumes, worldwide, increase in the same proportion as milk destined to make cheeses. Lactose, the major component of whey, is probably the least valuable component and most difficult to utilize.

2.5. Pre-feasibility study for the development of food products from whey

Whey is primarily composed of lactose, a relatively insoluble sugar with low sweetness, which may not always be absorbed by the human digestive system. Thus, lactose hydrolysis is vital for the use of effluent in the food industry, as it produces glucose and galactose, a mixture with greater solubility, higher sweetness, and easier absorption by the digestive mucosa (Zadow, 1984; Barnes, 1994; Cuellas, 2005).

The characteristics and composition of the effluent allow for the design of a range of options for the development of food products. Table 9 presents a series of products that can be obtained from cheese whey. The selection criteria for the production of some of these products must be tailored to the needs and capabilities of cheese establishments and consider fundamental aspects such as process cost, production time, and the possibility of introducing the resulting product into the market. This work includes a pre-feasibility study for the production of different products based on cheese whey and a second experimental stage in which the formulation of the selected product is developed.

PRODUCTS	DESCRIPTION	PROCESS	Volume used	Acceptance
Ricotta	Small coagulated protein granules. With a soft and granulated texture, and a slightly salty flavor.	Protein coagulation by adding rennet and Calcium Chloride. 30 minutes at 70°C	Low	Medium
Mysost	Whey and milk caramelized. Dark brown in color and with a sweet, slightly toasted flavor. Dense and creamy texture.	Concentration of solids at high temperatures and caramelization of lactose	High	Low
Yogurt	Dairy product obtained through bacterial fermentation of milk	Lactic fermentation by Streptococcus thermophilus and Lactobacillus bulgaricus, formation of lactic acid, which thickens the milk and gives yogurt its characteristic flavor and texture.	Medium	High
Drinks	Functional and isotonic beverages	Mixing of ingredients, homogenization, and addition of stabilizing compounds.	High	High

Table 9. Pre-feasibility study for the development of food products from whey

In the feasibility study, the analysis includes fundamental aspects for the production of food products from whey, such as: utilization level, production time and cost, pollution reduction, and acceptance for different products. In this sense, it has been determined that the products that present the greatest advantages and the highest acceptance potential are isotonic beverages and yogurt. However, the former involves the utilization of a larger volume of effluent. Similar products such as isotonic and energy drinks respond to the new trend of consumption of natural and functional foods, constituting an expanding market. On the other hand, the beverage production process fully utilizes resources, employs simple technology to implement, and almost entirely reduces the environmental impact generated by this effluent (Inda Cunningham, 2000).

2.6. Preparation of functional drinks from cheese whey

For the production of the isotonic beverage, the whey underwent an enzymatic hydrolysis process, resulting in a syrup with an 80% lactose conversion. Considering the solubility, sweetness, and digestibility of sugars, the hydrolyzed beverage presents nutritional and technological advantages. These benefits are due to the higher proportion of monosaccharides and lower sucrose and lactose content (which promotes rapid absorption and a lower insulin response) and a lower production cost.

The hydrolyzed whey was used as raw material for different formulations varying in flavor, aroma, and color. These samples underwent sensory and acceptance tests. In the formulations made, an isotonic beverage highly accepted by a sensory panel was obtained, with good organoleptic characteristics and wide possibilities for market insertion. The production process does not present technological difficulties, requires facilities commonly used in the dairy industry, and allows cheese producers to reduce the environmental impact caused by improper whey disposal. Considering that in the industrial development of countries in the region it is essential to implement management systems that combine comprehensive resource utilization and minimize environmental pollution, it is concluded that the production of this product enables more efficient processes, cleaner technologies, and higher profitability.

2.7. Preparation of a fermented product, simile yogurt, from cheese whey

Fermented dairy products are widely distributed in the market, with the most well-known being yogurt, which is defined according to the Codex Alimentarius as milk (usually from cows) that has been fermented with Streptococcus thermophilus and Lactobacillus bulgaricus under defined conditions of time and temperature. Each species of bacteria stimulates the growth of the other, and the products of their combined metabolism result in the characteristic creamy texture and slight acidic flavor. Yogurt also contains other additives such as dairy solids, sugars, fruits, etc.
The ability of these microorganisms to utilize lactose as a carbon and energy source and produce lactic acid allows for the production of this product, which possesses a series of compounds that give it its characteristic flavor, aroma, and texture, properties that characterize it (Walstra, et al., 2001). The presence of this sugar in cheese whey allows for the formulation of a similar product that preserves the characteristic properties of yogurt and takes advantage of the nutritional benefits of the cheese by-product.

In order to achieve the best product, different formulations were evaluated. The formulation was carried out taking into account fermentation parameters, the highest percentage of milk substitution with whey, the addition of hydrocolloids to achieve the appropriate consistency, and the preferences of a sensory panel. Once all parameters were determined, the final product underwent studies of sensory and microbiological shelf life.

In order to take advantage of the nutritional value of the whey and minimize the effects of contamination, a fermented dairy product was developed that contains a high proportion of whey in its formulation. Following the industrial protocol for the manufacture of yogurt, different percentages of milk replacement by whey and the effect of the addition of hydrocolloids on the organoleptic properties of the final product were evaluated. The experiments included the rheological, sensory and microbiological tests. Once all parameters were determined, the final product underwent studies of sensory and microbiological shelf life.

These trials allowed for the successful utilization of the effluent as a raw material in the development of a fermented dairy product. This yogurt exhibits excellent organoleptic characteristics and broad market insertion possibilities. The manufacturing process requires simple technology and equipment commonly used in the dairy industry, making it easily adaptable for incorporation into small and medium-sized industries and can be integrated into the production lines of cheese plants. Microbiological analysis results met the requirements of the Argentine Food Code, determining that the product obtained is suitable for human consumption. The use of this technology will assist cheese producers in reducing the environmental impact caused by improper whey disposal, thus justifying the investment through compliance with legislation affecting cheese factories and increasing profitability by adding new products to the plant's production line. Regarding the expected impact, in Latin America, there are numerous medium and small-sized cheese industries that lack the necessary means to make significant investments, making it impossible to incorporate drying technology with lactose plant and/or whey concentrate. The utilization of this effluent as a raw material for food products would then be a feasible and easily implementable solution for process improvement and obtaining products with higher added value.

2.8. *Incorporation of selenium (Se) yeast into functional beverages*

The relationship between diet and brain health is a rapidly growing and highly relevant field of study, especially when it comes to preventing neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease (Morris et al., 2021; Li et al., 2020).

Nutrition plays a fundamental role in the optimal functioning of the brain and can influence the prevention of cognitive decline (Fernández-Martínez et al., 2020). The brain is a highly metabolic organ and, to function properly, it requires a constant supply of micronutrients (Bourre, 2006). These elements play a vital role in energy production, neurotransmitter formation, protection against oxidative stress and inflammation, processes that are closely related to the development of neurodegenerative diseases (Calderón-Garcidueñas et al., 2021; Yaribeygi et al., 2020).

Presently, selenium (Se) has garnered significant interest owing to its noteworthy antioxidant role. The antioxidant activity of Se-proteins in the central nervous system (CNS) is widely acknowledged. This mineral serves as a crucial component in preserving nervous system health, playing a substantial role in the normal physiological function of the brain (Cardoso et al., 2019). Low selenium levels have been linked to brain damage in various neurodegenerative diseases, including Huntington's disease (Vickers et al., 2021).
As described in Part I of the document, yeast cultivated in a suitable medium is an excellent source of Se-proteins. The absorption of selenium by yeast is attributed to the presence of functional groups exhibiting a negative charge on the surface of the cell wall (Klis et al., 2002). The extent of this absorption depends on the hydrophobicity of the yeast cell walls, which in turn relies on the proportion between their components (Kordialik-Bogacka, 2011).

In this context, the incorporation of Se-yeast into the resulting beverages would be of utmost importance to help maintain adequate levels of this mineral in the body. Incorporating these products into the diets of patients with neurodegenerative diseases could potentially help prevent associated brain damage (Vickers et al., 2021).

On the other hand, we can leverage the versatility of whey and reuse it for various purposes simultaneously (Fig. 6). The production process of microbial protein and the parallel functional beverage becomes a promising option for obtaining high-value-added products with clean, sustainable, and environmentally friendly technologies. These procedures do not produce any additional waste besides the cleaning water from the installation, which has low pollution potential.

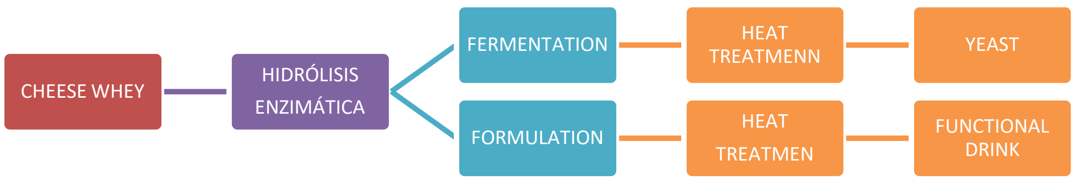


Fig 6 Simultaneous Process

For yeast to be incorporated into whey beverages, they must undergo a process of inactivation or autolysis; otherwise, their ability to ferment carbohydrates producing can cause gastrointestinal disorders. Additionally, they can deplete the body of vitamins, mainly B1 and B2, causing an effect contrary to the desired one. Currently, there are various methods to induce cell rupture, either mechanically or non-mechanically. For the incorporation of Se-yeasts into the product, they were subjected to a high-pressure homogenization process, causing cell rupture and micronization of the cell wall. Finally, emulsifiers and stabilizers were added to the formulation, achieving greater palatability and appropriate consistency.

2.9. Conclusions

The characteristics and composition of the effluent allow for a range of options, from expensive technologies to comprehensive solutions that enable the production of dairy products at a low cost. The selection criteria for the production of any of these products must be tailored to the needs and capabilities of cheese establishments and consider fundamental aspects such as process cost, production time, and the possibility of introducing the resulting product into the market.

In the formulations made, an isotonic beverage and yogurt were obtained, both products highly accepted by a sensory panel, with good organoleptic characteristics and wide possibilities for market insertion. The production processes of them, do not present technological difficulties, require facilities commonly used in the dairy industry, and allow cheese producers to reduce the environmental impact caused by improper whey disposal. Considering that in the industrial development of countries in the region, it is essential to implement management systems that combine comprehensive resource utilization and minimize environmental pollution, it is concluded that the production of this product enables more efficient processes, cleaner technologies, and higher profitability.

Finally, we can capitalize on the nutritional properties of whey and its multifunctionality. By incorporating Se-yeast into whey-based beverages, we can create a nutritional tool aimed at delaying symptoms of neurodegenerative diseases. The production process of microbial protein alongside the functional beverage offers a promising avenue for creating high-value-added products using clean, sustainable, and eco-friendly technologies. These methods minimize waste generation, with only the cleaning water from the facility posing minimal pollution potential.

ANEXO I: Activities carried out as part of the program.

In addition to the reported research activities, the following activities were carried out:

Kick-off Meeting and 1st International Congress on Whey and Milk Derivatives

In August 2019, the kick-off meeting was held in Quito, Ecuador, attended by researchers and consultants from the project "Development of Se-yeast for nutritional therapies in neurodegenerative disease [Se-Yeast]," Perez-Guerrero (PGTF), UNDP. In this context, the team was summoned by the Provincial Government of Pichincha and the Ecuadorian Center for Biotechnology and Environment (CEBA) to participate in the organization of the 1st International Congress on Whey and Milk Derivatives, held in Quito, Ecuador, on August 19th and 20th, 2019.
The general objective of the congress was to disseminate the scientific and technological advances in the utilization of whey, through the exchange of experiences and local, national, and international knowledge, allowing the maximum utilization of whey and milk derivatives in Ecuador and countries of Latin America and the Caribbean.
The project members presented the following conferences:

- Development of Se-yeast for nutritional therapies in neurodegenerative disease [Se-Yeast]. MSc. Anahi Cuellas, UNQ, Argentina.
- Immobilization of β-galactosidase for lactose hydrolysis. MSc. Anahi Cuellas, UNQ, Argentina.
- Yogurt and isotonic beverages based on whey effluent. MSc. Anahi Cuellas, UNQ, Argentina.
- Production of single-cell protein (SCP) from whey. Dr.C. Miguel Otero Rambla, PhD, ICIDCA, Cuba.
- Whey biorefinery: Biotechnological derivatives of whey. Dr. C. Amaury Álvarez, ICIDCA, Cuba.
- Ecuadorian Bioeconomy Strategy Horizon 2035. Dr. C. Julio Pineda Insuasti, PhD CEBA, Ecuador.
- Cheese whey as substrate for Se-yeast growth. Dr.C. Miguel Otero Rambla, PhD, ICIDCA, Cuba.

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