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FRACTIONATION OF SPENT BREWER'S YEAST FOR HIGH VALUE-ADDED BIOMOLECULES PRODUCTION.

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Thesis submitted to Bioenergy Research Institute, São Paulo State University UNESP, Rio Claro – SP, Brazil, as part of the requirements for obtaining a Doctor of Science degree.

Advisor: Prof. Dr. Pedro de Oliva Neto

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FRACTIONATION OF SPENT BREWER'S YEAST FOR HIGH VALUE-ADDED BIOMOLECULES PRODUCTION.

EDSON MARCELINO ALVES

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ABSTRACT

Currently, the yeast biomass is an underutilized waste product of brewing industry. Moreover, the use of this biomass can be an economical source for the extraction of several compounds such as yeast extract, proteins, *β*-fructofuranosidase, RNA and 5'-ribonucleotides, which are by-products with wide applications in pharmaceutical and food industry. Faced with these issues, the objective of this work was to study and design an integrated bioprocess methodology using spent brewer's yeast biomass as feedstock for obtaining several high added value biomolecules through fractionation and downstream techniques. For this, the biotechnological potentials of yeast utilization are reviewed in the Chapter 1. The Chapter 2 presents the results of the initial stages of preparation and characterization of yeast from the brewing industry as well as the evaluation of autolysis parameters in order to maximize RNA extraction for subsequent 5'-ribonucleotides production. The Chapter 3 describes the use of acid shock at the beginning of autolysis in order to accelerate the autolytic process and the extraction of proteins. The results of enzymatic hydrolysis of RNA to produce 5'-ribonucleotides using 5'-phosphodiesterase from residual malted barley roots is presented in the Chapter 4. The recovery of extracellular β-fructofuranosidase enzyme as the first by-product before autolysis and studies on its immobilization in sodium alginate and activated charcoal beads were described in the Chapter 5. Finally, an economic analysis as well as a bioprocess plant design for yeast processing is proposed in the Chapter 6 in order to determine the profitability and financial viability of the methodology developed in this work. The proposed approach indicates an excellent strategy, since a better use of spent yeast from fermentation processes in the proposed biorefinery plant can open up a range of applications and add value to this product, making the industrial sectors more profitable.

Keywords: Biorefinery. Spent brewer's yeast. RNA. 5'-Ribonucleotides. 5'-Phosphodiesterase. β-Fructofuranosidase.

RESUMO

Atualmente, a biomassa de levedura é um resíduo subutilizado da indústria cervejeira. Além disso, o uso dessa biomassa pode ser uma fonte econômica para a extração de vários compostos, como extrato de levedura, proteínas, β-frutofuranosidase, RNA e 5'-ribonucleotídeos, subprodutos com amplas aplicações na indústria farmacêutica e de alimentos. Diante dessas questões, o objetivo deste trabalho foi estudar e propor uma metodologia integrada para o fracionamento de levedura residual cervejeira em diversas biomoléculas de alto valor agregado. Para isso, os potenciais biotecnológicos da utilização de leveduras são revisados no Capítulo 1. O Capítulo 2 apresenta os resultados dos estágios iniciais de preparação e caracterização da levedura da indústria cervejeira, bem como a avaliação de parâmetros de autólise para maximizar a extração de RNA para subsequente produção de 5'-ribonucleotídeos. O Capítulo 3 descreve o uso de choque ácido no início da autólise, a fim de acelerar o processo autolítico e a extração de proteínas. Os resultados da hidrólise enzimática do RNA para a produção de 5'-ribonucleotídeos usando 5'-fosfodiesterase proveniente de radícula de malte de cevada são apresentados no Capítulo 4. A recuperação da enzima extracelular da β-frutofuranosidase como o primeiro subproduto antes da autólise e estudos sobre sua imobilização em microesferas de alginato de sódio e carvão ativado são descritas no Capítulo 5. Por fim, é proposta no Capítulo 6 uma análise econômica, bem como um projeto de planta de bioprocesso para o processamento de leveduras, a fim de determinar a rentabilidade e viabilidade financeira da a metodologia desenvolvida neste trabalho. A abordagem proposta indica uma excelente estratégia, uma vez que um melhor uso de levedura residual de processos fermentativos pode abrir uma gama de aplicações e agregar valor a esse produto, tornando os setores industriais mais rentáveis.

Palavras-chave: Biorrefinaria. Levedura residual cervejeira. RNA. 5’-Ribonucleotídeos. 5'-Fosfodiesterase. β-Frutofuranosidase.

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1 GENERAL INTRODUCTION

Brazil occupies the third place in the world ranking of beer production, just behind China and the United States, and this sector contributes to generate development, jobs, income and prosperity. According to data from Brazilian Beer Industry Association (CERVBRASIL), which brings together the four largest brewers in the country and account for about 96% of the market, domestic production of beer has grown at an average rate of 5% per year over the last ten years (CERVBRASIL, 2016). The latest published bulletin shows that beer production was consolidated in 14.1 billion liters in 2018 (CERVBRASIL, 2018). Beer yields data for 2019-2020 has not yet been released but should remain around 14.1 billion liters beer per year. Due to this high production, the brewing industry is one of the most important of the Brazilian economy, which corresponds to 1.6% of the Brazilian Gross Domestic Product (GDP) and collects more than R$ 23 billion in taxes, contributing greatly to the development of the country (CERVBRASIL, 2016).

During brewing process, yeast biomass is an important by-product and is produced in large quantities. For each cubic meter of lager beer produced after wort fermentation, about 1.7 to 2.3 kg of spent yeast biomass are recovered on a dry basis (FERREIRA et al., 2010). Since Brazil annually produces approximately 14 billion liters of beer, the estimated yeast production is about 28 thousand tons just by the brewing sector. The bakery and alcohol sectors contribute to increase this number even more (RAMOS et al., 2011). Most of the dry yeast biomass produced, however, is sold at a low price by breweries and ethanol distilleries as a nutritional supplement in animal feed formulations (OLIVEIRA AND OLIVA NETO, 2011).

In many countries, on the other hand, yeasts are recognized as a high added value product, being the raw material for the extraction of several biomolecules. Yeast derivatives represent a rich source of nutrients such proteins, amino acids, minerals, vitamins, particularly those of the B complex, as well as carbohydrates, enzymes, lipids and nucleotides with important physiological properties (PÉREZ-TORRADO et al., 2015). Taking into account the abundance, the low cost and the richness in nutrient constituents, it becomes an excellent strategy the improvement of the technology of fractionation and purification of the yeast biomass.

Nowadays, the main challenges related to the beer and ethanol industry are the search for a more suitable destination for one of its main waste products: spent yeast.

If discarded incorrectly, such residues can cause environmental contamination besides representing a waste of material rich in nutrients from the biochemical point of view (RAKOWSKA et al., 2017). If used as a supplement in food products, the amount should be limited by the nucleic acid content, since in humans, RNA is metabolized to uric acid, which can cause gout (HUIGE, 2006). The change of this scenario, of spent yeast underutilization, began to be implemented in Brazil only in 1998 by the Cooperative of Sugar, Sugar and Alcohol Producers of the State of São Paulo (Copersucar) and by the Institute of Food Technology (Ital) in a project financed by FAPESP under the Partnership for Technological Innovation program (PITE). Preliminary results reported by Sgarbieri et al. (1999) indicated that yeast could be utilized more effectively in animal feed as well as it was possible to formulate four derived substances: autolysate, yeast extract, cell wall and protein concentrate.

Time passed and despite the residual yeast have gained more notoriety because of its great potential, the development of technologies geared to reuse this raw material keeps walking at a slow pace. South and southeast Brazil already have industries that develop other applications for yeast, however just last year, in 2018, Alagoas became the first state in the Brazilian northeast to own an alcohol distillery that dries yeast for sale as an animal supplement. Meanwhile, Japan use yeast as raw material for nucleic acids extraction and production of drugs with pharmaceutical applications since the 1980s. It is clear: for Brazil to become more competitive, innovation and research are needed. This path can be achieved more quickly by the partnership between research institutes that develop new technologies and bioprocess industries that invest in this market that has not been much explored in the Brazilian context.

The use of residual yeast from beer industry, for example, may be a promising approach to overcome the challenges related to RNA obtainment and hydrolysis in large scale for the production of 5'-ribonucleotides. The yeast biomass obtained by primary fermentation for the sole purpose of RNA extraction aiming to produce nucleic acid derivatives presents high costs and makes the product more expensive. The unique Brazilian company that produced RNA for 25 years, Omtek (Iracemápolis-SP), recently ended its activities due to the obsolete and expensive methodology used. Its process was based in primary fermentation to obtain yeast biomass and presented low yield of RNA extraction (only 55%), data not published. One of the alternatives to overcome this issue, object of the research in this work, would be to obtain RNA from

a more profitable starting point. Also, in the context of RNA extraction, another challenge to be overcome that deserves to be highlighted is the efficiency of its extraction once the biomass of yeast has been obtained. The current extraction techniques consist of the use of partial hydrolysis of the yeast cell wall at high temperatures, as well as the use of salts to osmotically force the RNA out into the extracellular environment (DIMOPOULOS et al., 2018). The use of acids such as H_2SO_4 and H_3PO_4 in addition to salts such as NaCl and KCl make the process expensive and not well seen by the food industry due to the high salt content in the final product (RAKOWSKA et al., 2017).

Yeast biomass is undoubtedly an underutilized waste from brewing and its use can be considered an economical source for the production of 5'-ribonucleotides and other compounds on a large scale in order to meet the growing demand for new inputs for food and pharmaceutical industries, as well as for the development of biorefineries, using simple and efficient strategies that guarantee economic viability. Therefore, it is necessary and justifiable the development of multidisciplinary and integrated research, aiming, in the medium and long term, to develop new products with more diversified functional and nutritional characteristics from this biomass. In view of the exposed characteristics, the present work appears as an attempt to answer the question: it is possible to establish a methodology capable of fractioning efficiently and with low cost the spent brewer's yeast into by-products of high added value? The yeast fractionation approach is promising because the byproducts have a higher value than whole yeast. The extract, for example, can be marketed in powder or paste to flavor meat in soups. The sale of the extract can yield up to R$ 8.00 per kilo, while the current price, with unprocessed yeast, reaches the value of R$ 0.80 per kilo. Yeast processing can at least double its marketing value or even reach 10 times more for other uses in food or pharmaceuticals.

In order to answer the posed question, the present work reviews the biotechnological potentials of yeast utilization in the Chapter 1. The Chapter 2 presents the results of the initial stages of preparation and characterization of yeast from the brewing industry, as well as the evaluation of yeast autolysis parameters in order to maximize RNA extraction for subsequent 5'-ribonucleotides production. The Chapter 3 describes the use of acid shock at the beginning of autolysis in order to accelerate the autolytic process and the extraction of proteins. The results of enzymatic hydrolysis of RNA to produce 5'-ribonucleotides using 5'-phosphodiesterase from residual malted

barley roots is presented in the Chapter 4. The recovery of extracellular β-fructofuranosidase enzyme as the first by-product before autolysis and studies on its immobilization in sodium alginate and activated charcoal beads were described in the Chapter 5. Finally, an economic analysis, as well as a bioprocess plant design for yeast processing were presented in the Chapter 6 in order to determine the profitability and financial viability of the methodology developed in this work. The proposed approach indicates an excellent strategy, since a better use of spent yeast from fermentation processes in the proposed biorefinery plant can open up a range of applications and add value to this product, making the industrial sectors more profitable.

2 GENERAL OBJECTIVE

The general objective of this work was to study and design an integrated bioprocess methodology using spent brewer's yeast biomass as feedstock for obtaining several biomolecules through fractionation and downstream techniques: yeast extract rich in proteins, *β*-fructofuranosidase, RNA and 5'-ribonucleotides.

2.1 SPECIFIC OBJECTIVES

- Reviewing the possible biotechnological applications of spent brewer's yeast;
- Evaluation of different cell disruption methods such autolysis and acid hydrolysis regarding the efficiency to obtain maximum RNA yields;
- Evaluation of the addition of acid at the beginning of the autolytic process aiming to accelerate the autolysis and high yields of yeast extract;
- Production of 5'-ribonucleotides by enzymatic hydrolysis of RNA using 5'-phosphodiesterase from spent malted barley roots.
- Recovering of extracellular β-fructofuranosidase enzyme before autolysis and evaluate its immobilization in sodium alginate and activated charcoal beads.
- Designing a bioprocess plant for spent brewer's yeast processsing and performing an economic analysis in order to determine the profitability and financial viability of the methodology developed under optimized conditions.

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CHAPTER 1 BIOTECHNOLOGICAL POTENTIAL OF SPENT BREWER'S YEAST - A BRIEF REVIEW

ABSTRACT

The recovery and reuse of spent brewer's yeast to extract functional compounds and develop innovative ingredients has been the object of several research over the last few years. Thus, this review aims to explore the biotechnological potential of spent yeast biomass in different applications of food science, pharmaceutical science and technology, as well as various applications in industrial and environmental biotechnology. Breweries are among the largest waste producers, thanks to the high quantity of malt bagasse and spent yeast produced per year. Due to the brewing process being done all year round, there is a good availability of this raw material unequivocally called waste, which could be used for obtaining new valuable products. Different cell disruption techniques are also described such autolysis, hydrolisys, plasmolysis and other mechanical methods. These various methods have been assessed over the time in order to up-grade this by-product through fractionation and isolation of valuable compounds. Lastly, future trends in new applications exploring the biotechnological potential of spent yeasts are very welcome. It can fulfill the dual function of being a pro-healthy, important flavouring and functional additive as well as simultaneously playing an important role leading to sustainable lifestyles regarding waste management. Innovative approaches like these are compatible with the concept of biorefinery and the limitation of resources available on the planet.

Keywords: Spent brewer's yeast. Biotechnological applications. Yeast extract. Flavor enhancers. 5'-Ribonucleotides. Yeast cell disruption techniques.

1 BREWER'S YEAST BIOMASS

Yeast used in beer production is a type of unicellular fungus known as *Saccharomyces cerevisiae*. Along with other species of *Saccharomyces,* including *Saccharomyces bayanus, Saccharomyces cariocanus, Saccharomyces kudriavzevii, Saccharomyces mikatae* and *Saccharomyces paradoxus,* brewer's yeast is used to ferment beer and some types of breads (KURTZMAN AND ROBNETT, 2003). Basically, it consumes the sugar extracted from the malt, turning it into alcohol and carbon dioxide, as well as contributing aroma, flavor and color (DOS SANTOS MATHIAS; DE MELLO; SERVULO, 2014).

The most abundant element in yeast cells is carbon, which accounts for just under 50% of the dry weight. Other major elemental components are oxygen (30–35%), nitrogen (5%), hydrogen (5%) and phosphorus (1%) (MUSSATTO, 2009). The centesimal composition of brewer's yeast biomass (*Saccharomyces sp*) is shown in Table 1.

Table 1 - Centesimal composition of integral brewer's yeast (%) reported by different authors.

Components	**Caballero-Córdoba, Pacheco and Sgarbieri (1997)**	**Tu, Farnum and Cleland (1975)**	**Guzmán-Juarez (1983)**
Protein	48.51	49.80	45 - 49
RNA	7.52	8.40	8 -12
Lipids	3.44	4.91	4 - 7
Ashes	8.33	5.10	5 - 10
Total carbohydrates	32.86	-	26 - 27
Soluble fiber	9.59	-	-
Insoluble fiber	2.60	-	-

Protein is the main class of macromolecules present in residual yeast, reaching levels of 35-60% on dry basis, which includes all the essential amino acids (Table 2) (CHAE; JOO; IN, 2001). Therefore, yeast is an excellent source of high-quality protein, comparable in value with soy protein (OTERO et al., 2000), besides being an inexpensive nitrogen source with good nutritional characteristics and generally recognized as safe (GRAS) (BRIGGS et al., 2004). Carbohydrates represents the second highest compound, 35-45% of dry basis. Yeast is also considered a rich source of other nutrients such minerals (Table 3), vitamins, particularly those of the B complex, as well as enzymes and lipids (Table 4).

Table 2 - Amino acid composition (g of amino acids / 100g of protein) of integral brewer's yeast reported by Caballero-Córdoba, Pacheco and Sgarbieri (1997).

Non-essential amino acids	Yeast biomass	Essential amino acids	Yeast biomass	Standard reference*
Cys	0.34	Lys	7.13	5.8
Tyr	4.68	Leu	8.84	6.6
Glu	13.15	Ile	5.64	2.8
Asp	11.98	Thr	6.16	3.4
Ser	6.13	Try	1.10	1.1
Pro	4.45	Val	6.20	3.4
Ala	7.07	Met + Cys	2.84	2.5
Gly	4.93	Phe + Tyr	9.98	6.3
Arg	4.11	His	2.06	1.9
Phe	5.30	Met	2.50	

* Standard reference from FAO/WHO/UNU (1985).

Table 3 - Mineral composition (macro and microelements) of integral brewer's yeast reported by Caballero-Córdoba, Pacheco and Sgarbieri (1997).

Macroelements	mg/100g	Microelements	mg/100g
Phosphorus	16.94	Selenium	24.21
Potassium	13.56	Manganese	15.91
Sodium	8.95	Lead	9.69
Magnesium	2.10	Chromium	9.63
Aluminum	0.95	Nickel	7.22
Calcium	0.73	Lithium	5.89
Iron	0.10	Zinc	4.56
		Copper	4.54
		Vanadium	0.63
		Cadmium	0.29

Table 4 - Composition of fatty acids from total lipids of integral brewer's yeast reported by Caballero-Córdoba, Pacheco and Sgarbieri (1997).

Fatty acids	Structure	Concentration (% of the total)
Caprylic acid	C8:0	0.28
Capric acid	C10:0	6.13
Lauric acid	C12:0	1.29
Myristic acid	C14:0	0.88
Myristoleic acid	C14:0	0.44
Palmitic acid	C16:0	31.37
Palmitoleic acid	C16:1	3.78
Stearic acid	C18:0	9.24
Oleic acid	C18:1	10.69
Linoleic acid	C18:2	4.42
Linolenic acid	C18:3	0.57

In addition to various nutrients and enzymes of commercial interest such as proteinases and invertase, the yeast still has a high content of nucleic acids and

nucleotides with important physiological properties (DOS SANTOS MATHIAS; DE MELLO; SERVULO, 2014; PÉREZ-TORRADO et al., 2015). The content of RNA in *Saccharomyces* species accounts for more than 95% of the total nucleic acid content and is typically in the range of 7-12% (m/m), reaching concentrations up to 13% (w/w) in mutant strains (KUNINAKA, 2008).

It is common practice in brewing industry to reuse cell mass generated for inoculation of new fermentation tanks (VIEIRA; BRANDÃO; FERREIRA, 2013). The number of reuses depends on species, type of beer produced, content of the wort extract, and it may be between 3 and up to 10 times as long as it does not compromise the sensory quality of the beverage. However, when the possibility of cells recycling runs out, those cells must be removed from the process, generating new solid waste. This residue is the second largest by-product of brewing, the other two main by-products are spent grains and spent hops, and is considered very important due to its rich biochemical composition and its large amount production (MUSSATTO, 2009). The global beer production amounted to about 195 billion liters in 2017 (STATISTA, 2018) and China, the United States and Brazil are the leading countries in beer production. From this data, the world yeast biomass production can be estimated at around 390 thousand tons per year once 1.7 to 2.3 kg of cell are recovered on a dry basis as a residue after one cubic meter beer fermentation (FERREIRA et al., 2010).

The current major destination of brewery residual yeast is to formulate animal feed and to mix it with spent grain generated in the process to increase their nutritional value. However, in order to up-grade this by-product, fractionation and isolation of compounds has been tentatively assessed. The release of the intracellular content is required and of great importance in order to obtain the yeast extract and other compounds aiming new applications as nutritive complements and ingredients for formulations in food and pharmaceutical industry (DOS SANTOS MATHIAS; DE MELLO; SERVULO, 2014). The yeast fractionation can be achieved by cell disruption techniques, which, in general, can be classified as mechanical and nonmechanical (MIDDELBERG, 1995). The main techniques used for yeast processing are described below.

2 CELL DISRUPTION TECHNIQUES

According to Middelberg (1995), mechanical methods of cell disruption are non-specific and can be applied to most cell types. On an industrial scale, mechanical methods are restricted mainly to bead mill and high-pressure homogenization. Smoother breakage methods may also be employed. These are subdivided into physical, chemical and enzymatic methods. Physical methods (eg, osmotic shock and thermolysis) are rarely used because of their low efficiency. Chemical methods (eg, antibiotics, chelating agents, detergents, solvents, alkalis and acids) have been developed for bacteria such as *Escherichia coli*, but are not used in cells with thick cell walls (eg, yeast). For these cells, enzymatic methods (eg, lytic enzymes and autolysis) have been employed with greater success. Methods that combine one or more techniques (eg, enzymatic processes, followed by mechanical processes) are increasingly being used.

2.1 AUTOLYSIS

The term autolysis was first used by Salkowski in 1875 and can be understood as a lithic and irreversible event that occurs in cells caused by the action of intracellular enzymes (ALEXANDRE, 2011). More specifically, the cell's proteolytic activity is concentrated in the vacuole (HECHT; O'DONNELL; BRODSKY, 2014) and is mainly attributed to the protease A, protease B and carboxypeptidase Y (BĚHALOVÁ and BERAN, 1979). The development of autolysis usually occurs at the end of the stationary phase of growth, and is often associated with cell death (ALEXANDRE, 2011). It is characterized by a loss of cytoplasmic membrane permeability, altered cell wall porosity, decreased respiratory enzyme activity, gradual hydrolysis of cytoplasmic material by endogenous enzymes, and subsequent release of degraded products into the extracellular environment (POZO-BAYÓN et al., 2009).

The autolytic process requires careful application and control of heat to kill cells without inactivating the yeast enzymes. This process is usually carried out under moderate agitation and temperatures between 30 and 60 °C for 12 to 48 h. It has some disadvantages such as low extraction yield, difficulty in solide liquid separation due to high content of residue in autolysate, poor taste characteristics as a flavour enhancer, and risk of deterioration due to microbial contamination (FERREIRA et al., 2010).

Although it is a naturally occurring event, autolysis in yeast can be artificially induced by physical inducers, such as an increase in temperature (40-60 °C) and osmotic pressure; chemical inducers such addition of plasmolizers, antibiotics, detergents and organic solvents; in addition to mechanical intervention and other procedures that facilitate the breakdown of the cell wall, cytoplasmic membrane and activate intracellular enzymes (MALEKKHAHI et al., 2016). The addition of a pre-autolysate, for example, generally contributes to increase the yield of autolysis, since in it, the enzymes are already released and assist in breaking new cells. The autolytic process typically solubilizes about 62% solids and has a maximum yield of 80% of the original yeast protein content (ALEXANDRE and GUILLOUX-BENATIER, 2006).

2.2 ACID HYDROLYSIS

Hydrolysis refers to the use of acids to hydrolyze the yeast's constituents and does not rely on the cell's own lytic enzymes. For the production of yeast extract, this procedure requires the use of strong acids, such as hydrochloric acid, in order to degrade proteins, carbohydrates, and nucleic acids in their respective subunits with greater solubility (DALLIES; FRANCOIS; PAQUET, 1998). Acid hydrolysis is considered the most efficient technique for yeast solubilization; however, it is also considered the least practical for commercial scale production (REED and NAGODAWITHANA, 1991).

Unlike the autolytic process, acid hydrolysis does not require the use of yeast with high cell viability. In order to achieve maximum efficiency, typical hydrolysis can be initiated with resuspended dry yeast at a solid concentration of 65-85%, followed by hydrochloric acid treatment, pH of 1-5, at a temperature of 60-100 °C, where the degree of hydrolysis can be controlled by the reaction time. At the end of the hydrolysis, the highly acidic material is then neutralized with sodium hydroxide to pH 5-6, filtered and concentrated as a syrup or dried at 5% moisture content (REED and NAGODAWITHANA, 1991).

Although the acid hydrolysis procedure is known for its high yield and productivity, it has certain drawbacks that make it unattractive (HUIGE, 2006). Equipment, for example, must be made of non-reactive materials, due to the corrosive nature of hydrochloric acid. Other disadvantages include the high salt concentration in

the final product and high probability of containing carcinogenic compounds like monochloropropanol and dichloropropanol (REED and NAGODAWITHANA, 1991; HUIGE, 2006).

2.3 PLASMOLYSIS

Plasmolysis is a process that is triggered when yeast cells undergo osmotic stress caused by compounds such as sodium chloride salt and ethyl acetate added to the surrounding medium (BELOUSOVA et al., 1995; BREDDAM and BEENFELDT, 1991). The osmotic shock leads to membrane rupture and leakage of cellular contents, facilitating the degradation process. This method requires removal of the added compounds, making the process more complicated, while increasing the salt content of the final product. This process has limited use, since there is a growing demand for processed foods with low salt content.

2.4 BEAD MILL

The bead mill is a simple and effective equipment to promote cell rupture and release of cytoplasmic material. This equipment consists of a horizontal or vertical grinding chamber filled with glass or stainless-steel beads, generally less than 1,5 mm in diameter. The beads occupy between 80 and 85% of the chamber volume. During the milling process, the cell suspension and glass beads are vigorously stirred while cooling water runs through a blanket around the breaching chamber so as to dissipate the heat generated during stirring (HARRISON, 1991). By means of a rotational axis equipped with multiple discs, kinetic energy is transmitted to the beads, which leads to cellular disruption. Although an exact understanding of the mechanism of cell disruption is still unclear, cell disruption is believed to occur by direct cell collision with the beads and the action of combined cavitation and shear forces. Bead mill performance is influenced by agitator velocity, bead size, flow rate through the mill in continuous mode, cell concentration, temperature and equipment design (HARRISON, 1991). In general, large cells such as yeast and filamentous fungi, which have a rigid cell wall composed of polysaccharides, are efficiently disrupted by the bead mill (RICCI-SILVA; VITOLO; ABRAHAO-NETO, 2000).

2.5 OTHER DISRUPTION TECHNIQUES

Other methods of cell disruption have been employed in recent years, in order to increase the extraction yield of yeast intracellular products. Dimopoulos et al. (2018) investigated the effects of pulsed electric fields on the progress of yeast autolysis. They found that this treatment led to an increase of final amino acid and total solids release of 37% and 20% respectively. Furthermore, autolysis was described mathematically by a first-order fractional model through which pulsed electric fields treatment was found to accelerate the progress of autolysis up to 78% compared to untreated samples. Liu et al. (2013) investigated the effect of ultrasonic disruption for the extraction of intracellular proteins from yeast cells. Protein extraction increased with increasing treatment intensity and cell debris shifted to smaller particle sizes. Verduyn, Suksomcheep and Suphantharika (1999) reported the use of High-Pressure Homogenization for disruption of yeast prior to autolysis. Although yields were initially increased, the resulting extracts suffered of poor separability of cell debris and required clarification steps which led to the loss of solids. Finally, it is important to note that, the type of technique chosen to break the cell wall and release the intracellular compounds depends on the type of product to be obtained. Thus, there is no optimum efficient technique for hydrolyzing the yeast in all compounds of interest. The choice of the cell disruption method is important and must be done carefully, once it will influence the next stages of substance recovery, purification and application.

3 BIOTECHNOLOGICAL APPLICATIONS OF SPENT BREWER'S YEAST

The recovery and reuse of the spent brewer’s yeast to extract functional compounds and develop innovative ingredients has been the object of several research over the last few years (AMORIM et al., 2016). Different applications of spent yeast biomass in the food science, pharmaceutical science and technology, as well as various applications in industrial and environmental biotechnology can be seen in Table 5.

The search for innovation is related to overcoming challenges in current food science and technology as well as to deal with issues of environmental protection and a better waste management perspective. Brewer’s yeast is recognized as being a

beneficial dietary ingredient making up healthy and nutritious feed for farmed animal health in terms of increased immunity and decreased morbidity rates (RUMSEY; WINFREE; HUGHES, 1992; BURRELLS et al., 2001; SAKAI et al., 2001; LI and GATLIN, 2003). As a protein feedstuff, brewer's yeast has been included in commercial diet formulations for several fish species, including salmonids. It has been recognized to have potential as a substitute for live food (NAYAR et al., 1998) or as a potential replacement for fishmeal (OLIVA-TELES and GONÇALVES, 2001). Brewer's yeast is also used as microbiological media. Its autolysates and hydrolysates are used as a nutrient source for the growth of fastidious microorganisms or related product formation. The use of yeast biomass of *Saccharomyces* is also reported to be a suitable biosorbent of metal ions like lead, zinc, copper and nickel (FERREIRA et al., 2010). Moreover, heat-killed cells showed a higher degree of heavy metal removal than live cells, being more suitable for bioremediation works (FERREIRA et al., 2010).

Table 5 - Biotechnological applications of spent brewer's yeast.

Application	**References**
Animal feed and human nutrition	IN; KIM; CHAE, 2005.
Biogas production	ZUPANČIČ; ŠKRJANEC; LOGAR, 2012.
Biosorption and precipitation of heavy metals for remediation of soils and aqueous media	CHEN and WANG, 2008; MARQUES et al., 1999; FERRAZ and TEIXEIRA, 1999; BUTT, 1993; FERRAZ; TAVARES; TEIXEIRA, 2004.
Enzymes obtaininment (invertase)	HOUGH, 1990.
Ethanol production	YORK and INGRAM, 1996.
Filter elements for beverages clarification	REINOLD, 2007.
Fish nutrition	OLIVA-TELES and GONÇALVEs, 2001; RUMSEY et al., 1991.
Flavoring agent production: 5'-GMP and 5'-IMP	VIEIRA et al., 2012; FERREIRA et al., 2010, CHAE; JOO; IN, 2001.
Formulation of microbiological growth media.	FERREIRA et al., 2010; CHAMPAGNE; GAUDREAU; CONWAY, 2003; JONES and INGLEDEW, 1994.
Functional additive in a form of beverages, power or tablets	RAKIN; BARAS; VUKASINOVIC, 2004.
Nutraceuticals and pharmaceuticals	NIELSEN and JEWETT, 2008.
Positive effect on immune response, disease resistance and intestinal microbial community.	BURR et al., 2008.
Production of succinic acid for preparation of biodegradable polymers	JIANG et al., 2010.
Single cell protein production	CHANDA and CHAKRABARTI, 1996.
Substrate for microalgae cultivation	RYU et al., 2013.

Nevertheless, the applications of spent yeast in human nutrition have some limitations. High consumption levels of yeasts may cause certain diseases due to their high nucleic acid content (RUMSEY; HUGHES; KINSELLA, 1990; RUMSEY et al., 1991; RUMSEY; WINFREE; HUGHES, 1992; PODPORA et al., 2015). The borderline dose of nucleic acids that has no impact on uric acid levels in blood is 2 g/day, corresponding to an average consumption of 30 to 50 g of dried yeast (RAKOWSKA et al., 2017).

The major by-products that can be obtained from yeast correspond to the soluble fraction (yeast extract) and insoluble fraction (cell wall) beside nucleic acids and derivatives. Their characteristics and principal applications are described below.

3.1 YEAST EXTRACT

Yeast extract is a common food additive used to flavor broths, soups and condiments because of its ability to impart a characteristic umami flavor and meaty aroma (DIMOPOULOS et al., 2018). It also has played an important role in the food industry as a protein enricher, due to high protein content, high concentration of B-complex vitamins and excellent amino acid balance (PÉREZ -TORRADO et al., 2015).

It is obtained from autolysate. The autolysate corresponds to the total content obtained after the autodigestion of the cells in a process by which the cellular components are solubilized through the activation of degradative enzymes present inside the cell itself (ŠUKLJE et al., 2016). During this process, carefully controlled conditions of temperature, pH, time, and the addition of certain agents that enhance autolysis are required. The soluble part is called yeast extract and corresponds to the protein concentrate, released naturally by the cell after degradation of the intracellular content (POZO-BAYÓN et al., 2009). Yeast extracts have diverse flavors depending on the methods used for their manufacture and are affected by the interactions between amino acids, nucleotides, carbohydrates and peptides present in the extracts. By controlling the manufacturing process, different flavors such as those of chicken soup, meat, cheese, mushrooms and others can be obtained. Meat flavors in extracts are produced, inter alia, by the reaction of 5’-nucleotide glutamic acid and cysteine (RAKOWSKA et al., 2017).

The global yeast extract industry has been expanding dynamically, presenting annual market growth rates of more than 8% between 2009 and 2015 (ULRICH, 2014). Yeast extract manufacturers today are looking for environmentally friendly and cost-effective solutions to provide a high quality, maximum yield product, ensuring process reliability and minimal waste.

3.2 YEAST CELL WALL

The cell wall corresponds to the insoluble part of the autolysate, being constituted by a fraction rich in carbohydrates like mannans, β-glucans and glycoproteins. β-glucans constitute the building blocks of yeast cell walls and can thus be used in human nutrition as a non-caloric food, dietary fiber, thickeners of sauces and soups or serving as food additives in functional foods as water-holding or oil-binding agent and emulsifying stabilizer (THAMMAKITI et al., 2004).

β-Glucan can be prepared from yeast cell walls recovered after spent brewer's yeast autolysis. Briefly, the cell walls are homogenized with alkali and the extraction occurs followed by an acid and washing step with distilled water. The obtained β-glucan preparations usually present a light-tan colored paste having a pH value near 4.3 and approximate composition in percentage (w/w) as followed: moisture 93.37, fat 0.07, ash 0.04, protein 0.38, and carbohydrate 6.13 (WORRASINCHAI ET al., 2006).

Worrasinchai et al. (2006) studied the application of β-glucan prepared from spent brewer's yeast as a fat replacer in mayonnaise. In their work, fat was partially substituted by β-glucan at levels of 25, 50, and 75%. The reduced fat mayonnaises exhibited higher storage stability than the full fat sample used as a control experiment. According to Rakowska et al. (2017), β-glucans products obtained via post-fermentation of beer also exhibit a high and multi-faceted biological activity where they improve the blood's lipid profile, enhance immunological status and have both prebiotic and anti-oxidant properties. Moreover, it also has attracted attention because of its bioactive and medicinal properties such as anti-inflammatory, anti-microbial, anti-infective, anti-viral, anti-tumoral, cholesterol-lowering, radioprotective and wound-healing (KOGAN, 2000; STONE and CLARKE, 1992).

3.3 RIBONUCLEOTIDES

Ribonucleotides can be extracted from the yeast biomass by hydrolysis, plasmolysis, and autolysis. The most widely used method of extraction is autolysis (KIM; LEE; LEE, 2002). During this process, RNA is degraded releasing 2 '-, 3'- or 5'-ribonucleotides, by endo-enzymes of the microorganism, mainly by proteases and nucleases (AUSSENAC et al., 2001; ALEXANDRE, 2011). Ribonucleotides with 2'- and 3'-phosphate groups show no flavoring characteristics and are of little commercial interest. On the other hand, 5'-ribonucleotides are high value-added molecules and are widely used in the food and pharmaceutical industries because of their bioactive properties (OLMEDO et al., 1994).

5'-ribonucleotides can be obtained by chemical or enzymatic hydrolysis of RNA (OLMEDO et al., 1994). The chemical hydrolysis makes it possible to obtain all ribonucleotides RNA components, but without any specificity. On the other hand, the enzymatic treatment produces specific ribonucleotides, this being the main method for the production of ribonucleotides for food and pharmaceutical uses (KIM; LEE; LEE, 2002). The enzymes responsible for the decomposition of RNA are phosphatases, which hydrolyze the nucleotide sequences, breaking the phosphodiester bonds and releasing the corresponding nucleosides in addition to the phosphoric acid. According to Deoda and Singhal (2003), enzymatic hydrolysis is less complex, more economical and has higher yields compared to other methods of 5'-ribonucleotide production.

Yeast extracts rich in 5'-ribonucleotides, especially 5'-inosine monophosphate (5'-IMP), 5'-guanosine monophosphate (5'-GMP) and monosodium glutamate (GMS), are traditionally known to enhance flavors and produce softness in soups and sauces (LÖLIGER, 2000). Extracts rich in 5'-cytosine monophosphate (5'-CMP), 5'-uridine monophosphate (5'-UMP) and 5'-adenosine monophosphate (5'-AMP) have applications in the pharmaceutical industry, antiviral, anti-tumor and immunostimulatory properties (SOARES; DE SOUZA; FERREIRA, 2001). Glutamate is the most important flavor enhancing substance and its impact threshold lies at a concentration of 100-300 ppm, whereas for 5’- GMP and 5’-IMP this threshold stands respectively at 35 and 120 ppm (concentrations in aqueous solution). Glutamate’s impact on flavor may however be 10 to 15 times greater when used in conjunction with the 5’-nucleotides (RAKOWSKA et al., 2017).

4 FINAL CONSIDERATIONS

Breweries are among the largest waste producers, thanks to the high quantity of malt bagasse and spent yeast produced per year. Due to the brewing process being done all year round, there is good availability of raw material unequivocally called waste which could be used for obtaining new valuable products. According to a new report by Grand View Research, Inc (2019), global demand for nucleotides is expected to reach $ 809.3 million by 2022. World leaders in the production of ribonucleotides are in the Asia-Pacific region, where it is the region of the planet where the fastest growth is expected, with an average annual growth rate of 9.7% by 2022. The substantial growth of this market over the period of prediction, contributes to the Brazilian brewing sector to better exploit the potential of yeast biomass and reach other market niches besides beer, thus becoming more competitive.

Finally, the spent yeast reuse through its fractionation into several biomolecules can be an alternative to obtain add value compounds from a cheap source. This process could be applied as an effective approach in many industrial applications for technological purposes with great benefits. Furthermore, future trends in new applications exploring the biotechnological potential of spent yeasts are very welcome. It can fulfill the dual function of being a pro-healthy, important flavouring and functional additive as well as simultaneously playing an important role leading to sustainable lifestyles regarding waste management. Innovative approaches like these are compatible with the concept of biorefinery and the limitation of resources available on the planet.

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CHAPTER 2 SPENT BREWER'S YEAST AUTOLYSIS OPTIMIZATION FOR RNA AND DERIVATIVES RECOVERY

ABSTRACT

Currently, the main challenges related to the production of nucleic acid and derivatives are linked to RNA obtainment in large scale as well as its breaking down into small molecules. Considering the large amount of spent yeast produced after beer fermentation, the challenge proposed here is to use this biomass as a cheap source to extract RNA aiming to decrease the costs related to the obtainment of cell biomass. Thereby, the goal of this work was to evaluate autolysis parameters as cell disruption method regarding the efficiency to obtain maximum RNA yields. The parameters analyzed were temperature, pH, cell concentration and addition of inducers like salt. After autolysis optimization, further studies on acid hydrolysis and RNA recovery and purification through downstream processes were also object of study. Our results showed that autolysis was more effective than acid hydrolysis to extract RNA. However, in relation to protein extraction, the acid hydrolysis at pH 2 presented performance 71% higher than the autolysis. Spontaneously major formation of 5'-GMP and 5'-AMP was observed during autolysis at 60 °C. The results achieved are very promising and the possibility of obtaining flavor enhancers, proteins and β-D-fructofuranosidase with the strategy discussed here increases the potential for application of spent yeast as well as to add value to this residue. Further studies on 5'-ribonucleotides purification are suggested as future perspectives and challenges that still need to be overcome.

Keywords: Autolysis. Acid hydrolysis. RNA extraction. Flavor enhancers, 5'-Ribonucleotides.

1 INTRODUCTION

Currently, the main challenges related to the production of nucleic acid and derivatives are linked to RNA obtainment in large scale as well as its breaking down into small molecules. Yeast species such *Saccharomyces cerevisiae* and *candida utilis* present high RNA content (7-12% on dry basis) and are used over the time for yeast extract and other compounds production (KUNINAKA, 2008). Other challenge to be overcome is the extraction of RNA from inside yeast cell. The current extraction techniques consist of autolysis, partial hydrolysis of the yeast cell wall at high temperatures with concentrated acids as well as the use of salts to osmotically force the RNA out into the extracellular environment (DIMOPOULOS et al., 2018). The use of acids such as H_2SO_4 and H_3PO_4 in addition to salts such as NaCl and KCl make the process expensive and not well seen by the food industry due to the high salt content in the final product (RAKOWSKA et al., 2017).

Considering the large amount of spent yeast produced after beer fermentation, the challenge proposed here is to use this biomass as a cheap source to extract RNA aiming to decrease the costs related to the obtainment of cell biomass. Thereby, the goal of this work was to evaluate autolysis parameters as cell disruption method regarding the efficiency to obtain maximum RNA yields. The parameters analyzed were temperature, reaction time, biomass concentration and addition of inducers like salt and acid. After autolysis optimization, further studies on the recovery and purification of RNA and derivatives were also proposed. The following topics present a brief review of the mechanism involved during autolysis as well as the principles involved in downstream steps like ultrafiltration and ion exchange liquid chromatography, which were used in this work.

1.1 THE AUTOLYTIC PROCESS

Autolysis is defined as a cellular lytic event caused by the action of enzymes and has been studied since the middle of 1875, initially by Salkowski (ALEXANDRE, 2011). From Salkowski's primary experiments, a considerable amount of knowledge accumulated in the area, through observation of the various phases of autolysis. It was observed that autolysis occurs naturally by the action of endogenous enzymes commonly at the end of the stationary phase due to natural aging of cells. However,

autolysis process can be induced and accelerated according to physical factors (increase in temperature, osmotic pressure and variation between freezing and melting state), chemical (pH, detergents and antibiotics) and biological (aeration and nutrient deprivation) (ALEXANDRE, 2011).

According to Babayan and Bezrukov (1985) and Alexandre, (2011), four steps could be proposed to describe yeast autolysis: a) degradation of cell endo-structures releasing vacuolar proteases in the cytoplasm; b) inhibition of released proteases by specific cytoplasmic inhibitors followed by an activation phase due to degradation of these inhibitors; c) hydrolysis of intracellular polymer components and accumulation of the hydrolysis products in the space restricted by the cell wall; and d) release of hydrolytic products when their molecular mass is small enough to cross the cell wall pores.

The control of autolysis parameters such as pH and temperature are fundamental for the optimization of yeast extract production. The effect of temperature on yeast cells is quite complex and affects the synthesis and activity of endogenous enzymes, mechanism of cell control, transfer of anabolic information from genes to ribosomes, absorption of ions and molecules and, most important, composition and integrity of cell membranes (REED and NAGODAWITHANA, 1991). Cell membranes have lipids and proteins, as well as cations for their stabilization. If the incubation temperature of a yeast suspension is increased from 30 to 40 °C rapidly, two simultaneous effects occur: the enzyme activity of a number of enzymes increases and the membranes become more permeable due to an increase in the ratio unsaturated/saturated fatty acid.

In addition to the cell membrane, yeasts have a cell wall consisting mainly of rigid polymers of glucans, mannans and chitin, which requires more specific conditions of cellular disruption. During autolysis, the biochemical mechanism leading to the rupture of the yeast cell wall is related to the synergistic action of endogenous glucanases, mannanases and chitinases enzymes (CHARPENTIER et al., 1986). The process is irreversible and can be accelerated by the use of inducers such as fresh yeast autolysate, in the order of 15% (v/v), sodium chloride and specific temperature and pH (KOLLÁR; ŠTURDÍK; FARKAŠ, 1991).

The understanding and study of this factors that lead to changes in the cell during autolysis are important because they will influence the efficiency of RNA extraction. Thus, the goal is to optimize parameters such as temperature and other

inductors so that the membrane and cell wall become semipermeable enough for the RNA to be flushed out.

1.2 DOWNSTREAM PROCESSES

After RNA extraction, downstream steps characterized primarily by the recovery and purification of the product are necessary. Downstream processes are defined based on some characteristics such as: intracellular or extracellular product location, the required final purity, the market price, the physical and chemical properties and the concentration of the product in the medium among others (CHISTI, 1998).

Generally, the main steps in downstream process consist of the remove solid residues, if the product is intracellular, a cellular breakdown is necessary, then a primary isolation of the product, a purification and concentration step and finally the isolation and final treatments of the final product (ZYDNEY, 2016). Removal of solids may involve filtration, centrifugation or precipitation. Two phase system can be used to liquid-liquid extraction using immiscible substances, where the product will remain in only one phase due to its own characteristics. For a product that requires concentration and purification of high resolution, ultrafiltration and ion exchange liquid chromatography can be used. Finally, after the product is isolated, it will go to the final treatments, which may consist of a lyophilization to obtain the powder of the product, or a spray dryer (STANBURY; WHITAKER; HALL, 2013; GOLDBERG, 2012).

For the purposes of RNA and ribonucleotides recovery and purification, ultrafiltration and ion exchange liquid chromatography steps were considered in this work as the idea is to obtain a high-quality product as fast as possible, with an efficient recovery rate, using minimum investment and minimum costs.

1.3 ULTRAFILTRATION

Ultrafiltration is one of the membrane filtration processes that aims to separate solutes dissolved in liquid streams. Ultrafiltration membranes have pore sizes in the range of 20 to 100 nm, and are generally characterized in terms of the Nominal Molecular Weight Cut off (NMWC), which is the molecular weight of the largest globular protein that can pass through the membrane. NMWC values range from 1 to 100 kD

in ultrafiltration range (ZEMAN and ZYDNEY, 2017). These filters are used for concentrating and fractionating protein streams, virus concentration, desalting and buffer exchange.

Generally, the configuration used in ultrafiltration is cross flow filtration instead of dead-end filtration, where the starting material is simply passed through the filters. A key feature of cross flow filtration is the flow of fluid along the membrane surface that sweeps away the buildup of material on the filter surface and reduces fouling of the filter. In addition, retentate solution can easily be recirculated, allowing thorough processing of large volumes of solution (ZYDNEY, 2016).

Ultrafiltration has been widely used in several sectors, for example for the removal of proteins and other macromolecules in biological sample analyzes (ALELE and ULBRICHT, 2016), in water purification (MA et al., 2017) and solutes fractionation technologies (JIANG et al., 2018). Harvesting and cell clarification applications involve separation of relatively large particles (cells and/or cell debris) from macromolecules, so that high selectivity is generally not required. Lysate clarification, on the other hand may make more stringent demands, since the yeast extract will contain a wide range of proteins and other macromolecules like RNA, ribonucleotides e nucleosides (STANBURY; WHITAKER; HALL, 2013; GOLDBERG, 2012).

The objective of ultrafiltration processes in this work is to retain soluble macromolecules such as proteins above a certain size, while allowing smaller molecules such as 5’-ribonucleotides, nucleosides and small RNA chains to pass through the membrane.

1.4 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography for the separation of biomolecules was introduced in the 1960s and continues to play a major role in the separation and purification of biomolecules (AHMAD et al., 2019). The technique is capable of separating molecular species that have only minor differences in their charge properties. It involves the separation of ionizable molecules based on their total charge from an ion exchange, a process that exchanges ions of the same signal between a solution and a resin (HALAN et al., 2019). The principle of separation is an ion exchange of the solution with the ions set in the ion exchanger. The separation is

influenced by a number of factors, for example, the way in which the net surface charge of each protein in the sample varies with pH, the pH and ionic strength of buffers, and the elution conditions (JIAO et al., 2017). Understanding the role and importance of each parameter ensures that every separation can be performed with the required resolution, throughput and speed.

The ion exchange chromatography has been used in several separation and purification processes, having applications in the food and pharmaceutical industries (FEKETE et al., 2015), agriculture and environmental soil research (QIAN and SCHOENAU, 2002), as well as biotechnological processes such as for purification of proteins and peptides (REGNIER, 1982) and other charged biomolecules like nucleotides (RANDERATH and RANDERATH, 1964), offering high resolution and group separations with high loading capacity. These features and its wide use make ion exchange chromatography well suited for 5'-ribonucleotides intermediate purification.

2 OBJECTIVES

This work aims to optimize autolysis parameters as cell disruption method regarding the efficiency to obtain maximum RNA yields. The parameters analyzed were temperature, pH, cell concentration and addition of inducers like salt. Acid hydrolysis was also object of study as an alternative to obtain RNA. The recovery and purification of RNA and other by-products such as β-D-fructofuranosidase (invertase) and 5'-ribonucleotides were performed, since a better use of spent brewer's yeast can open a range of applications and add value to this product, making the industrial sectors more profitable.

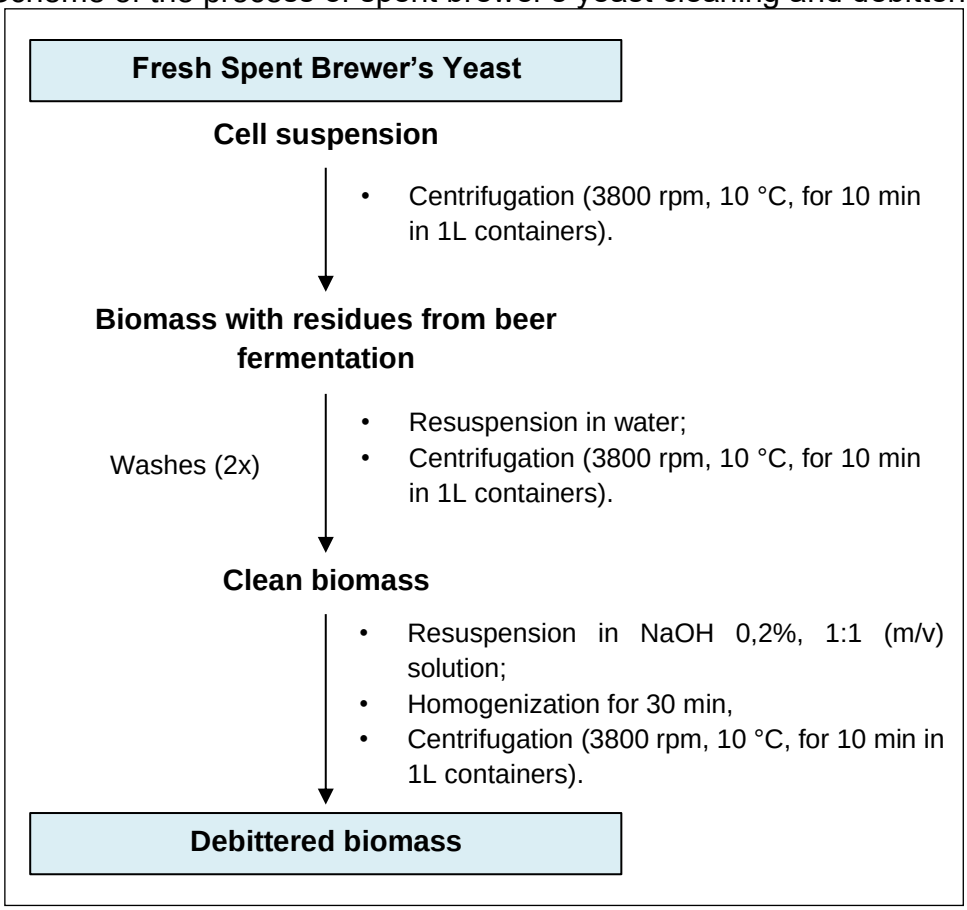
3 MATERIAL AND METHODS

3.1 SPENT BREWER'S YEAST CLEANING AND DEBITTERING

Fresh yeast cells of *Saccharomyces cerevisiae*, in the form of aqueous suspension, kindly provided by the company Cervejaria Malta (Assis-SP) were used in this work. Approximately 1 liter of yeast cream was subjected to a series of 3 washes

for the removal of culture medium residues from the fermentative brewing process. The biomass was centrifuged in 1 L containers at 3800 rpm (RCF = 4794 x G) for 10 min at 10 °C (Heraeus Cryofuge 6000 Centrifuge, Thermo Scientific, Osterode, Germany). The supernatant was discarded, the cells were resuspended in 1 L of water and then centrifuged again for a second and third wash.

Figure 1 - Scheme of the process of spent brewer's yeast cleaning and debittering.



(SGARBIERI et al., 1999, modified)

After cleaning, the cells were homogenized for 30 minutes with addition of 0.2% NaOH solution 1: 1 (m/v) to promote the biomass debittering. This stage is important once the residual yeast from breweries shows a markedly bitter taste due to the adsorption on the surface of the cells of bitter components such as iso-alpha acids, resins and hop tannins. The main method of removing bitterness from residual yeast consists in the hydrolysis of these components in the presence of a catalyst (sodium hydroxide). Figure 1 present a diagram of the methodology used for cleaning and debittering. After debittering, the cells were characterized in terms of RNA and protein content according to section 3.6 and 3.7.

3.2 AUTOLYSIS PARAMETERS OPTIMIZATION FOR MAXIMUM RNA YIELDS

Cellular suspensions constituted of cleaned and debittered yeast cells were submitted to autolysis under controlled conditions for 24 hours. Experiments were carried out in order to optimize the following parameters: temperature (varying from 40 to 65°C), pH (varying from 5 to 7), cell concentration (varying from 5 to 12.5%, dried yeast w/v) and amount of salt (varying from 5 to 15%, w/w dried yeast) added at the beginning of the autolysis. The design of experiments was carried out using the on-factor-at-a-time method (FREY; ENGELHARDT; GREITZER, 2003). This technique consists of a traditional approach in which factors or variables are tested one at a time while maintaining the other factors or variables at fixed levels instead of testing multiple factors simultaneously. The first parameter evaluated was the temperature, which was studied in the range of 40 to 65 °C while maintaining pH 5, cell concentration at 10% (dried yeast w/v) and 0% salt (w/w dried yeast). The subsequent evaluated parameters were pH, cell concentration and salt content respectively. The assays were performed in triplicates using 2g (dry mass) of yeast in 50mL falcon tubes. Thermostatic bath was used to control the temperature.

3.3 ACID HYDROLYSIS STUDY FOR RNA EXTRACTION

In addition to the autolysis optimization, the acid hydrolysis process was studied as an alternative to obtain RNA. The acid hydrolysis was carried out using H_2SO_4 for 60 minutes, at 10% (w/v) cell concentration, pH varying from 1 to 3 and temperature varying from 50 to 100 °C.

3.4 RNA RECOVERY

At the end of the autolysis and acid hydrolysis, the samples were centrifuged at 3800 rpm (RCF = 4794 x G) for 10 min at 10 °C (Heraeus Cryofuge 6000i Centrifuge, Thermo Scientific, Osterode, Germany) in order to obtain the insoluble fraction (cell wall) and the yeast extract (soluble fraction). The soluble fraction was assessed regarding total soluble solids, protein and RNA content.

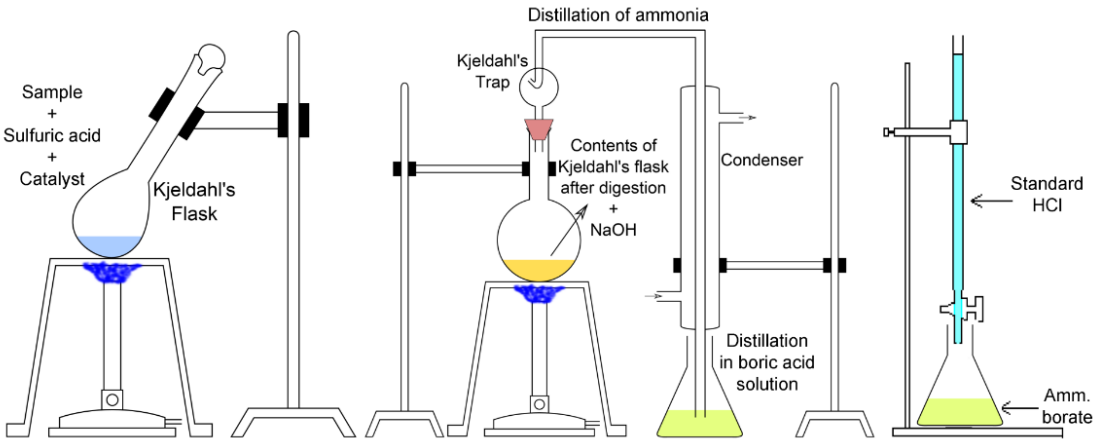
3.5 TOTAL SOLUBLE SOLIDS ASSAY

Soluble fractions were characterized as total soluble solids content. 10 mL of each yeast extract were dried at 105 °C for 24 hours in a pre-weighed petri dish in order to determine the mass of total soluble solids in the yeast extract. The analysis was performed in triplicate considering the initial dry mass of yeast used at the beginning of autolysis.

3.6 PROTEIN QUANTIFICATION

The determination of the nitrogen and total protein parameters was achieved using the classical Kjeldahl method, according to the methodology described by AOAC (2000), which basically comprised three steps (Figure 2): digestion of the sample with sulfuric acid and a catalyst at high temperatures, which resulted in the conversion of ammoniacal nitrogen; distillation of ammoniacal nitrogen into a catch solution; and finally the quantification of ammonia by titration.

Figure 2 - Scheme for total nitrogen and protein determination using the classical Kjeldahl method (AOAC, 2000).



(1) Digestion (2) Neutralization and distillation (3) Titration

For the analysis, approximately 0.1 g (for dry samples) or 1 mL (for liquid samples) was digested with 2 mL of conc. H_2SO_4, 1 g of catalytic mixture ($CuSO_4$ and K_2SO_4 in the ratio 4:96) in Kjeldahl tubes heating up to 350 °C gradually for about 2 hours until the appearance of dense white fumes and solutions became clear. After

cooling to room temperature, the solutions were diluted with 10 mL of distilled water and five drops of phenolphthalein was added. Each Kjeldahl flask was coupled to the nitrogen distillation device, approximately 5 mL of 12.50 M NaOH solution was added until the solution turning to pink and the distillation process was performed by receiving the distillate in previously filled 125 mL Erlenmeyers with 20 mL of 0.32 M boric acid solution, 2 drops of methyl red and 2 drops of bromocresol green. The distillation was stopped at about 100 mL. Then the samples were titrated (light gray to light red) with 0.02 M HCl and the volume of the titration was recorded. The blank test was done using distilled water instead of the sample. Equations 1 and 2 were used to determine the total protein nitrogen content of each sample:

$$TN = \frac{(Va - Vb) * M * f * 0.014 * 100}{m} \quad (1)$$

Where:

TN – total nitrogen content in the sample (%);

Va – volume of the hydrochloric acid solution spent in the titration of the sample, in mL;

Vb – volume of the hydrochloric acid solution spent in the blank titration, in mL;

M – molarity of the hydrochloric acid solution (0,02 mol/L);

f – correction factor for hydrochloric acid;

m – mass of the sample in grams (dry basis). For liquid samples, the total volume of extract obtained was considered in the accomplishment of the calculations.

$$\mathrm{TP} = TN * Fn \quad (2)$$

TP – crude protein content in the sample (%);

Fn = Nitrogen to protein conversion factor (for yeasts Fn=5.8).

The conversion factor 5.8 was used to calculate the protein content (SGARBIERI, 1996). According to Reed & Nagowithana (1991), the use of the universal factor 6.25 overestimates the value of the yeast protein, since about 14% of the total nitrogen corresponds to the non-protein nitrogen from the purine and pyrimidine bases.

3.7 RNA QUANTIFICATION

The determination of RNA concentration in the samples was performed according to Herbert, Phipps, and Strange (1971). In this method, the pentoses are converted to furfural during treatment with hot acid and then converted to hydroxymethylfurfural. In the reaction catalyzed by iron salts, the pentoses (ribose), after heating and reaction with orcinol, produce greenish coloration, which is read in a spectrophotometer at 670 nm.

3.8 DOWNSTREAM PROCESSES

3.8.1 Protein and RNA separation through ultrafiltration

Ultrafiltration technique was evaluated in order to separate the RNA from the protein portion after autolysis. The obtained yeast extract was submitted to an ultrafiltration process using an ultrafiltration system (TE-198, Tecnal, Piracicaba, Brazil) (Figure 3) equipped with ultrafiltration membrane with 10 kDa molecular weight cut off and 290 cm^2 area (UFP-10-C-3X2MA, GE Helthcare Bio-Sciences Corp., Westborough, USA) in order to concentrate large molecules such as proteins and enzymes in the retentate.

Figure 3 - Ultrafiltration system used in this work.

The parameters used during ultrafiltration are presented in Table 1. They are important for future scale-up studies.

Table 1 - Operational parameters during ultrafiltration.

Parameters	Values
Feed pressure	17 psi
Retentate pressure	16 psi
Flux	2.17 mL/s
Flux through the fiber's lumen	0.16 mL/s
Sample initial volume (yeast extract)	98 mL
Eluent volume (deionized water)	500 mL
Filtrate volume	500 mL
Retentate volume	98 mL

After ultrafiltration, analyzes of the protein content, RNA and β-D-fructofuranosidase activity were performed in the filtrate and retentate in order to evaluate the efficiency of the ultrafiltration. The enzyme activity was determined by the concentration of reducing sugars produced after reaction with sucrose. The quantification of reducing sugars was performed according to Miller (1959), with adaptations, using 3,5-dinitrosalicylic acid (DNSA) as a color reagent. 1 unit of β-D-fructofuranosidase activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing groups, measured as glucose per minute, under the conditions used in the reaction.

3.8.2 5-Ribonucleotides quantification

During autolysis, part of the RNA is naturally degraded by yeast nucleases with 5'-ribonucleotides production (ZHAO and FLEET, 2005). High Performance Liquid Chromatography (HPLC) was used to verify and quantify the presence of these naturally produced molecules. Standard solutions containing 10µg/mL of 5'-GMP (G8377-5G, Sigma-Aldrich Brazil Ltda), 5'-CMP (C1006-5G, Sigma-Aldrich Brazil Ltda), 5'-UMP (U6375-5G, Sigma- Aldrich Brazil Ltda.), 5'-IMP (I4625-5G, Sigma-Aldrich Brazil Ltda) and 5'-AMP (01930-5G, Sigma-Aldrich Brazil Ltda) were prepared and used for standard curve preparation. The separation of ribonucleotides was achieved on reverse phase column Acclaim C30 (2.1 x 150 mm, 3µm) at 15 °C using an HPLC system (2475 - Multi λ Fluorecence Detector, Waters, 2012) with UV detector at 260 nm. The mobile phase flow rate was 0.25 mL/min with gradient elution. The mobile phase consisted of 3 components: A) deionized water; B) 100 mM ammonium acetate buffer, pH 5 and C) Methanol. Component B was kept constant along the

gradient to maintain the concentration of mobile phase buffer at 20 mM. Methanol was maintained in 0% from 0 to 5 minutes, increased to 10% in 3 minutes, increased to 40% in 5.5 minutes, maintained at 40% for 4 minutes, decreased to 0% in 0.1 minute followed by 10 minutes of column reequilibration to the initial condition. The entire run lasted 28 minutes. The elution gradient events are detailed in Table 2. From this methodology, chromatograms of each ribonucleotide were obtained to determine the retention time of the molecules.

Table 2 - Elution gradient.

Time (min)	% de A	% de B	% de C	Flux (mL/min)
0	80	20	0	0.25
5	80	20	0	0.25
8	70	20	10	0.25
13.5	40	20	40	0.25
17.5	40	20	40	0.25
17.6	80	20	0	0.25
28	80	20	0	0.25

3.8.3 Isolation of 5'-ribonucleotides by ion exchange chromatography

After ultrafiltration, the filtrate was subjected to ion exchange chromatography for the separation of 5'-ribonucleotides fractions. AKTA Start chromatograph (GE Healthcare Bio-science, New Jersey, USA) (Figure 4) was used for the purification assay.

Figure 4 - AKTA Start chromatograph used to purify 5'-ribonucleotides.

Column (model XK 16/20) with a bed volume of 30 mL was filled with 25 mL of strong anion exchange resin containing quaternary ammonium groups (Q Sepharose® Fast Flow, 17-0510-10, GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Deionized water pH 7 (A) and 0.5 M NaCl solution (B) were used as mobile phase during the elution. The chromatographic run was performed at room temperature with a flow velocity of 100 cm/hr. The column was equilibrated for 30 minutes with solution (A), then 200 ml of the ultrafiltrate was injected into the system in order to saturate the adsorptive capacity of the column. Thereafter, the unbound molecules were removed from the system in the wash step, in which a volume of 125 mL of (A) was used. Finally, during the elution step (500mL), the ionic force of mobile phase was increased linearly from 0 to 100% of the solution (B), aiming to separate 5'-ribonucleotides according to the force with they were adsorbed on the resin.

4 RESULTS AND DISCUSSION

4.1 BIOMASS CLEANING AND DEBITTERING

Yeast cleaning and debittering are important steps for removing residues from the fermentative medium as well as to remove the bitter taste. Figures 5 shows the visual appearance of spent brewer's yeast before and after cleaning and debittering.

Figure 5 - Visual aspect of the fresh spent brewer's yeast containing residues from beer fermentation (a) and after the cleaning and debittering procedure (b).

a)  **b)** 

A color clarification of the biomass and a fresh yeast smell without bitterness taste was observed after the cleaning process. According to Kutsakova et al. (2012) and Simard and Bouksaim (1998) alkali and iso-alpha acids react to form soluble salts that are easily removed with water, and can promote a reduction of bitterness by up to

98% without affecting cell viability or protein content. The results of yeast cell characterization in terms of RNA and protein content are presented in Table 3.

Table 3 - Centesimal composition of integral brewer's yeast.

Compounds	**% on dry basis**
Proteins	53.43
RNA	9.47
Others	37.10

The values found for protein and RNA content in integral cells are in agreement with the literature. According to Chae, Joo and In (2001), brewer's yeast cells have about 40-60% protein. The results for RNA content are also as the expected for *Saccharomyces* species, which is typically in the range of 7-12% (w/w) (KUNINAKA, 2008).

4.2 OPTIMIZATION OF AUTOLYSIS PARAMETERS

The autolysis optimization aimed high yields of RNA extraction and was performed by evaluating each parameter of temperature, pH, cell concentration and NaCl content separately. Figure 6 shows the influence of each parameter on the RNA extraction yields.

The first parameter evaluated was the temperature, which was studied in the range of 40 to 65 °C maintaining fixed pH 5 and cell concentration at 10%. It was observed that, under these conditions, the temperature has great influence under RNA extraction (Figure 6a), with the best yields being obtained in the range of 60 to 65 °C ($p<0.05$). For pH evaluation (Figure 6b), the temperature was set at 60 °C and the cell concentration was maintained at 10% (w/v). It was observed that the extraction of RNA showed little sensitivity to pH variations in the range of 5.5 to 7. From this observation, it can be concluded that there is no need to correct the pH during autolysis, since the yeast cell suspension when prepared has pH in the range of 5, 8 to 6.2. Regarding the optimization of cell concentration (Figure 6c), the best value was found for a cellular concentration of 7.5% (w/v) ($p<0.5$) at pH 6 and 60 °C for 24 hours. The addition of 10% NaCl (w/v) is reported in the literature (OLIVEIRA and OLIVA NETO, 2011) as an element that increases the extract yield during autolysis. However, it was observed in

this study (Figure 1d) that addition of salt at the concentrations range studied did not significantly influence RNA extraction (p>0.5).

Figure 6 - Influence of each parameter regarding the percentage of RNA extracted in relation to the dry mass of yeast used. Autolysis lasted 24 hours for all the assays. In (a), the temperature was varied, keeping fixed the parameters pH at 5, 10% (w/v) cell concentration and 0% salt. In (b), the pH was varied, keeping fixed temperature at 60°C, cell concentration at 10% (w/v) and 0% salt. In (c), the cell concentration was varied, keeping fixed temperature at 60 °C, pH at 6 and 0% salt. In (d), the NaCl content was varied, keeping fixed temperature at 60 °C, pH at 6 and cell concentration 7.5% (w/v).

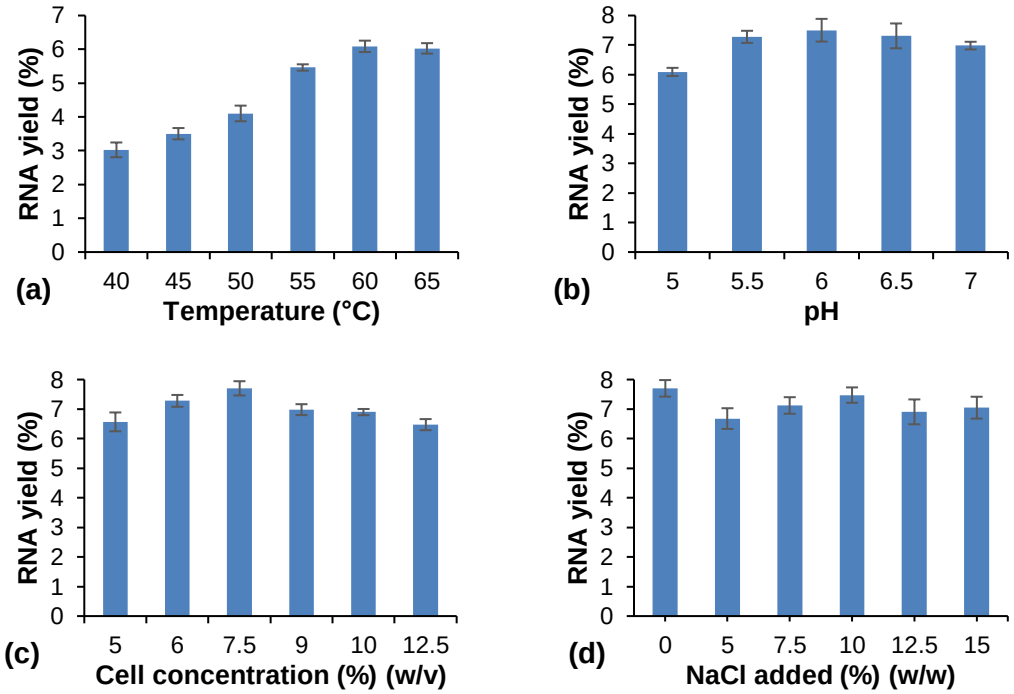


Table 4 - Yields of RNA, protein and total soluble solids present in the yeast extract in relation to the dry mass of yeast used in the autolysis.

Compound	Autolysis without salt (standard deviation)	Autolysis with salt (standard deviation)
RNA %	8.12 (0.24)	8.16 (0.52)
Protein %	24.40 (1.27)	23.62 (1.21)
Soluble solids %	35.90 (0.52)	36.38 (0.31)

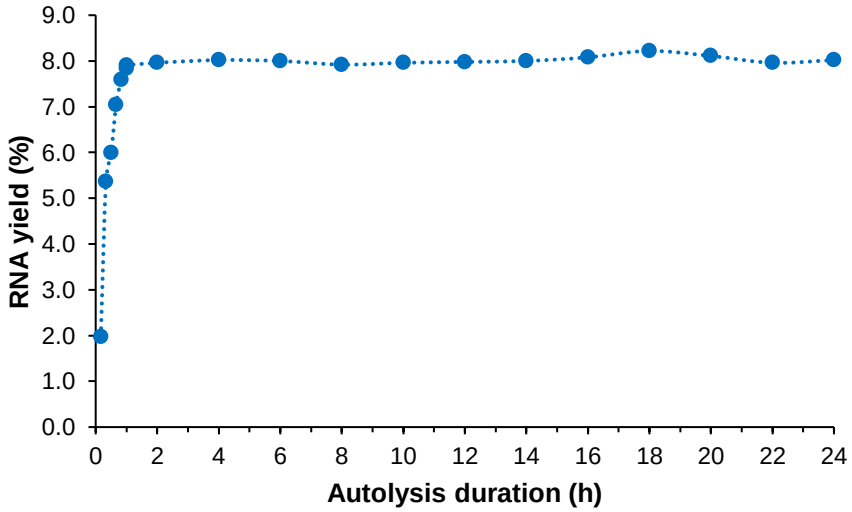
The influence of the salt under the extraction of other components can also be observed in Table 4, which shows the yield of the salty and salt-free autolysis assays under optimized conditions of temperature 60 °C, pH 6.0 and cell concentration of 7.5% (w/v) for 24 hours. There were no statistically significant differences (p> 0.05) between the means of the three components analyzed. These results show that there is no need

of NaCl addition in the autolysis under the conditions here proposed. This result is welcome since the final product will not have increasing in the salt content.

Finally, a kinetic study of RNA releasing profile to the extracellular medium over autolysis is presented in Figure 7. It was observed that the percentage of RNA extracted in relation to the yeast dry mass increased rapidly at the beginning of the autolysis from 10 to 60 min. It stabilizes around 8% and no significant increase was observed after this period. Thus, it is possible to define 60 minutes as the optimal autolysis duration time for RNA extraction. This result of RNA extraction is very promising since it was believed that a longer time interval for RNA extraction would be required.

Previous studies on yeast autolysis pointed out periods from 12 to 36 hours for a good extraction yield of proteins and soluble solids (OLIVEIRA and OLIVA NETO, 2011). However, the best time for RNA extraction was not studied. In this work, it was possible to extract 82.7% of the total RNA present in the cells in only 60 minutes of autolysis. The high yield allied to the short time autolysis and the absence of salt addition are shown as an excellent strategy to obtain RNA from residual brewer's yeast. From the findings of the present work it is possible to establish an RNA extraction methodology that saves time and reduces the costs inherent in the process.

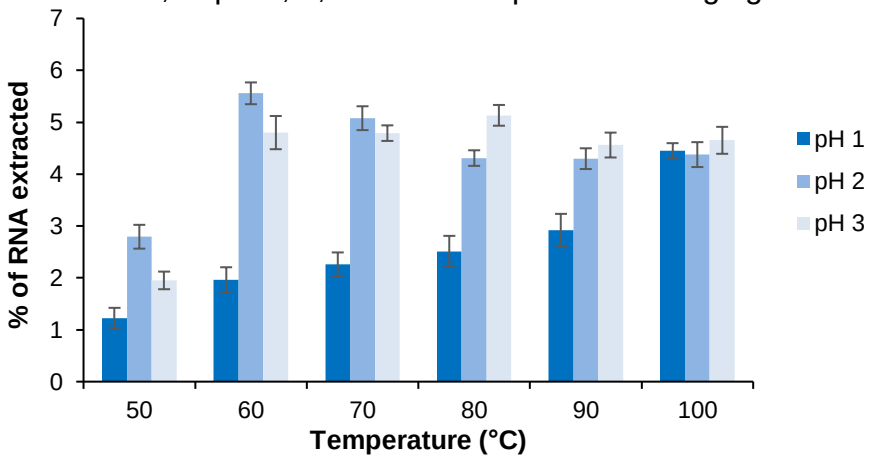
Figure 7 - Kinetics of RNA releasing profile into the extracellular medium during autolysis at 60 °C, pH 6.0, cell concentration of 7.5% and without addition of salt.



4.3 ACID HYDROLYSIS STUDY FOR RNA EXTRACTION

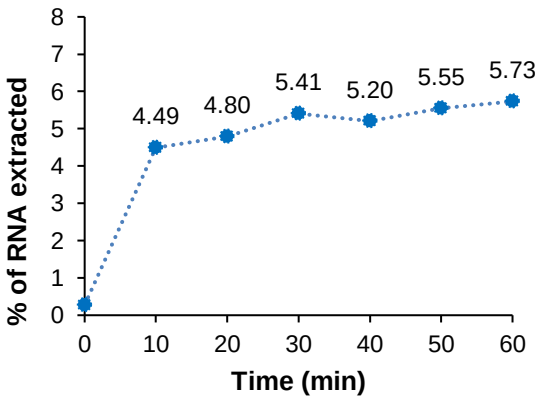
In addition to the autolysis, acid hydrolysis process was studied as an alternative to obtain RNA from spent brewer's yeast. Figure 8 presents the results of the acid hydrolysis study under different pH and temperature conditions.

Figure 8 - Study of the acid hydrolysis in different pH and temperature conditions related to % RNA extracted. The acid hydrolysis was carried out for 60 minutes at 10% (w/v) cell concentration, at pH 1, 2, 3 and at temperatures ranging from 50 to 100 °C.



From the results presented in Figure 8 it is possible to verify that acid hydrolysis using sulfuric acid at 60 °C and pH 2, presented the best yield for RNA extraction. It was further observed that at 100 °C, acid hydrolysis showed similar yields at the three pHs studied. In addition to the evaluation of pH and temperature during acid hydrolysis, a kinetic study was performed to verify the behavior of RNA extraction over time (Figure 9).

Figure 9 - Kinetics of RNA extracted to the extracellular medium during acid hydrolysis at 60 °C, pH 2 and 10% cell concentration.



The behavior was similar to that of autolysis, and it is possible to observe that the % of RNA extracted stabilizes around 60 minutes of reaction, but with a lower yield when compared to autolysis. The low yield of RNA extraction using the acid hydrolysis can be explained as a consequence of the low pH value. According to Sherbet, Lakshmi and Cajone (1983), RNA has isoelectric point value of 5.2 and its solubility is affected by pH. At pH below 2.5 the RNA precipitates as can be observed in Figure 10 (this test was performed only to observe this behavior using standard RNA solution 1% and adding sulfuric acid until pH 2). The precipitation compromises de RNA passage through the membrane and cell wall and makes the extraction have lower yields.

Figure 10 - Standard RNA solution 1% precipitated at pH 2 with sulfuric acid.

4.4 COMPARISON BETWEEN AUTOLYSIS AND ACID HYDROLYSIS REGARDING RNA EXTRACTION

After the study and definition of the best operating conditions of autolysis and acid hydrolysis, an optimized test was performed in order to compare the efficiency of each technique (Table 5).

Table 5 - Protein and RNA extraction yields under different treatments.

Treatment	RNA % average (standard deviation)	Protein % average (standard deviation)
Acid hydrolysis pH 1	1.54 (0.23)	15.22 (1.19)
Acid hydrolysis pH 2	6.26 (0.19)	**22.09 (1.11)**
Acid hydrolysis pH 3	5.29 (0.11)	20.39 (1.17)
Autolysis	**7.76 (0.24)**	12.86 (1.21)

In relation to RNA extraction, the process of autolysis was more effective than the other studies. However, in relation to protein extraction the acid hydrolysis at pH 2 presented performance 71% higher than the autolysis. According to Reed and Nagodawithana (1991), acid hydrolysis is considered the most efficient technique for the solubilization of yeasts, however, it is also considered the least practical for the production of yeast extract on a commercial scale due to the use of strong acids. In view of the advantages and simplicity, autolysis can be considered the best method for RNA extraction. The optimal conditions achieved in this study were temperature at 60 °C with 7.5% cell concentration during 60 minutes.

4.5 ULTRAFILTRATION RESULTS

Table 6 presents the results of total RNA and proteins content as well as β-D-fructofuranosidase activity in each fraction obtained after yeast extract ultrafiltration.

Table 6 - Composition of the yeast extract, ultrafiltrate and retentate fractions after the ultrafiltration procedure.

Sample	Total RNA (g)	Total proteins (g)	β-D-fructofuranosidase activity
Yeast extract	0.33	1.11	24.09
Filtrate	0.32	0.42	0.00
Retentate	0.01	0.69	24.00

It was observed a difference in the amount of total proteins present in the retentate and ultrafiltrate. This result indicate that ultrafiltration was efficient in separating molecules and proteins with molecular weight cut off greater than 10 kDa. Among these retained proteins, the β-D-fructofuranosidase activity was observed only in retentate. According to Kulshrestha et al. (2013), *Saccharomyces cerevisiae* yeasts are large β-D-fructofuranosidase producers, which exist in more than one form. The extracellular form has molecular weight cut off of 135 kDa and the intracellular form 270 kDa. Thus, the presence of invertase only in the retentate is in agreement with the expected one. The ultrafiltration procedure was efficient to retain 62.16% of the total proteins in the retentate. Thus, a bioprocess using this technique can be adopted as a pre-stage of RNA purification.

4.6 QUANTIFICATION OF 5'-RIBONUCLEOTIDES

Figure 11 - UV-260 nm chromatogram obtained for each standard 5'-ribonucleotide.

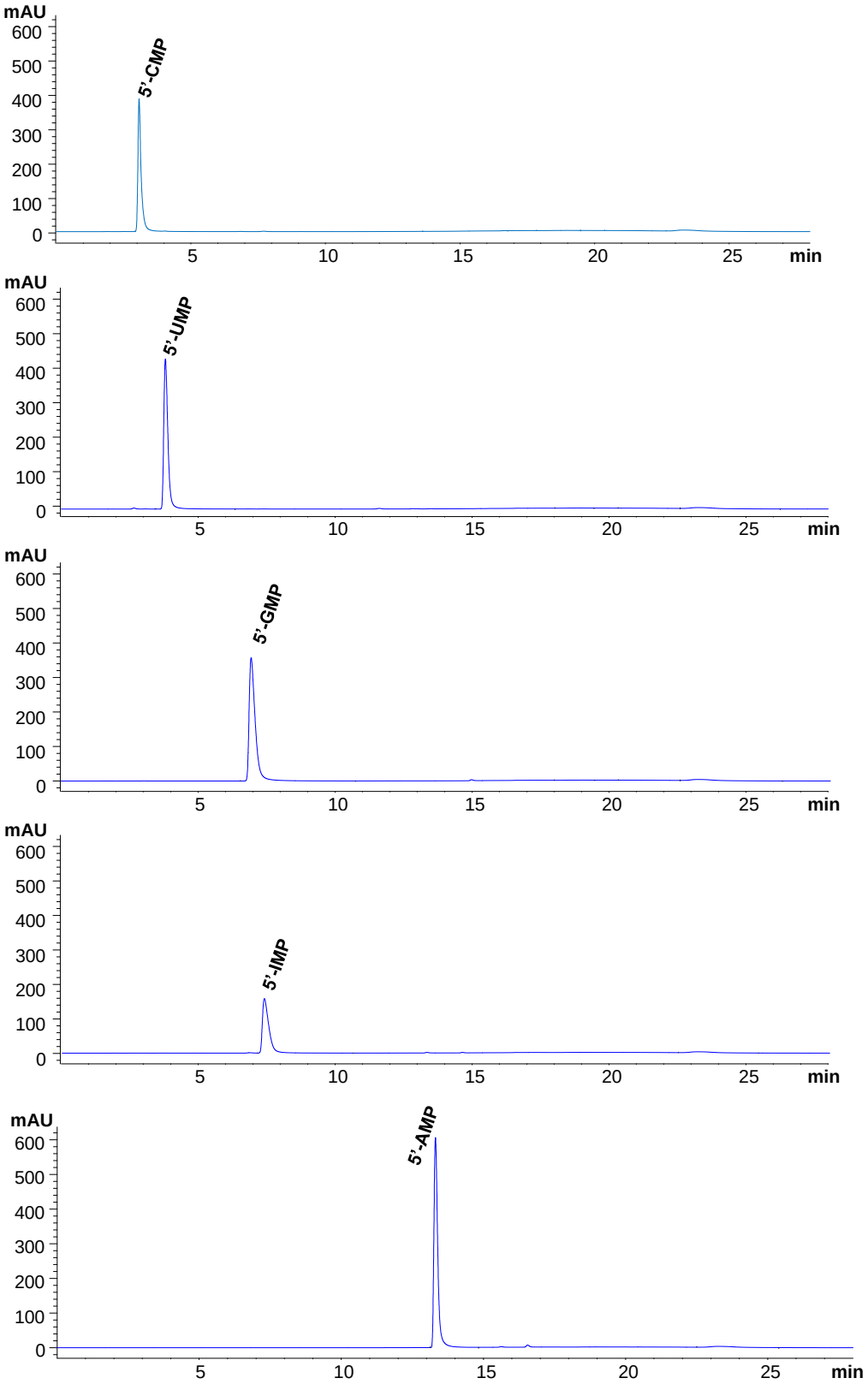
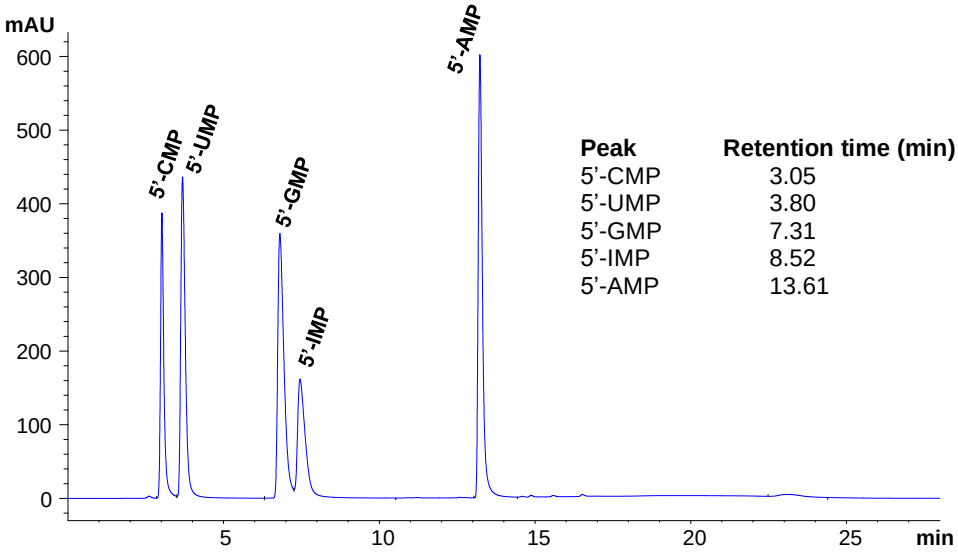


Figure 11 shows the elution sequence of 5'-ribonucleotide standards used to define the retention time of each molecule and to calibrate a standard curve. As noted, 5'-ribonucleotides composed of pyrimidine bases (5'-CMP and 5'-UMP) have a lower retention time than those having in their composition purine nitrogen bases (5'-GMP, 5'-IMP and 5 '-AMP). As the column used is reverse phase (C18) the stationary phase has nonpolar characteristics. Polar substances elute faster than nonpolar substances because nonpolar compounds have higher interactions with the stationary phase (nonpolar). In addition, substances with higher molecular weight and the same polarity elute more slowly than smaller analogues. Figure 12 shows the chromatogram of 5'-ribonucleotide standards together in a mixture as well as each respective retention time.

Figure 12 - UV-260 nm chromatogram and retention time for 5'-ribonucleotides.

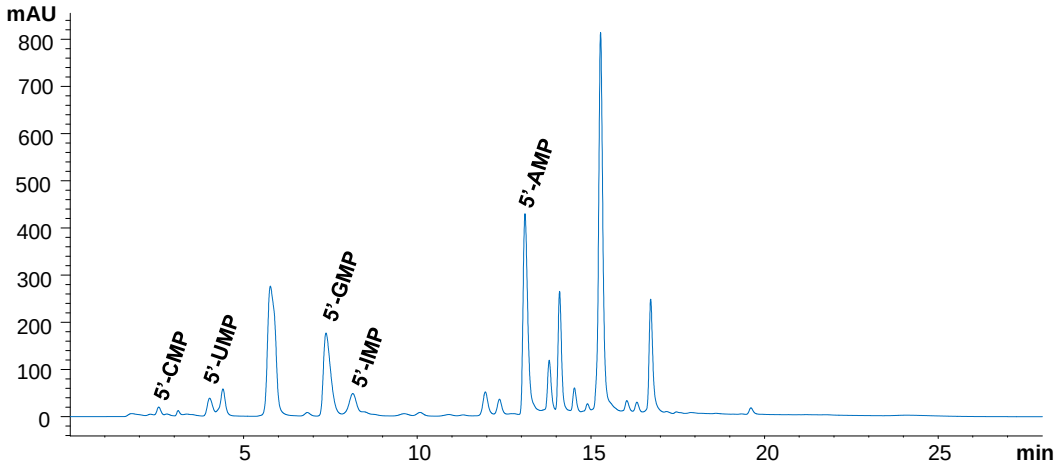


Standard curves were constructed for the quantification of each 5'-ribonucleotide from the areas of their peaks. The equations for each curve are presented in Appendix A.

4.7 5'-RIBONUCLEOTIDES IN THE ULTRAFILTRATE

The amount of 5'-ribonucleotides present in the ultrafiltrate after autolysis was determined by high performance liquid chromatography according to item 3.8.2 and the obtained chromatogram is presented in Figure 13.

Figure 13 - UV-260nm HPLC chromatogram obtained for the ultrafiltrate.



From the chromatogram of Figure 13 and Table 7 it is possible to confirm the presence of the 5'-ribonucleotides screened in the ultrafiltrate. This result confirms the formation of these substances naturally during the process of autolysis as described by Zhao and Fleet (2005).

Table 7 - 5'-ribonucleotides content in the ultrafiltrate due to spontaneous formation during autolysis

Ribonucleotide	**Mass (mg)**	**% ***
5'-CMP	0.77	0.01
5'-UMP	2.01	0.04
5'-GMP	**25.28**	0.55
5'-IMP	1.32	0.02
5'-AMP	**24.85**	0.54
Total	54.23	1.16

* % in relation to the dry mass of yeast used in the autolysis.

Higher formation of 5'-GMP and 5'-AMP during autolysis at 60 °C was observed in this work, which is similar to the findings of Zhao and Fleet (2005), that also observed a higher formation of 5'-AMP during autolysis. The production of these two compounds is welcome, since 5'-GMP is a potent flavor enhancer and 5'-AMP is a precursor of 5'-IMP, another potent flavor enhancer. The enzyme 5′-adenylate deaminase can be used for easy conversion of 5'-AMP to 5'-IMP. Yamaguchi (1967) studied the synergistic taste effect of flavor enhancing and found that activity of monosodium glutamate and 5'-IMP mixtures is more than 100 times greater than that isolated ones. The results achieved here are very promising and opens up perspectives and strategies that can be used by the food industry. An approach for the separation and

purification of 5’-ribonucleotides was evaluated using ion exchange chromatography as the next step downstream process.

4.8 5'-RIBONUCLEOTIDES PURIFICATION BY ION EXCHANGE CHROMATOGRAPHY

Figure 14 shows the ion exchange chromatogram obtained in an attempt to purify the 5'-ribonucleotides. The samples were collected every 10 mL in test tubes by the equipment fractionator and then subjected to characterization and quantification of 5'-ribonucleotide content by HPLC (Figure 15).

Figure 14. Chromatogram obtained using AKTA Start system with emphasis on fractions A and B, which contain 5'-ribonucleotides.

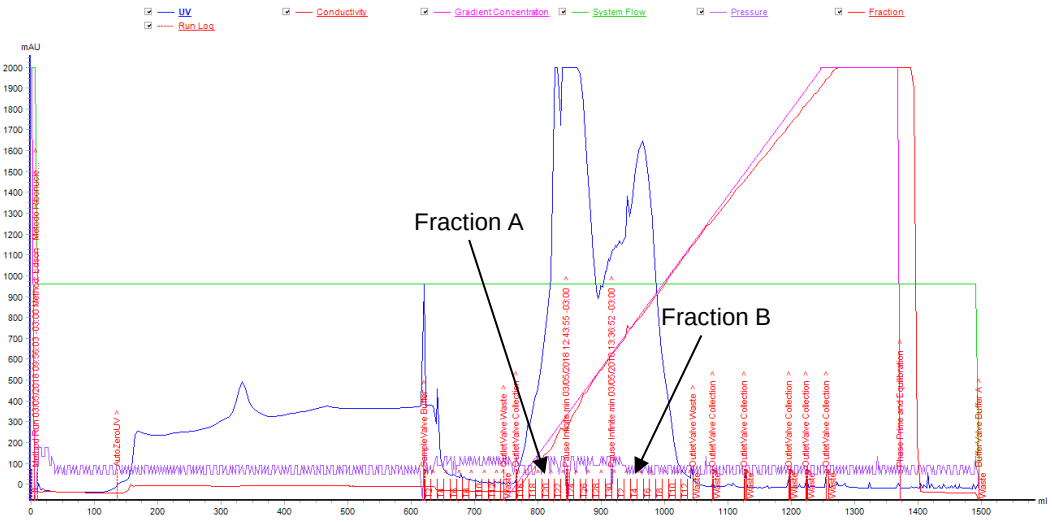
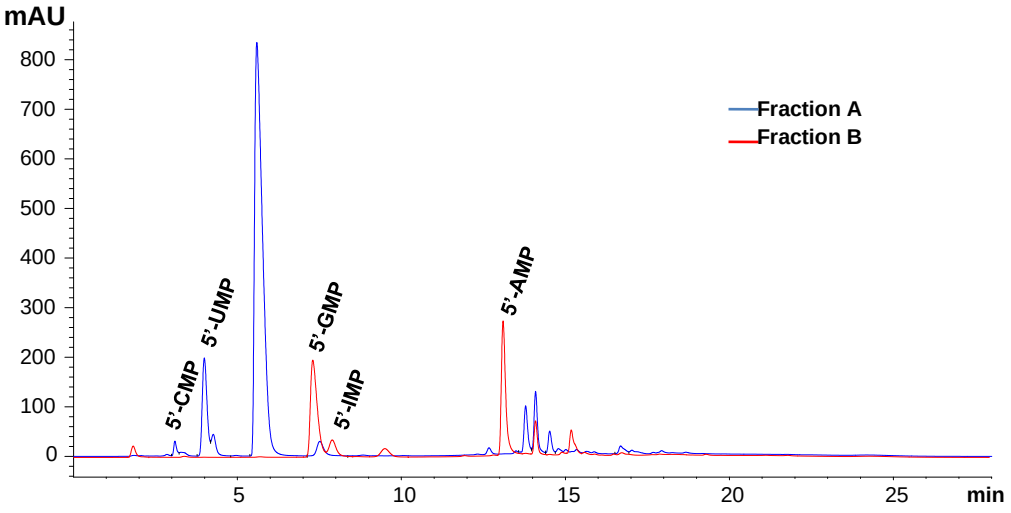


Figure 15. Overlay of UV-260nm chromatogram of fraction A (blue) and B (red) obtained by HPLC.



The presence of 5'-ribonucleotides was observed in 2 distinct fractions, A and B. With this methodology, it was possible to partially isolate 5'-ribonucleotides, having 5'-CMP and 5'-UMP in fraction A and 5'-GMP, 5'-IMP and 5'-AMP in fraction B (Figure 15). The results of the ion exchange chromatographic separations are partial and it is intended for future works to continue the studies of isolation and purification of 5'-ribonucleotides as well as the study of the enzymatic hydrolysis of RNA using 5'-phosphodiesterase in order to increase yield of 5'-ribonucleotides.

5 CONCLUSION

From the tests performed in this work it was possible to observe the use of residual brewer's yeast as a good alternative to obtain RNA. The autolysis showed higher yields for obtaining RNA when compared to acid hydrolysis, generating good results quickly and with few costs inherent to the process. The possibility of obtaining flavor enhancers and β-D-fructofuranosidase with the strategy here achieved increases the potential for application of spent yeast as well as adds value to this residue. Further studies on 5'-ribonucleotide production using 5'-phosphodiesterase and subsequent next steps of purification are suggested as future perspectives and challenges that still need to be explored.

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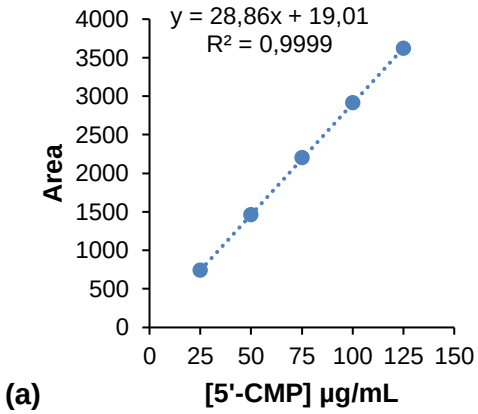
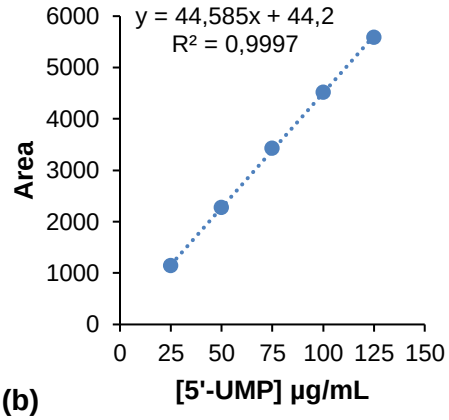
APPENDIX A

Standard curves for 5'-CMP (a), 5'-UMP (b), 5'-GMP (c), 5'-IMP (d) and 5'-AMP (e) constructed for HPLC quantification of 5'-ribonucleotides. R^2 coefficient showed a value close to 1 in all the regressions, indicating that 99% of the peak area values can be explained by the variation of the concentration of each 5'-ribonucleotide.



(a)



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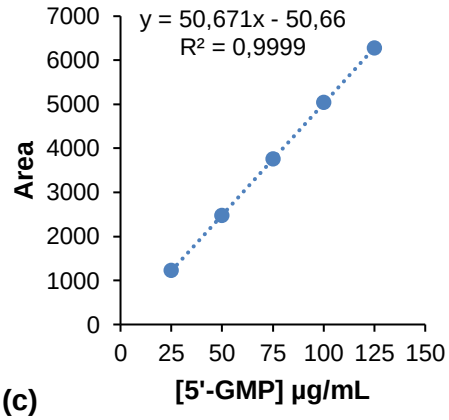
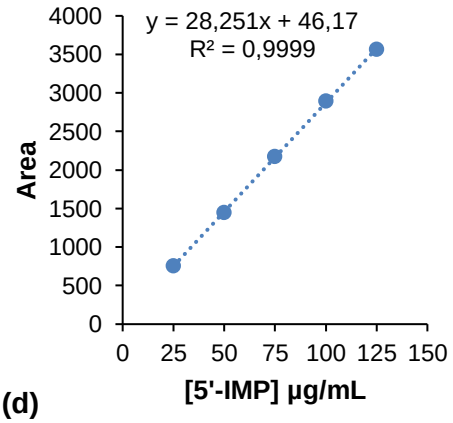
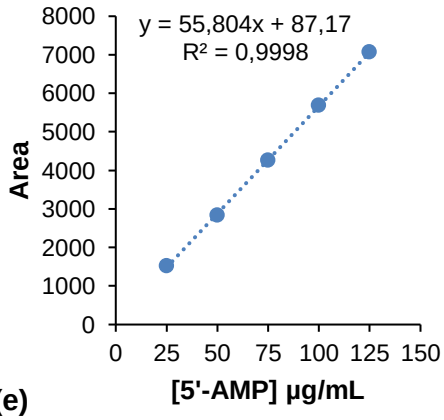


(c)



(d)



(e)

CHAPTER 3 SPENT BREWER'S YEAST AUTOLYSIS BY FASTER AND SAFER PROCESS FOR YEAST EXTRACT PRODUCTION

ABSTRACT

The ever-growing demand for nutritional food has enhanced the production of yeast extracts and autolysates across the globe. The main challenges faced by the yeast extract companies are regarding to autolysis parameters optimization for increase profits and reduce costs. Considering these challenges, the present work comes up with an attempt to accelerate the autolysis with focus on the high yield of yeast extract production. The proposed strategy is based on decreasing the pH of the yeast suspension at the beginning of autolysis through an acid shock to activate the cell's autolytic system under stressful conditions of temperature and pH. The influence of cell concentration, temperature, time and acid shock at the beginning of the autolysis on yeast extract yields were studied. The best yields of proteins (84% and 41.20%) and total solids (47% and 58.48%) were observed for autolysis treated with acid shock and gradual increase of temperature. The present study reached satisfactory and positive results while the relationship between acid and conventional autolysis, taking into account only as the parameter the efficiency, being the acid shock more productive. In addition, it was able to demonstrate how the alteration of parameters such as time and temperature affects the production of the autolysate and can generate a significant improvement in efficiency by controlling the temperature for the action of the enzyme in its optimum temperature range.

Keywords: Autolysis. Acid shock. Yeast extract. Parameters optimization.

1 INTRODUCTION

1.1 YEAST EXTRACT MARKET

The ever-growing demand for nutritional food in the developing and developed countries has enhanced the production of yeast extracts and autolysates across the globe. As principal ingredients, they contain proteins, amino acids, nucleotides, minerals, vitamins and are used in diverse sectors of food and pharmaceutical industry (KELLERSHOHN and RUSSELL, 2015). Another boosting factor for yeast extracts and autolysates is the increasing demand of flavor enhancers like monosodium glutamate (MSG). Studies have highlighted that yeast extract is an adequate replacement of MSG, which contributes to rising even more its market (JO and LEE, 2008).

Basically, the global yeast market is segmented on the basis of type (yeast extract, autolysate yeast, yeast *β*-glucan, and others yeast derivatives) and application (feed, food, others). Both products, autolysate yeast and yeast extract are produced by self-digestion of yeast constituents by endo enzymes (ŠUKLJE et al., 2016). However, two major aspects differ yeast extract from autolysate yeast: autolysate yeast contains cell wall whereas yeast extract does not and second, as compared to yeast extract, autolysis stage of the production process is shorter in autolysate yeast (POZO-BAYÓN et al., 2009). Lead by its strong application scope, yeast extract is used in several food and beverage segments, sweet or savory (STAM; HOOGLAND; LAANE, 1998). Snacks and prepared meals are the main food items produced using yeast extracts (Table 1).

Table 1 - Main food products launched using yeast extracts.

Product	**Example**	**Yeast extract destination**
Snacks	Chips	28%
Prepared meals	Bolognese spaghetti, pumpkin risotto	23%
Sauces and seasonings	Soy sauce, stock cube, ketchup, BBQ sauce	16%
Fish based prepared meals	Salmon lasagnas	12%
Soups	Instant or liquid soup	10%
Others	Side dishes (noodles), bakery (crackers), spreads (hummus, organic pâté), dairy products (processed cheese), sweet products (granola cereals), drinks (soda)	11%

Source BIOSPRINGER (2018).

Nowadays, with an increasing demand for ready-to-eat food items global food industry manufacturers are committed to reducing the sodium content of their products (DESMOND, 2006). Yeast extract stands out in this scenario because it provides an excellent solution that is different from the MSG and 5'-IMP + 5'-GMP, in low-salt foods. It is gives good flavor and also enhances taste. Adding a certain amount of yeast extract in food and flavoring with a salt content of less than 4%, could significantly improve and enhance good flavor and taste, which MSG and 5'-IMP + 5'-GMP fail to do (JO and LEE, 2008). So, the usage of yeast extract can reduce the consumers' salt intake.

Currently, yeast extracts manufacturers are concentrated in China, USA, Europe and Japan. Some of the key players of global yeast extracts and autolysates market are Chr. Hansen A/S, Synergy (High Wycombe) Ltd., Alltech Inc., Leiber Gmbh, Angel Yeast Co. Ltd., Royal Dsm N.V., Associated British Foods Plc, Oriental Yeast Co. Ltd., Archer Daniels Midland Company, Lesaffre Group, and Lallemand Inc. In addition, the market is characterized by accreditation of product, capacity expansion, capital expansion, and substantial investment decisions to improve market share of manufacturers (BIOSPRINGER, 2018).

Finally, the main challenges faced by the yeast extract production sector are regarding autolysis parameters optimization for increase profits and reduce costs. In this way, the study and understanding of the physical and chemical parameters involved during autolysis are important because the effects and interactions between them and additives may reflect positively or negatively on the expected results, as well as the chemical composition of the final product. The following section presents the main mechanisms and parameters that influence autolysis.

1.2 PROCESS PARAMETERS AFFECTING YEAST EXTRACT PRODUCTION

The autolysis of yeasts can be understood as an endogenous and irreversible lytic event, which occurs in cells caused by the action of intracellular enzymes, proteases and carbohydrases (PODPORA et al., 2015). The cell will activate this mechanism when it encounters a physiological stress. The phenomenon is characterized by a loss of permeability of the cytoplasmic membrane, altered porosity of the cell wall, decreased respiratory enzyme activity, gradual hydrolysis of the

cytoplasmic material by endogenous enzymes and subsequent release of the degraded products (nutrients) in the extracellular environment (ALEXANDRE, 2011).

Small variations in physical, chemical, and biological factors like an increase in temperature, osmotic pressure, pH, or cell starvation can significantly influence the results of autolysis (REED and NAGODAWITHANA, 1991). Yeast autolysate, for example, can be obtained with different characteristics depending on the incubation temperature.

Autolysis performed at 30 °C for 48 hours allows the obtainment of an autolysate with a lower content of nitrogen and nucleotides. Feuillat and Charpentier (1982) demonstrated that, in a pH 5 buffer solution, the release of nitrogen from yeast cells is faster in the higher temperature ranges (44 °C to 55 °C). On the other hand, it was also observed that after heating at 55 °C for a period of 4 hours, the concentration of amino acids in the cell suspension did not increase. However, at 36 °C, autolysis extends for 48 hours or longer and the nitrogen concentrations obtained may be as high as those obtained at 44 °C. This type of kinetics suggests that a passive release of nitrogenous compounds from the cells to the medium occurs first, followed by an active (enzymatic) release, which is rapidly inhibited at high temperatures (FEUILLAT and CHARPENTIER, 1982).

In addition to temperature, the autolytic process can be accelerated by the use of auxiliary products such as plasmolising agents (sodium chloride, ammonia, organic solvents, proteolytic enzymes and acids) (MALEKKHAHI et al., 2016). The use of ammonia, for example, reduces process time, however, produces a dark extract. Other disadvantages of the use of additives to accelerate autolysis also include alterations in the final composition of the extract, which may present high salt concentration. Toxic compounds to animals and humans also can be formed from the use of hydrochloric acid to optimize the autolysis (OLIVEIRA and OLIVA NETO, 2011).

1.3 RECENT CHALLENGES

Industrial autolysis process for yeast extract production can generally take from 24 hours to a few days at relatively high temperatures (40 to 55°C). However, contamination by microorganisms such as proteolytic and pathogenic bacteria such as *Clostridium sp* may occur in this period (ANDERSSON; RONNER; GRANUM, 1995).

Bacteria of this genus prefer environments richer in proteins, pHs close to neutrality and low oxygen content, conditions found in the environment of yeast autolysis. In addition to cause diseases, these bacteria can also cause a putrid odor and dark color, which decreases the quality of the yeast extract produced (ANDERSSON; RONNER; GRANUM, 1995).

Productivity is an important parameter when considering the production of low value-added compounds where profitability is given by the scale of production. Thus, a time-consuming process becomes more costly, considering energy and labor costs. Considering these challenges, the present work comes up with an attempt to accelerate the autolysis with focus on the high yield of yeast extract production. The proposed strategy is based on decreasing the pH of the yeast suspension at the beginning of autolysis through an acid shock to activate the cell's autolytic system under stressful conditions of temperature and pH.

2 OBJECTIVES

The present work aims to evaluate the influence of cell concentration, temperature, time and acid shock at the beginning of the autolysis on yeast extract yields.

3 MATERIAL AND METHODS

3.1 YEAST BIOMASS

Fresh spent brewer’s yeast cells of *Saccharomyces cerevisiae*, in the form of aqueous suspension were used in this work. The cells were homogenized for 30 minutes in NaOH solution (0.2%) in the ratio of 1:1 (v/v) for biomass debittering and then centrifuged for the removal of residues from the culture medium of the fermentative process. After washing, the cells were subjected to the autolysis studies.

3.2 INFLUENCE OF CELL CONCENTRATION ASSAY

Approximately 10 g of centrifuged spent brewer's yeast, having a moisture content of 75%, were weighed and then added a suitable volume of water so that the autolysis was performed at a ratio of yeast/water (w/v) ranging from 5% to 15%, on yeast dry basis (Table 2). Subsequently, 25 µL of H_2SO_4 98% was added and homogenized. It was observed previously that the addition of 10 µL sulfuric acid per gram of dry yeast is sufficient to reduce the pH of the cell suspension to near pH 2.2. After homogenization, the cell suspension was subjected to autolysis for 60 minutes at temperature of 60 °C. A duplicate of the autolysis under the same time and temperature conditions, however without addition of the acid, was performed in order to evaluate the influence of the acid treatment on the autolysis yield.

After the end of autolysis, the samples were centrifuged and the supernatant was evaluated for the total protein content extracted using the classical Kjeldahl method, according to the methodology described by AOAC (2000), and total soluble solids by evaporation of the yeast extract at 105 °C to constant weight according to Baird, Eaton and Rice (2017).

Table 2 - Parameter conditions for autolysis cell concentration influence assay.

Cell concentration (%)*[1]	**Yeast (g) *[2]**	**Water (mL)**	**Control H_2SO_4 98% (µL)**	**Acid treatment H_2SO_4 98% (µL)**
5	10	42.5	0	25
7.5	10	25.8	0	25
10	10	17.5	0	25
12.5	10	12.5	0	25
15	10	9.1	0	25

*[1] Yeast/water ratio (w/v), on yeast dry basis.
*[2] Wet yeast mass after centrifugation, presenting moisture content of approximately 75%.

3.3 INFLUENCE OF ACID SHOCK AND TEMPERATURE ASSAY

Fresh cells of *Saccharomyces cerevisiae* obtained from the brewing industry were subjected to the same cleaning and debittering process described in Section 3.1. Approximately 10 g of wet yeast (75% moisture content) were weighed and then 25.8ml of distilled water was added (autolysis with yeast/water ratio of 7.5%, w/v on yeast dry basis). After homogenization, suitable volumes of H_2SO_4 98% were added so that autolysis was performed in a concentration ranging from 0 to 100 (µL H_2SO_4/g yeast, on dry basis) and at temperatures of 50, 60, 70, 80, 90 and 100 °C (Table 3).

Table 3 - Parameter conditions for acid shock and temperature influence assay.

H_2SO_4 (%)*[1]	Yeast (g)*[2]	Water (mL)	H_2SO_4 98% (µL)	Temperature (°C)
0	10	25.8	0	50, 60, 70, 80, 90 and 100
1	10	25.8	25	50, 60, 70, 80, 90 and 100
2	10	25.8	50	50, 60, 70, 80, 90 and 100
4	10	25.8	100	50, 60, 70, 80, 90 and 100
10	10	25.8	250	50, 60, 70, 80, 90 and 100

*[1] H_2SO_4/yeast ratio (v/w), on yeast dry basis.
*[2] Wet yeast mass after centrifugation, presenting moisture content of approximately 75%.

After 60 minutes of autolysis, the samples were centrifuged and the supernatant were also evaluated for protein and total soluble solids content as described in Section 3.2.

3.4 INFLUENCE OF ACID SHOCK, TEMPERATURE AND TIME ASSAY

Approximately 50 g of wet yeast (75% moisture content) were weighed and then added 129 mL of distilled water (autolysis with yeast/water ratio of 7.5%, w/v on yeast dry basis) and 125 µL H_2SO_4 98%, 1% (v/w) in relation to the yeast mass. Control groups without the acid addition were also prepared. After homogenization, the suspensions were subjected to the different autolysis treatments according to Table 5 for 120 hours. Throughout the autolysis, 10 mL of the samples were collected at time intervals of 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120h. The samples were centrifuged and the supernatant was evaluated for protein and total soluble solids content as described in section 3.2.

Table 4 - Parameter conditions for acid shock, temperature and time influence assay.

Treatment	Yeast (g)*	Water (mL)	H_2SO_4 98% (µL)
45 °C for 120 h	50	129	0
45 °C for 120 h	50	129	125
60 °C for 120 h	50	129	0
60 °C for 120 h	50	129	125
45°C (0-2h), 50°C (2-4h), 55°C (4-8h), 60°C (8-120h)	50	129	125
60°C (0-2h), 55°C (2-4h), 50°C (4-8h), 65°C (8-120h)	50	129	125

* Wet yeast mass after centrifugation, presenting moisture content of approximately 75%.

4 RESULTS AND DISCUSSION

4.1 INFLUENCE OF CELL CONCENTRATION ON YEAST EXTRACT AND PROTEIN YIELDS

The results of cell concentration influence on yeast extract yields are presented in Table 5.

Table 5 - Total soluble solids and proteins content in yeast extract after 60 min at 60 °C.

Cell content (%)*[1]	Control Autolysis			Autolysis with acid treatment*[2]		
	Total soluble solids (g)	Proteins (g)	Protein content in the yeast extract (%)	Total soluble solids (g)	Proteins (g)	Protein content in the yeast extract (%)
5.0	0.5092	0.3209	63.01	0.6966	0.4813	69.09
7.5	0.5587	0.3473	62.16	**0.7698**	**0.5379**	**69.87**
10.0	0.5543	0.3346	60.36	0.6906	0.4706	68.15
12.5	0.5437	0.3228	59.38	0.6244	0.4283	68.60
15.0	0.4280	0.2508	58.60	0.5362	0.3451	64.36

*[1] Yeast/water ratio (w/v), on yeast dry basis.
*[2] 1% H_2SO_4/yeast ratio (v/w), on yeast dry basis.

The addition of acid at the beginning of the autolysis resulted in an increase of total soluble solids and proteins extracted. The best result was achieved with acid treatment and 7.5% yeast/water ratio (w/v), on yeast dry basis. Under these conditions, yeast extract presented 69.87% of proteins, which represents an increase of 12.4% in relation to the control group. On the other hand, Figures 1 and 2 present the percentage of total soluble solids and proteins extracted in relation to the initial dry mass of yeast used in the autolysis.

Figure 1 - Total soluble solids content in yeast extract in relation to the initial yeast dry mass after 60 minutes autolysis at 60 °C. *Yeast/water ratio (w/v), on yeast dry basis.

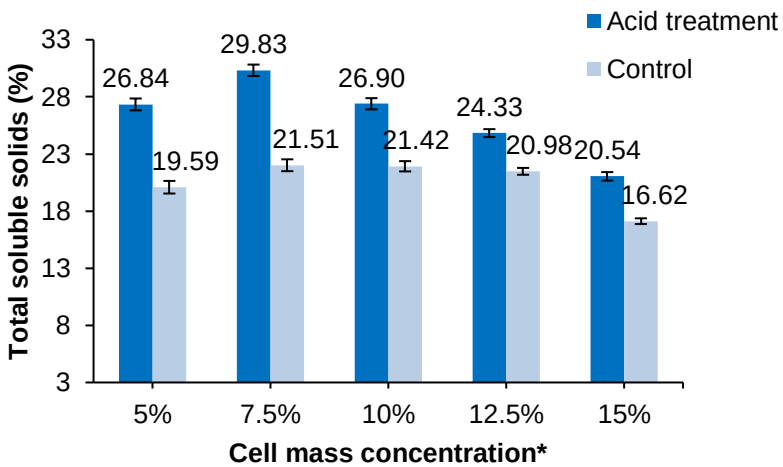
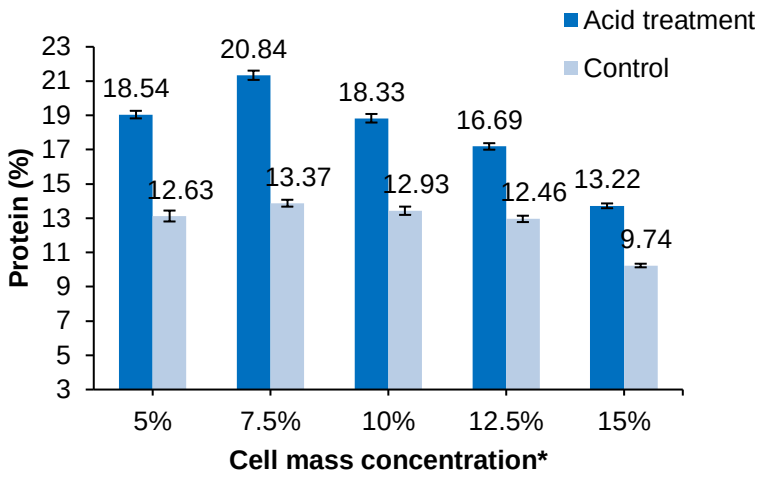


Figure 2 - Proteins content in yeast extract in relation to the initial yeast dry mass after 60 minutes autolysis at 60 °C. *Yeast/water ratio (w/v), on yeast dry basis.



The results presented in this way allow to evaluate the overall efficiency of the self-digestion process. The highest yield of total soluble solids and protein extraction were also observed with 7.5% yeast/water ratio (w/v). Under this condition, the acid treatment showed increases of 38.63% for total soluble solids and 55.83% for protein extraction when compared to the control group.

4.2 INFLUENCE OF ACID SHOCK AND TEMPERATURE ON YEAST EXTRACT AND PROTEIN YIELDS

The results of acid shock and temperature influence on yeast extract yields are presented in Table 6 and 7.

Table 6 - Observed responses for total soluble solids in yeast extract.

Temperature °C	**H_2SO_4 (%)***				
	0%	**1%**	**2%**	**4%**	**10%**
50	11.63	25.09	18.37	14.07	11.98
60	24.47	**30.43**	21.57	16.75	15.40
70	24.79	22.00	19.88	27.05	14.29
80	22.91	21.84	17.16	15.82	20.27
90	23.89	21.10	10.08	18.36	24.42
100	23.51	22.87	20.55	22.10	27.47

* H_2SO_4/yeast ratio (v/w), on yeast dry basis.

Table 7 - Observed responses for protein content in yeast extract.

Temperature °C	H_2SO_4 (%)*				
	0%	**1%**	**2%**	**4%**	**10%**
50	7.21	17.30	13.14	10.35	10.03
60	14.78	**20.05**	15.47	12.70	13.38
70	15.19	15.01	10.81	12.25	11.72
80	14.55	14.35	11.45	12.11	17.28
90	14.88	12.99	11.32	12.29	20.67
100	14.16	13.31	12.39	14.65	22.56

* H_2SO_4/yeast ratio (v/w), on yeast dry basis.

The best result for soluble solids and protein extraction were achieved with 1% acid treatment at 60°C. Under this condition, the acid treatment showed increases of 24.37% for total soluble solids and 35.65% for protein extraction when compared to the control group.

4.3 INFLUENCE OF TIME AND TEMPERATURE

Figures 3 and 4 show the values of proteins and soluble solids released in the treatments performed under different conditions according to Table 4.

Figure 3 - Percentage of proteins extracted in relation to the yeast dry mass during autolysis under different conditions. In control experiments there was no addition of acid. 1% sulfuric acid (v/w) was added in relation to the dry mass of yeast in acid shock treatments.

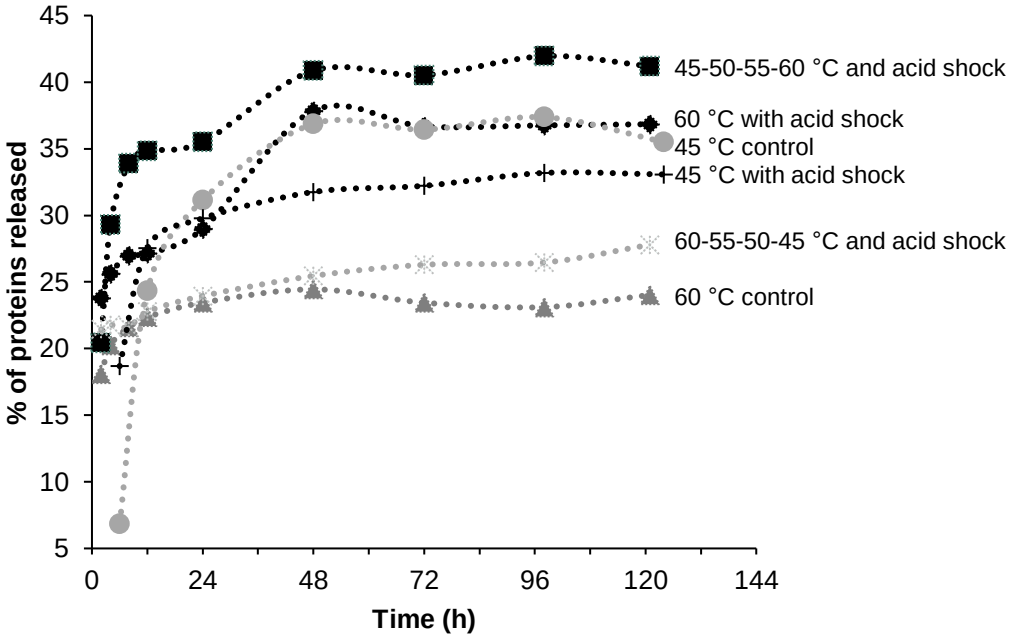
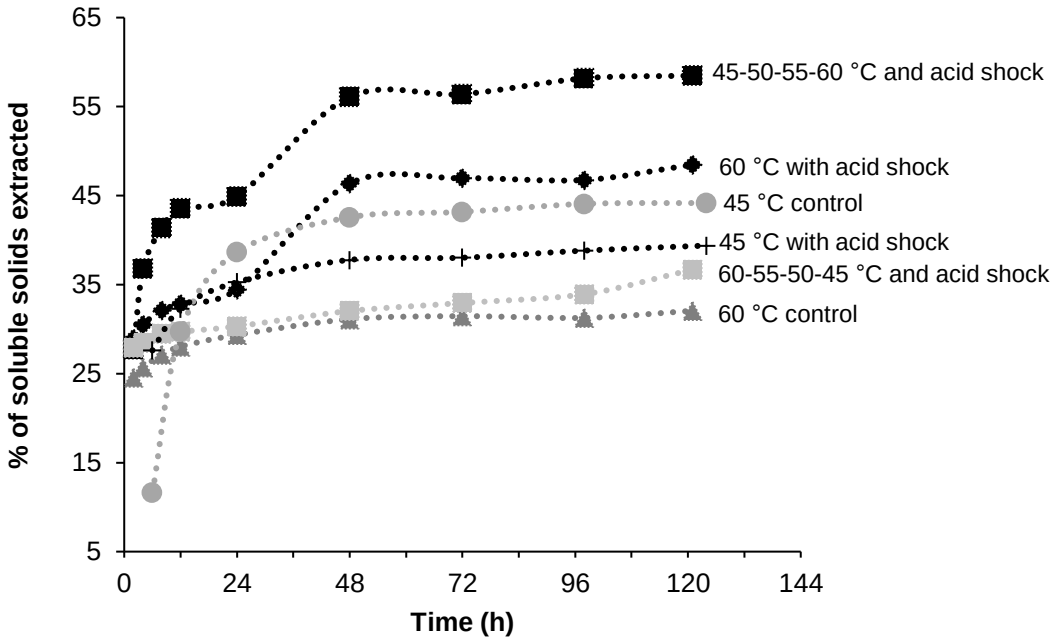


Figure 4 - Percentage of soluble solids extracted in relation to the yeast dry mass during autolysis under different conditions. In control experiments there was no addition of acid. 1% sulfuric acid (v/w) was added in relation to the dry mass of yeast in acid shock treatments.



It is observed that at the temperature of 45ºC the control obtained better results than in autolysis with acid shock. However, the experiments performed at the temperature of 60 °C showed to be much higher in relation to the control at the same temperature in relation to the extraction of proteins and soluble solids. The best yields of proteins and total solids were observed for autolysis treated with acid shock and gradual increase of temperature.

At low pH the thermotolerance mechanism of *S. cerevisiae* is inhibited and in addition, it still affects the activity of enzymes responsible for autolysis, which reach the ideal pH for the reaction. This explains how pH associated with extreme temperature are factors that accelerate yeast autolysis. The results obtained here are according to Oliveira and Goméz (2005) and Oliveira and Oliva Neto (2011) regarding pH, which obtained better results at a pH in the range of 3.8 to 5, varying a little in the temperature only, since they obtained better results in temperature of 48 to 55 ºC.

The biochemical mechanism that leads to the rupture of the yeast cell wall is related to the synergistic action of endogenous enzymes (glucanases, mannases and chitinases) of the cell itself (CHARPENTIER et al., 1986). The effect of temperature on yeast cells is quite complex and affects the synthesis and activity of these enzymes,

as well as cell control, transfer of anabolic information from genes to ribosomes, absorption of ions and molecules, and membrane integrity (REED and NAGODAWITHANA, 1991). Yeast autolysis is characterized by the degradation of cellular proteins, nucleic acids, lipids and polysaccharides, leading to cellular inviability and solubilization of cellular biomass. This process causes cell death, but should not inactivate the responsible enzymes such as proteases, lipases and nucleases considered necessary for the autolytic process (HERNAWAN and FLEET, 1995). Four of the important proteolytic enzymes that play a role during lysis are: protease A, protease B, carboxypeptidase Y and carboxypeptidase S (REED and NAGODAWITHANA, 1991).

Among the enzymes that participate in this process in yeast, protease A has a crucial function, being responsible for 80% of the nitrogen released during autolysis under optimal conditions (ALEXANDRE, 2011; CHARPENTIER et al., 1986). According to data from Maddox and Hough (1970), this protein has optimum pH between 2-6 and optimal temperature of 35-40 ºC. Protease B has an optimum pH of 6-10 and optimum temperature of 45-55 °C, carboxypeptidase Y has its best conditions at pH 4-7 and temperature of 45-55 °C, whereas carboxypeptidase S the pH is 7 and the temperature of 60 °C. These data justify the increase of the level of extracted proteins and soluble solids with the gradual increase of the temperatures in the obtained results. However, when dealing with pH only some proteases had their optimal conditions reached, such as proteinase A and carboxypeptidase Y.

The experiment with the gradual decrease of temperature presented results close to those found for the control autolysis at 60 °C. The results obtained from acid autolysis at 60 °C and gradual temperature increase showed the highest percentages of proteins (36, 84% and 41.20%) and solids (48, 47% and 58.48%) extracted at the end of the experiment (121 hours).

These results demonstrate some disparities in relation to the percentage of proteins found by Tanguler and Erten (2008). When evaluating temperatures separately, after 8 hours of incubation, they obtained a percentage of approximately 40% of proteins at 45 °C and 30% at 60 °C, whereas in the present study the percentage was obtained below 30% at 45 °C and greater than 30% at 60 °C in autolysis with acid shock treatment.

Likewise, significant differences were observed when compared to studies of autolysis in brewer's yeast performed by Bayarjargal et al. (2011). At temperatures of

45 and 50 °C, the percentage of solids extracted was less than 15% after 30 hours of incubation, whereas in the present study, acid autolysis at 45 °C already showed higher values in the first 2 hours.

The influence of temperature on the extraction of proteins and solids with significant increase in the first 24 hours and subsequent stability in the following hours, observed in this study for the temperature of 45 °C, was observed by Tanguler and Erten (2008) and Liu et al. (2008) when evaluating yeast autolysis and by Bayarjargal et al. (2011) and Chae, Joo and In (2001) when evaluating the enzymatic hydrolysis of the same microorganisms at the same temperature. However, even with the same pattern for other temperatures in these studies, in acidic autolysis with a gradual increase in temperature and 60 °C it is possible to observe another significant increase in the extraction of solids and proteins between 24 and 48 hours until the process in fact stabilizes, with little variation, until the end of the incubation period.

The autolysate may have a variable composition and, therefore, these differences may not only be related to the process of autolysis itself, but especially to the species of yeast to be autolysed. Podpora et al. (2015) attributes the difference to the fact that brewer's yeast is obtained from a suspension of the brewer's must, rich in protein, peptides and amino acids from barley malt (which may be variable) and from other additives present in the the beverage production process.

Another determinant factor may be related to the conditions of autolysis, since some yeast proteases have optimal temperature and pH to perform their proteolytic functions (REED and NAGODAWITHANA, 1991). Thus, the more positive results found with acid autolysis and gradual increase in temperature demonstrate that there may be a relationship with the optimal ranges of these parameters for the action of each of the enzymes in the process of autolysis.

5 CONCLUSION

The present study reached satisfactory and positive results while the relationship between acid shock treatment and conventional autolysis, taking into account only as the parameter the efficiency, being the acid more productive. In addition, it was able to demonstrate how the alteration of parameters such as time and temperature affects the production of the autolysate and can generate a significant

improvement in efficiency by controlling the temperature for the action of the enzyme in its optimum temperature range.

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CHAPTER 4 5'-RIBONUCLEOTIDES PRODUCTION USING 5'-PHOSPHODIESTERASE FROM SPENT MALT ROOTS

ABSTRACT

5'-ribonucleotides are high value-added molecules and are widely used in the food and pharmaceutical industries because of their bioactive properties. However, obtaining 5'-ribonucleotides it is not so easy because requires the hydrolysis of the crude RNA by means of a very specific enzyme, 5'-phosphodiesterase (5'-PDE). The present work aims to produce a composition of 5'-ribonucleotides using spent brewer's yeast as cheap source of RNA and barley malt rootlets as cheap source of 5'-PDE. This strategy is very promising because both are residues of the brewing process and are closely linked in a cycle that until now is not yet commercially exploited due to lack of studies. The results of this work showed that extraction of 5'-PDE was mainly influenced by the fineness of the rootlets and amount of extraction solvent (water). The optimum reaction temperature and pH for 5'-phosphodiesterase was found to be 65-70 °C and 5 respectively, with yeast RNA as the substrate. The main molecules formed during RNA hydrolysis were 5'-ribonucleotides, which represented 85.86% of the total hydrolyzed molecules. Finally, the extraction of 5'-PDE from malt roots is simple and economical and can be adapted to industrial production of 5'-PDE. Finally, the results of the approach here proposed can generate a new perspective for the brewing industry in relation to the management of its wastes in order to generate from them products of high added value and with a wide range of applications.

Keywords: RNA hydrolysis. 5'-Ribonucleotides. 5'-Phosphodiesterase. Bbarley malt rootlets.

1 INTRODUCTION

5'-ribonucleotides and nucleosides are high value-added molecules and are widely used in the food and pharmaceutical industries because of their bioactive properties (OLMEDO et al., 1994). Nucleosides are composed of a nitrogenous base (purine or pyrimidine) and a pentose (ribose for RNA or 2'-deoxyribose for DNA) (Figure 1). When one or more phosphate groups are present, the compound is known as nucleotide and is the most important class of nucleic acid derivatives (LEHNINGEr et al., 2005). Phosphodiester bonds connect the 5' carbon of one nucleotide to the 3' carbon of another nucleotide forming polymers known as polynucleotides or nucleic acids. The selective, chemical or enzymatic hydrolysis of ribonucleic acids produces a composition of nucleosides and nucleotides (Table 1).

Table 1 - Ribonucleic acid derivatives (MURRAY et al., 2014).

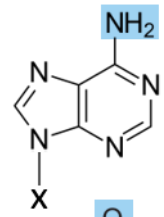
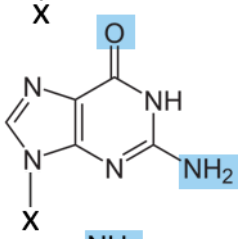
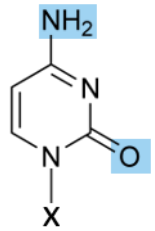
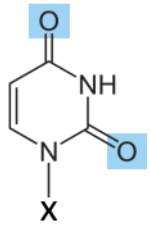
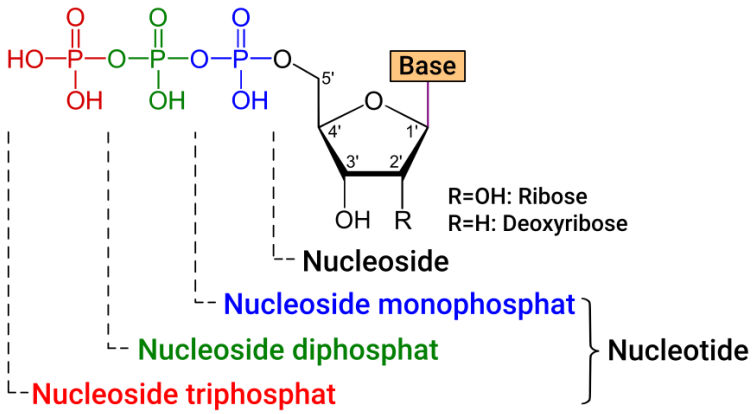
Structure	Base	Nucleosides	Nucleotides
	Adenine (A) (Purine)	Adenosine	Adenosine 5'-monophosphate (5'-AMP)
	Guanine (G) (Purine)	Guanosine	Guanosine 5'-monophosphate (5'-GMP)
	Cytosine (C) (Pyrimidine)	Cytidine	Cytidine 5'-monophosphate (5'-CMP)
	Uridine (U) (Pyrimidine)	Uridine	Uridine 5'-monophosphate (5'-UMP)

Figure 1 - General structure of nucleotides and nucleosides (MURRAY et al., 2014).



The process to be chosen for RNA hydrolysis deserves due attention because it directly affects the characteristics of the molecules produced. Chemical RNA hydrolysis can be carried out under alkaline conditions but is considered limited because RNA is degraded to lower molecular weight compounds without any specificity (BOCK, 1967). In this process, ribonucleotides with 2'- and 3'-phosphate groups are obtained mostly. These molecules show no flavoring characteristics and are of little commercial interest. 5'-ribonucleotides, on the other hand, are involved in several basic biological processes and are considered high value-added molecules. The production of 5'-ribonucleotides requires the hydrolysis of the crude RNA by means of a very specific enzyme, 5'-phosphodiesterase (5'-PDE). This enzyme hydrolyzes the RNA ester bonds at the 3' position, promoting the release of 5'-ribonucleotides in which the phosphate group is attached to the 5' carbon of the pentose molecule (KUNINAKA et al., 1959).

Cohn and Volkin (1953) were the first to demonstrate the presence of 5'-PDE activity in snake venom. Despite its high efficiency, it cannot be used in the agri-food industry for obvious reasons. Other important sources of 5'-PDE include certain fungi such *Penicillium citrinum* (ZHU et al., 1996), certain *Actinomyces*, such as *Streptomyces aureus* (TAKETO and TAKETO, 1974), species of *Halobacterium* (FITT and BADDOO, 1979) and *Aspergillus niger* (FUJIMOTO, et al., 1977). However, this type of enzyme production requires industrial fermentation and quite complex purification steps, which ultimately results in an expensive enzyme. 5'-PDE from *Penicillium citrinum*, for example, is available commercially at Amano Enzyme among others, under the name Nuclease RP-1G (or EC3, 1.30.1). Because of its very high cost, this enzyme is mainly used on an industrial scale in an immobilized form. Its use

in a process for producing yeast extracts enriched in 5'-ribonucleotides induces very large additional enzyme costs. Animal tissues are other sources of 5’-PDE (RAZZELL, 1963), which can also be obtained from germs of some plants, such as rootlets of oats, barley, wheat, corn, rye, millet, sorghum rice and malt rootlets (BOWLES, 1991).

Compared with many other sources, barley malt rootlets are by-products of the brewing industry and therefore cheaper materials for the preparation of 5’-PDE and additionally, there is no doubt about the safety of the enzyme. According to Prentice (1983), 5'-PDE can be obtained by simple decoction of a malt root powder in an aqueous solution to extract the enzyme. Barley rootlets are good source of this enzyme among others including deoxyribonuclease, ribonuclease and adenosine-5’-phosphomono-esterase. As already said, this enzyme can be obtained from a variety of other sources such as plant (DEODA and SINGHAL, 2003; TANAKA et al., 1985), animal (GUO-QING et al., 2006) and microbial (STEENSMA; VAN DIJCK; HEMPENIUS. 2004; TAO, 1993; ZHU et al., 1996). However, considering economic aspects, preparations of 5'-PDE from a cheap source residue such malt roots is very promising. Besides, it could serve as good raw material for large scale production of 5’-PDE.

Based on the explanations above, the present work aims to produce a composition of 5’-ribonucleotides using spent brewer’s yeast as RNA source and barley malt rootlets as source of 5'-PDE. This strategy is very promising because both are residues of the brewing process and are closely linked in a cycle that until now is not yet commercially exploited for lack of studies.

2 OBJECTIVES

The present work aims to study the optimal conditions for the extraction of 5’-PDE from malt rootlets as well as its application for hydrolyzing RNA form spent brewer’s yeast. It was also objective to determine the enzyme characteristics, including optimal pH and temperature, heat stability and Michaelis’ constant (Km).

3 MATERIAL AND METHODS

3.1 EXTRACTION OF 5′-PDE

Malt roots were kindly provided by Agrária (Cooperativa Agrária Agroindustrial, Guarapuava, PR, Brazil). Approximately 1 kg of malt roots were sieved and fractioned according to their granulometry (Tamis mesh 16-100, Bertel, Caieras, Brazil) (Figure 2).

Figure 2 - Sieves used for granulometric fractionation of malt rootlets.

The influence of granulometry and water proportion for the enzyme extraction was evaluated. Malt rootlets (1g) of the different granulometry were soaked with 6 mL distilled water at 20°C for 20 min to extract crude 5'-PDE. For the water-rootlet proportion assay, 1g of malt rootlet were soaked with water in the following proportions (w/w): 4:1, 8:1, 12:1, 16:1 and 20:1. After extraction, the sample were filtrated with gauze and centrifuged for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The supernatant was collected and used for 5'-PDE activity assays.

3.2 5'-PDE ACTIVITY ASSAY

The 5'-PDE activity was determined as described by Fujimoto et al. (1974). It consists of the measure of the absorbance at 260 nm of nucleotides molecules released after RNA hydrolysis by the enzyme. Test tubes (13x100 mm) containing 0.9 ml of RNA solution 1% (w/v) (Sigma-R6625) in acetate buffer solution 0.125 M pH 5.3 and 3 mM Zn^{2+} ion were incubated in a water bath (MA-093, Marconi, Piracicaba,

Brazil) at 69 °C for 10 minutes to equilibrate the temperature. Then, 100 µL of crude enzyme solution was added and the tubes were incubated for 15 minutes at 69 °C with shaking every 5 minutes. After incubation, 2.0 mL of ammonium molybdate solution 0.25% (m/v) and perchloric acid 2.5% (v/v) (kept in a refrigerator) were added to inactivate the reaction and precipitate the unhydrolyzed RNA. The tubes were then incubated in an ice bath for 10 minutes and then centrifuged for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The supernatant was collected and diluted 50-fold with deionized water and the absorbance was measured at 260 nm in a spectrophotometer (UV-M51, Bel Photonics, Piracicaba, Brazil) using quartz cuvettes with 1 cm of optical path. The blank was prepared by substituting 100 µl of enzyme solution with deionized water in the reaction mixture, followed by the same procedures. The activity of the enzyme was calculated according to equations Eq. 1.

$$5'PDE\left(\frac{U}{mL}\right) = \frac{(A_{260nm}Test - A_{260nm}\,Blank)*2*50}{10.6*0.1*15} \qquad (1)$$

Where:
2 = volume (mL) of stopped reaction;
50 = dilution factor;
10.6 = millimolar extinction coefficient ξ (µmol/mL) of hydrolyzed ribonucleic acid at 260 nm;
0.1 = volume (mL) of enzyme used;
15 = time (in minutes) of assay as per the Unit Definition.

One unit of 5'-PDE activity was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute.

3.3 5′-PDE CHARACTERIZATION

In order to determine the optimum reaction temperature, the enzyme activity was performed under the same conditions previously mentioned, changing only the temperature values to 30, 40, 45, 50, 55, 60, 65, 70, 80 and 90°C. To determine the optimal pH conditions of the reaction, the following 0.125 mol/L buffers were used:

glycine-HCl (for pH values in the range of 2.0 to 3), sodium acetate (for pH values in the range of 4.0 to 5.5) and sodium phosphate (for pH values in the range of 6.0 to 8). The determination of the enzyme activity was performed as previously described altering only the buffer solution and the pH during the reaction.

The determination of Michaelis' constant (Km) of the enzyme was performed by varying the initial concentration of RNA between 0.1 and 30 mg/mL during the reaction. The kinetic model proposed by Michaelis-Menten with substrate inhibition (Eq. 2) was adjusted to the experimental results of enzyme activities during the reaction as a function of substrate concentration by means of nonlinear regression using MATLAB software R2017b (Mathworks®), so that the inhibition constant *Ki* was also determined.

$$V = \frac{Vmax * S}{Km + S + \frac{S^2}{Ki}} \qquad (2)$$

The effect of heating on the thermostability of 5'-PDE was investigated at 60, 65 and 70°C for different periods up to 24 hours.

3.4 ENZYMATIC RNA HYDROLYSIS

Enzymatic RNA hydrolysis for 5'-ribonucleotides production was performed by incubating RNA from spent brewer's yeast with the crude enzyme extract from malt rootlet.

Approximately 10 g of centrifuged spent brewer's yeast, having a moisture content of 75%, were weighed and then added water (25.8 mL) so that the autolysis was performed at a ratio of yeast/water (w/v) of 7.5%, on yeast dry basis. After homogenization, the cell suspension was subjected to autolysis for 60 minutes at temperature 60 °C and then centrifugated for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The RNA hydrolysis assay was performed by mixing the supernatant (7.2 mL) with the crude enzyme (0.8 mL) for up to 24 hours. Samples were collected at intervals for 5'-ribonucleotides and ribonucleosides content determination.

3.5 5'-RIBONUCLEOTIDES AND RIBONUCLEOSIDES DETERMINATION

High Performance Liquid Chromatography (HPLC) was used to verify and quantify the 5’-ribonucleotides and ribonucleosides formed during RNA hydrolysis. Standard solutions containing 10µg/mL of 5'-GMP (G8377-5G, Sigma-Aldrich Brazil Ltda), 5'-CMP (C1006-5G, Sigma-Aldrich Brazil Ltda), 5'-UMP (U6375-5G, Sigma-Aldrich Brazil Ltda.), 5'-IMP (I4625-5G, Sigma-Aldrich Brazil Ltda), 5'-AMP (01930-5G, Sigma-Aldrich Brazil Ltda), Guanosine (G6752-25G, Sigma-Aldrich Brazil Ltda), Cytidine (C4654-5G, Sigma-Aldrich Brazil Ltda), Uridine (U3750-1G, Sigma-Aldrich Brazil Ltda), Inosine (I4125-5G, Sigma-Aldrich Brazil Ltda) and Adenosine (A9251-5G, Sigma-Aldrich Brazil Ltda) were prepared and used for standard curve preparation. The separation of molecules was achieved on reverse phase column Acclaim C30 (2.1 x 150 mm, 3µm) at 15 °C using an HPLC system (2475 - Multi λ Fluorecence Detector, Waters, 2012) with UV detector at 260 nm and elution gradient. The mobile phase consisted of 3 components: A) deionized water; B) 100 mM ammonium acetate buffer, pH 5 and C) Methanol. The entire run lasted 28 minutes and the elution gradient events are detailed in Table 2. From this methodology, chromatograms of each compound were obtained to determine the retention time of the molecules.

Table 2. Elution gradient.

Time (min)	% de A	% de B	% de C	Flux (mL/min)
0.0	80	20	0	0.25
3.0	80	20	0	0.40
5.0	70	20	15	0.40
8.5	40	20	40	0.25
15.0	40	20	40	0.25
15.1	80	20	0	0.25
23.0	80	20	0	0.25
23.1	80	20	0	0.40

4 RESULTS AND DISCUSSION

4.1 ENZYME EXTRACTION

Figure 3 shows the visual appearance of the fractions obtained after malt rootlets sieving. The influence of granulometry, water proportion and time for the enzyme extraction are shown in Figures 4 and 5.

Figure 3. Fractions obtained after granulometric separation of malt rootlets: (a) mesh>16, (b) 16<mesh<35, (c) 35<mesh<48, (d) 48<mesh<60, (e) 60<mesh<80 and (f) 80<mesh<100

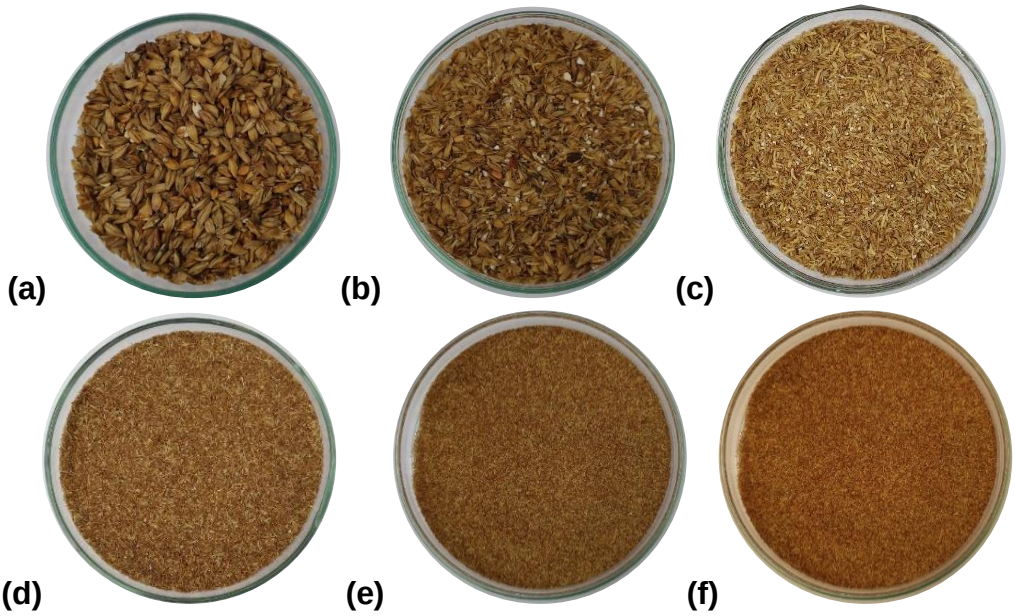


Figure 4 - Influence of malt rootlet granulometry on the 5'-PDE extraction.

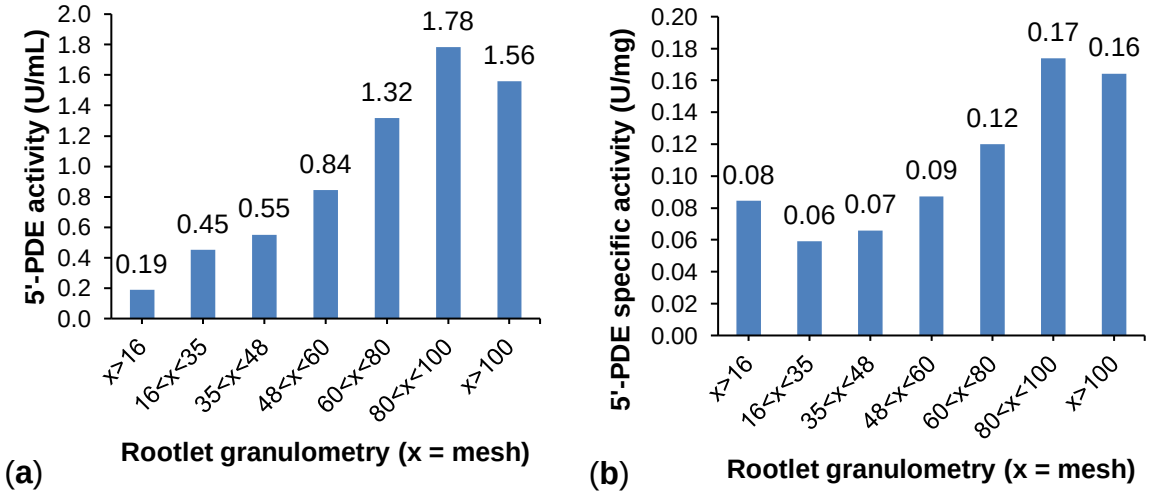
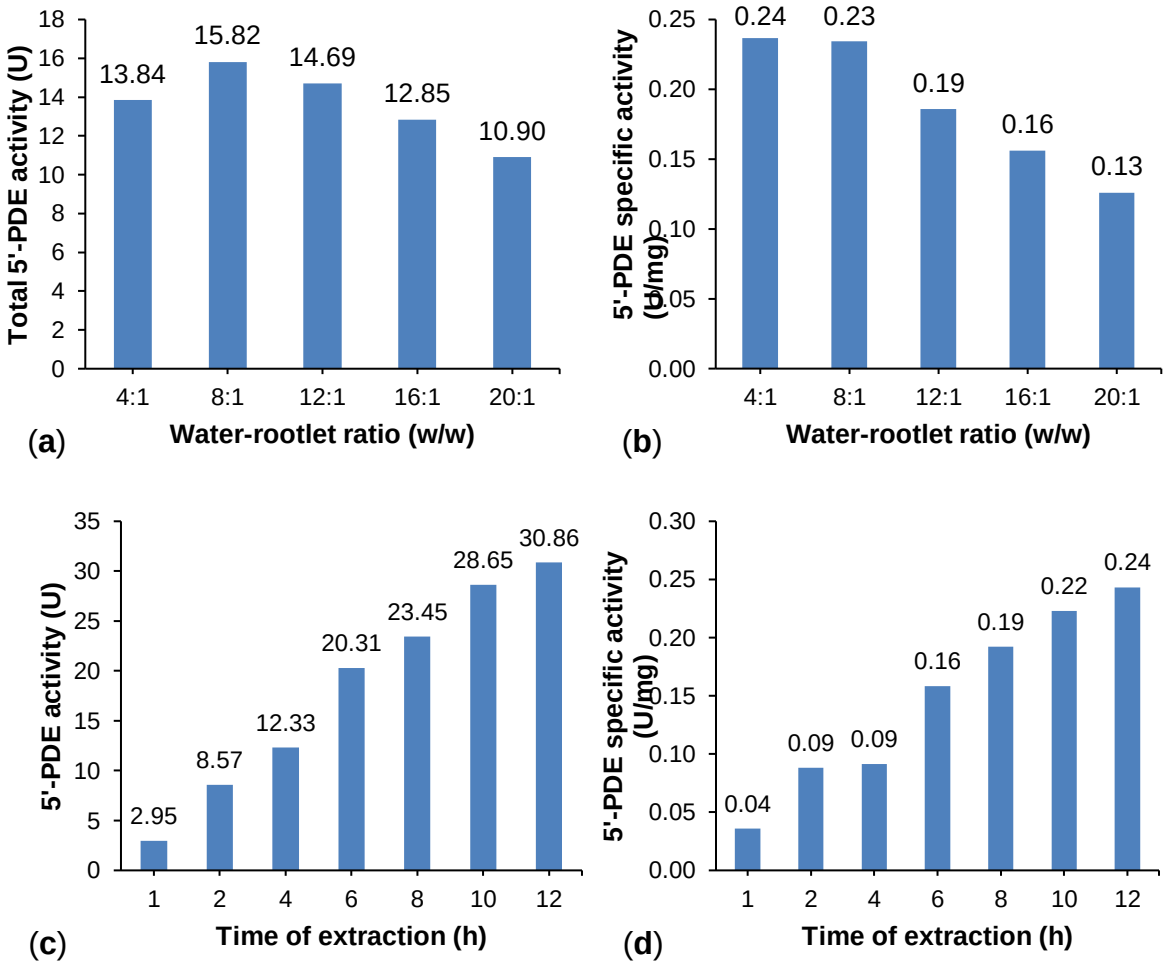


Figure 5 - Influence of water-rootlet ratio (a and b) and time of extraction (c and d) on the 5'-PDE extraction.



The extraction of 5'-PDE was mainly influenced by the fineness of the rootlets and amount of extraction solvent (water). The smaller the diameter of the rootlet, the greater the yield of the extraction. The best results found for malt rootlet granulometry and water ratio were 80<mesh<100 and 8:1 respectively. Hua and Huang (2010) studied 5′-PDE from barley malt rootlets and their results showed that maximum total enzyme activity were obtained with the fineness of the barley rootlets larger than 120 mesh, pH at 7, temperature at 20ºC, water-rootlet ratio of 16:1, and the extraction time of 7 hours. In the present work, with the increase in the extraction time, there was an increase in the extraction of the enzyme in a linear way. In addition, the specific activity also increased up to the maximum time studied (12h) (Figure 5d). Besides, the amount of water of the best water-rootlet ratio result (8:1) was lower in comparison to Hua and

Huang (2010) results, which gives a more concentrated crude extract and reduces the cost related to enzyme concentration.

4.2 ENZYME CHARACTERIZATION

The results of the influence of pH and temperature on the enzyme activity of 5'-PDE are shown in Figure 6 and 7 respectively. Analyzing Figure 6, it is noted that the pH value at which the enzyme activity is maximal is 5. 5'-PDE showed to be very sensitive to this parameter, thus small variations in the pH can contribute negatively to the enzyme activity being very important and necessary the use of buffer solution for better yield during the hydrolysis of RNA. As noted in Figure 7, the enzyme activity of 5'-PDE was favored by temperatures in the range of 60 to 70 °C. These results are in accordance with the results of Fujimoto et al. (1977) and Hua and Huang (2010), which also obtained an optimum 5'-PDE activity at temperatures in this range.

Figure 6 - Influence of pH on 5'-PDE activity.

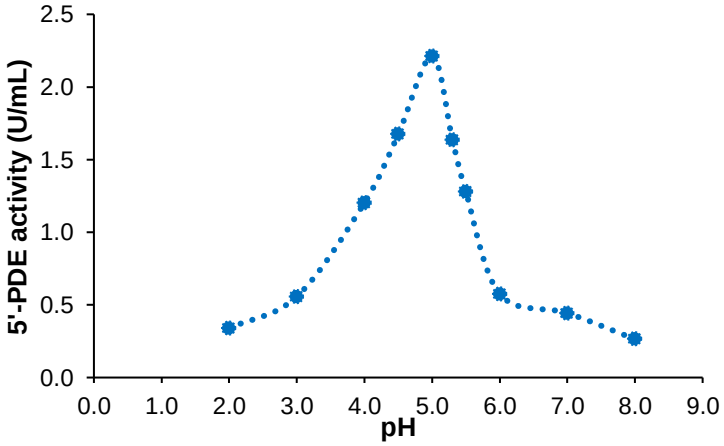
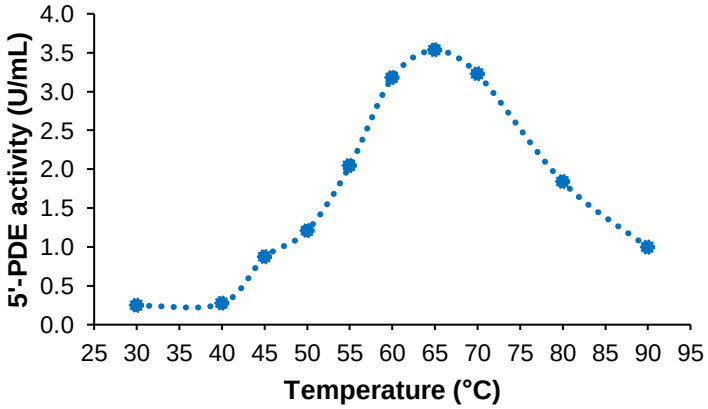
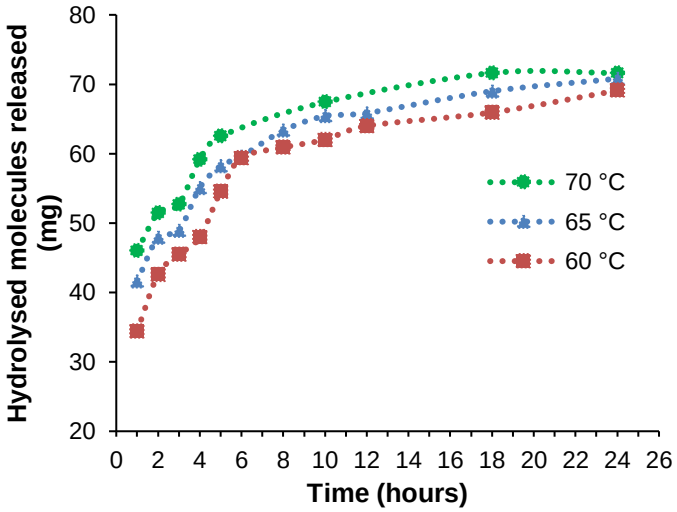


Figure 7 - Influence of temperature on 5'-PDE activity.



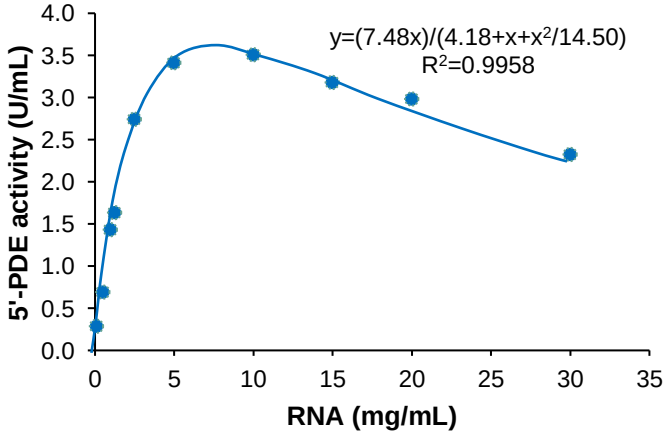
Furthermore, the effect of heating on the thermostability of 5'-PDE showed that this enzyme has excellent thermostable properties (Figure 8). The enzyme activity increased slightly with the increasing of the temperature but overall hydrolysis profile was similar up to 24 hours reaction.

Figure 8 - Effect of heating over the time on the thermostability of 5'-PDE.



As seen in Figure 9, the enzyme activity is also affected by the concentration of the substrate. 5'-PDE activity reaches a maximum value as the substrate concentration increases up to about 9 mg/mL and after this point, an addition of substrate inhibits the reaction. In order to mathematically model this behavior, the experimental results were adjusted to the substrate inhibition model according to Eq. 2 and the parameters of Vmax, Km and Ki were determined by a non-linear regression. A comparison between the experimental and predicted results obtained by the model can be observed in Figure 9.

Figure 9 - Nonlinear regression for the substrate inhibition model on 5'-PDE activity.



The adjustment of the model (Eq. 3) reached a coefficient of determination of 0.9958, indicating that 99.58% of the values of the enzyme activity obtained experimentally can be explained by the variation of RNA concentration.

$$V = \frac{7.48 * S}{4.18 + S + \frac{S^2}{14.50}} \qquad (3)$$

Where: *Vmax* = 7.48 U/mL;
Km = 4.18 mg RNA/mL;
Ki = 14.50 mg RNA/mL.

The Km value obtained is close to those found in the literature for the same enzyme. It is important to note that the choice of the regression method to determine parameters such as Vmax and Km is very important because it interferes with the reliability of the results. Several authors have criticized linear regression methods because they have become obsolete over the years. According to Tseng and Hsu (1990), for example, the Lineweaver-Burke graphs distort the measurements performed at low substrate concentrations and this may give rise to not very accurate estimates of Vmax and Km. On the other hand, Km is a characteristic of the enzyme and its substrate and its value is independent of the amount of enzyme used for its experimental determination, but the same is not true for Vmax. There is no absolute value of Vmax and its value depends on the amount of enzyme used.

In addition to Vmax and Km, Ki are of great importance in the study of enzyme kinetics, since it allows to obtain information about the way in which the activity of the enzyme is affected by the operating conditions, being possible to plan and optimize the parameters of operation in reactors enzymes. 5-PDE is an enzyme very sensitive to RNA inhibition, so it is important to study the ideal concentration of substrate that does not present a risk of inhibition. The Ki value found in this work indicates that RNA concentration above 14.50 mg/mL are very discouraged. The Ki value indicates how potent an inhibitor is, and can be defined as the concentration of inhibitor in which, under saturating substrate conditions, the reaction rate is reduced to half the maximum reaction rate Vmax. The lower the Ki value for a given enzyme, the greater the inhibitory capacity of the substrate.

4.3 ENZYMATIC RNA HYDROLYSIS

Figure 10 shows the UV-260 nm HPLC chromatogram obtained after 12 hours of reaction which allows to observe clearly the efficiency of the enzyme through the formed peaks in comparison with the RNA control, in which enzyme was not added. It was possible to obtain a good separation of the molecules as well as to calibrate a standard curve with correlation between area and amount of ribonucleotides and nucleosides close to 1 (Appendix A and B).

Throughout the enzymatic hydrolysis of RNA, it was observed that most of the compounds formed were 5'-ribonucleotides (Figure 11). Although obtained in smaller quantities with the proposed approach, nucleosides are important because they are involved in basic biological processes and can be used as precursors to other molecules with therapeutic activity. Some synthetic analogs of natural nucleosides often exhibit biological activities of great pharmaceutical value. Medical applications are related in cancer chemotherapy and AIDS, and as suppressors of the immune response during organ transplantation (SOARES; DE SOUZA; FERREIRA, 2001). Some examples of nucleoside analogs with therapeutic application are D-arabinofuranosylcytidine, effective against myelocytic leukemia; aciclovir, potent anti-herpetic agent, and 3'-azido-3'-deoxythymidine (AZT), an effective anti-HIV compound (COLLINS; FERRIER; BERLIN, 1995).

Figure 10 - UV-260nm HPLC chromatogram obtained after 12 hours of reaction.

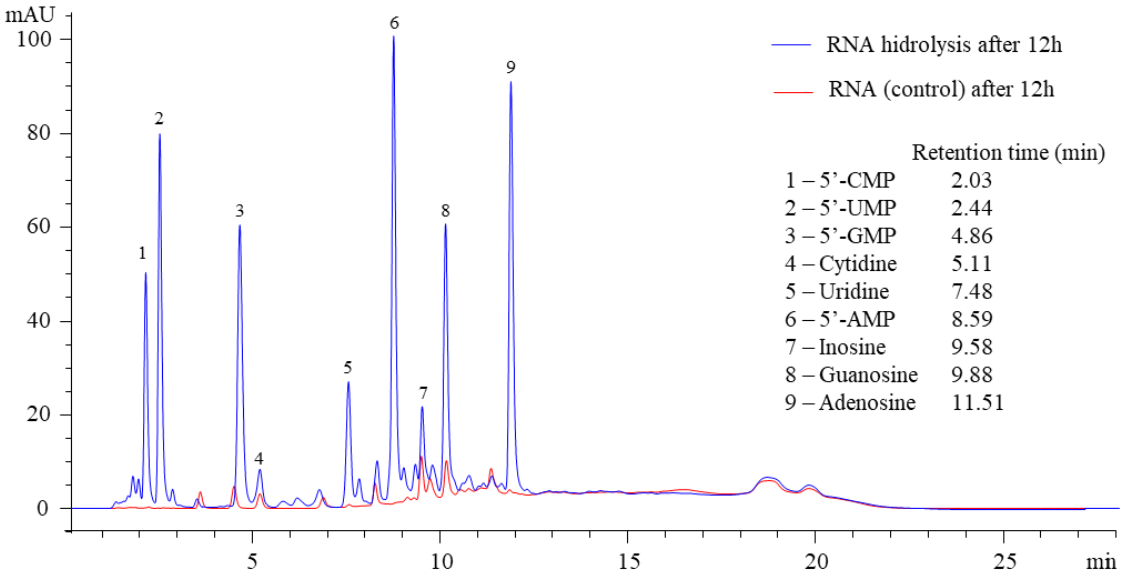
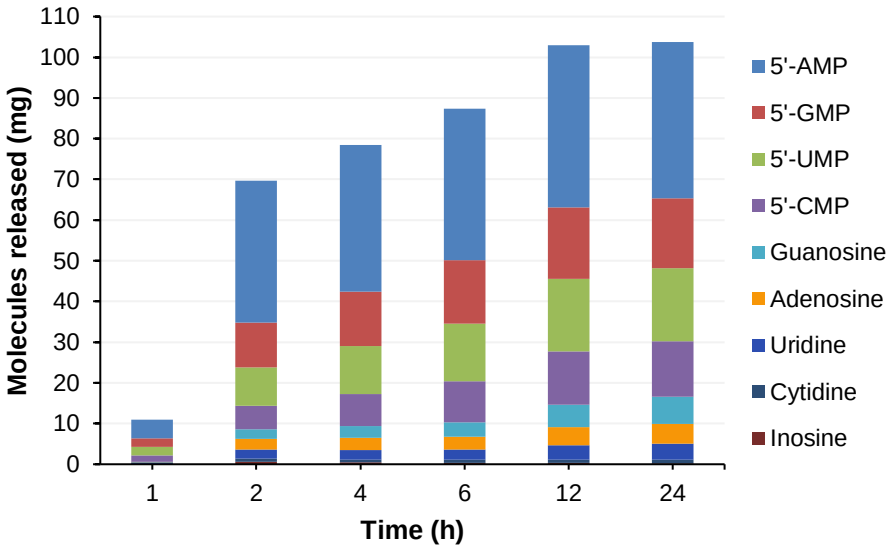
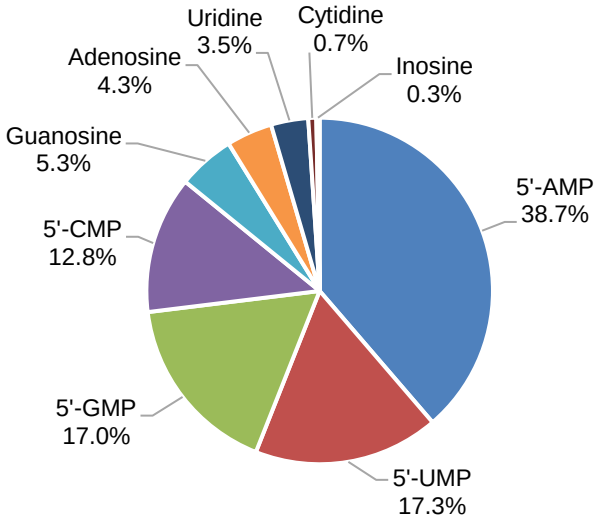


Figure 11 - 5'-Ribonucleotides and nucleosides content over the time of RNA hydrolysis using crude extract of 5'-PDE from barley rootlets.



Formation of 5'-IMP was not noted and this can be explained once 5'-IMP is just produced enzymatically by deamination of 5'-AMP using adenyl deaminase. Under the conditions of the reaction it was observed that the hydrolysis came to an end about 12 hours after the beginning, there was no increase in the number of hydrolyzed molecules after that. The highest yield of molecules formed were 5'-AMP, 5'-GMP and 5'-UMP (Figure 12). Ribonucleotides represented 85.86% of the total hydrolyzed molecules, which shows the potential of the methodology used to produce these compounds on a large scale.

Figure 12 - 5'-ribonucleotides and nucleotides content after 12h of RNA hydrolysis using crude extract of 5'-PDE from barley rootlets.



Nucleotides in general, as well as nucleosides, participate in several biochemical processes essential for the functioning of the organism (LEHNINGER et al., 2005). When bound to vitamins or their derivatives, the nucleotides constitute a portion of many coenzymes (FAD, NAD and CoA). As major donors and acceptors of phosphoryl groups in metabolism, tri- and diphosphate nucleosides, such as ATP and ADP, play the major role in energy transduction that accompanies metabolic interconversions and oxidative phosphorylation (ROSSI; XAVIER; RUTZ, 2007). Cyclic nucleotides cAMP and cGMP act as second messengers in hormone-regulated events, and both GTP and GDP play key roles in the cascade of events that characterize signal transduction pathways. Nucleotides, linked to sugars or lipids, are essential biosynthesis intermediates. Sugar derivatives, UDP-glucose and UDP-galactose, participate in the interconversions of sugars, as well as in the biosynthesis of starch and glycogen. Similarly, nucleotide-lipid derivatives, such as CDP-acylglycerol, are intermediates in lipid biosynthesis (ROSSI; XAVIER; RUTZ, 2007).

The range of application of nucleic acids derivatives is wide, which justifies the search for more effective ways of producing them. The molecules produced using the methodology described in the present work can be used in food applications. Nucleotides, when supplemented in the feed, have several beneficial therapeutic effects and are well evaluated as immunostimulatory agents, promoting protection against bacterial infection and enhancing the immunological function of the organism (BURMEITER and RAINSFORD, 1991). Studies to evaluate the effect of nucleotides in the immune system show that children vaccinated with antigens of protein T had the immune system increased (LERNER and RAANAN, 2000). In another study, there was an increase in immunoglobulin in children who received nucleotides in the diet (MARTÍNEZ-AUGUSTIN et al., 1997). Dietary nucleotides increase intestinal iron absorption, affect lipoproteins, and the polyunsaturated long chain fatty acid metabolism has a topical effect on the intestinal mucosa, liver and reduce the incidence of diarrhea (COSGROVE, 1998; SCHLIMME; MARTIN; MEISEL, 2000). Besides that**,** ribonucleotides such 5’-GMP and 5’-IMP are used as flavor enhancers in the food industry, in products such as corn chips, broth tablets, powdered soups, dehydrated seasoning mixes, among many others (JO and LEE, 2008).

5 CONCLUSION

Yeast biomass is undoubtedly an underutilized waste from brewing and its use can be considered an economical source for the production of 5'-ribonucleotides and other compounds on a large scale in order to meet the growing demand for new inputs for food and pharmaceutical industries, as well as for the development of biorefineries, using simple and efficient strategies that guarantee economic viability. The experimental results of this wok show that the extraction of 5'-phosphodiesterase from malt roots is simple and economical and can be adapted to industrial production of 5'-phosphodiesterase. The ribonucleotides and nucleosides produced have a wide range of application and can be used as raw materials for biochemical drugs, food additives, and health products. Finally, the approach here proposed can open up a new perspective for the brewing industry in relation to the management of its wastes, giving a better destination to spent brewer's yeast and malt rootlets.

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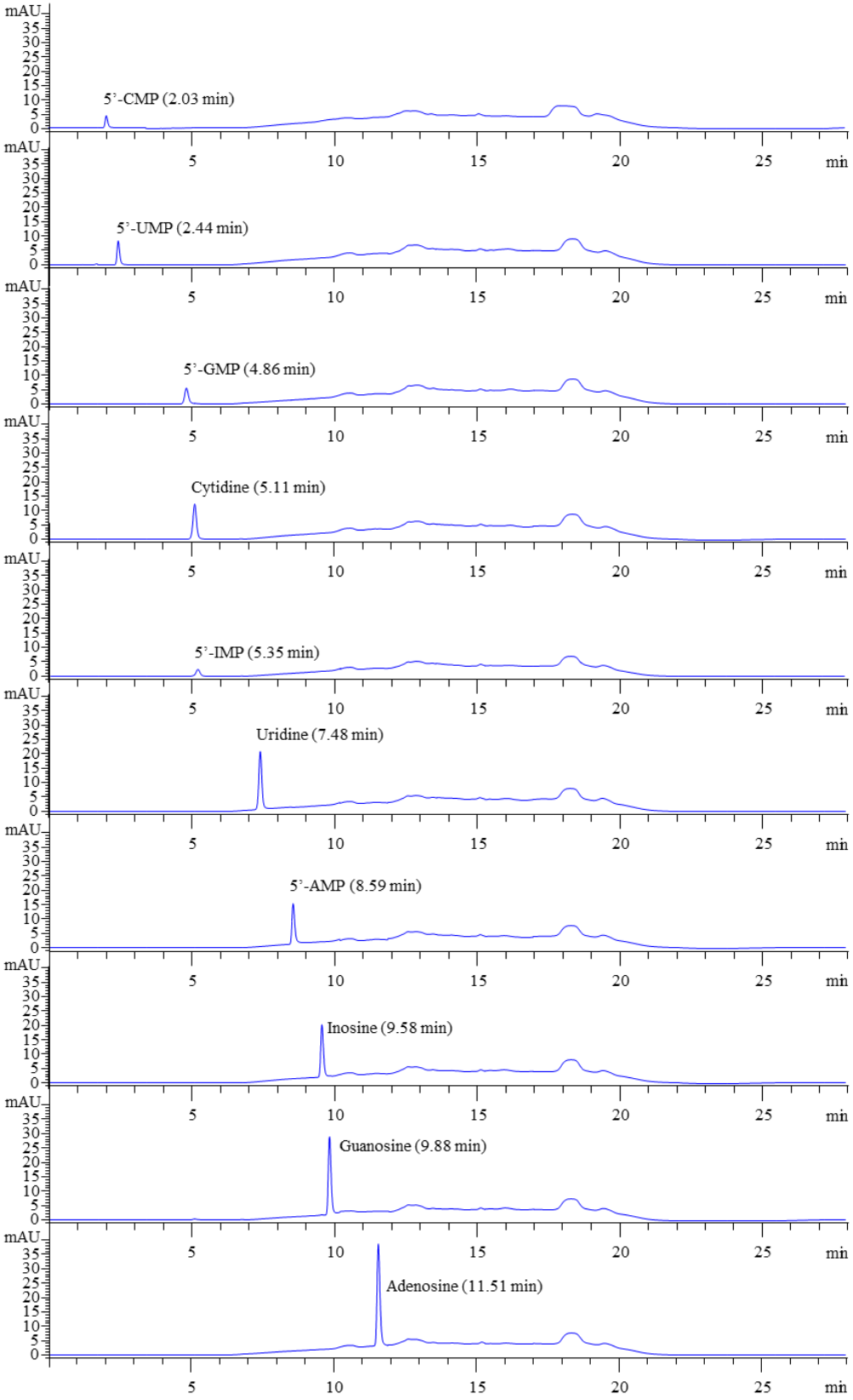
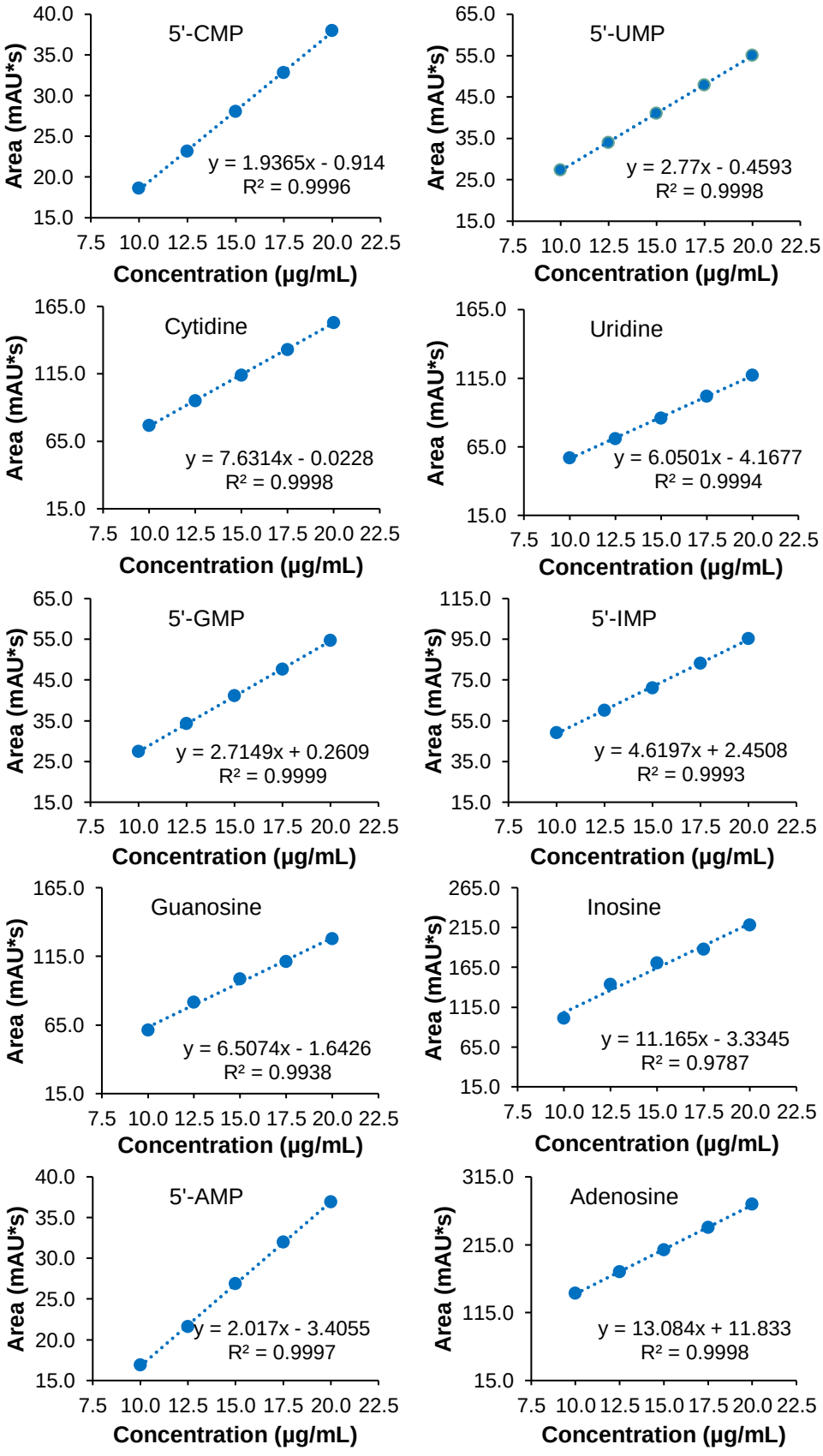
Appendix A

UV-260 nm chromatogram obtained for each standard 5'-ribonucleotide and nucleoside.



Appendix B

Standard curves for HPLC quantification of 5’-ribonucleotides and ribonucleosides.



CHAPTER 5 INVERTASE FROM SPENT BREWER'S YEAST: OBTAINMENT, CHARACTERIZATION AND APPLICATION

ABSTRACT

The present work aims to develop an innovative methodology based on a process of fast autolysis to obtain, concentrate and immobilize invertase from residual brewer's yeast. Autolysis of 113.83 g of yeast on dry basis was performed to obtain the extract containing invertase. After that, crude protein content was analyzed by Kjeldahl method, yield of invertase extraction and determination of the influence of pH, temperature and substrate concentration on the enzyme activity. The microfiltration, ultrafiltration and lyophilization methods were also evaluated for enzyme concentration, as well as their immobilization in calcium alginate for later application. The enzyme extraction method was shown to be effective in yielding 305.1 U per gram of dry yeast. The invertase concentration by ultrafiltration and microfiltration was not satisfactory, presenting great losses of enzyme activity during the process. On the other hand, the lyophilization method was effective, showing no loss of enzyme activity during the process. The immobilization of the enzyme in alginate presented losses of 32% and it was possible to achieve 100% hydrolysis of the sucrose 60 g/100 mL in a period of 17h and 30h for the reaction with free and immobilized enzyme respectively. Finally, it was possible to establish a methodology capable of better utilizing the yeast residue from fermentative processes, facilitating the discovery of new applications and the consequent increase of their added value.

Keywords: *Saccharomyces cerevisiae*. Invertase. Ultrafiltration. Lyophilization. Enzyme immobilization. Alginate.

1 INTRODUCTION

1.1 INVERTASE

Invertase or *β*-D-fructofuranosidase (EC 3.2.1.26) is an enzyme that catalyzes the hydrolysis reaction of sucrose. This enzyme is also capable, under some conditions, of transfructosylation reaction from fructooligosaccharides (NGUYEN et al., 2005). Invertase, acting as a catalyst in the sucrose breakdown, producing a mixture known as inverted sugar, with equimolar amounts of glucose and fructose (UHLIG, 1998). Sucrose has chemical properties other than glucose and fructose, being called non-reducing sugar. The glycosidic bond present in this molecule occurs between the carbon 1 of the glucose and the carbon 2 of the fructose, which makes it incapable of acting as a reducing agent (ZHU et al., 2010). The inverted sugar solution has free monosaccharides capable of reducing other compounds, besides is widely used in the food industry, mainly in the production of jellies, candies, sweets and syrups, due to its 40% higher sweetening factor than sucrose, and its slow crystallization, which improves the texture of the food (WHITE, 2014).

Invertase can be found in invertebrates, vertebrates, green algae, bacteria, vegetables, fungi and yeasts, mainly of the species *Saccharomyces cerevisiae* (NOVAKI et al., 2010). When used industrially, these enzymes are produced mainly by yeasts, presenting in two forms: an intracellular (20%) and an extracellular (80%) portion. Both fractions have similar catalytic activity, but differ in composition. The external form is found as glycoprotein with approximately 50% carbohydrates and has cysteine in its composition. The inner portion does not possess them (HEMMING 1995; PEBERDY 1994).

The factors that limit the production of invertase commercially are its obtention, extraction and purification, which raise its cost (NOVAKI et al., 2010). Thus, studies focusing on reduce the production costs such as the use of residual brewer's yeast could be viable strategies to obtain this enzyme with lower costs.

1.2 ENZYME KINETICS

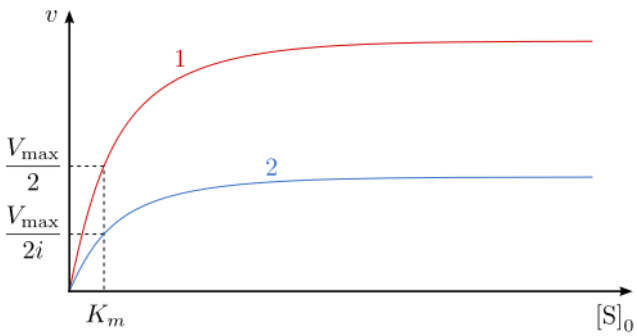
The enzyme kinetics can be defined as the study of the speed of the enzyme reaction and how it changes according to different parameters (CORNISH-BOWDEN, 2012). This is an important tool for understanding the mechanism of action of an enzyme. By studying the kinetics of a given enzyme, it is possible to measure the velocities of the transformations that are processed during the reaction, to study the influences of the working conditions, such as concentrations of activators, inhibitors and reagents, optimum pH and temperature, besides to establish criteria for process control and even design more suitable reactors (CORNISH-BOWDEN, 2012).

For various enzymes, the initial velocity, Vo, varies hyperbolically with the concentration of the substrate. The mathematical equation expressing this hyperbolic relationship between initial velocity and substrate concentration is known as the Michaelis-Menten equation (Eq. 1) (WILSON and WALKER, 2010).

$$Vo = \frac{Vmax*[S]}{Km+[S]} \qquad (1)$$

Where, *Vmax* is the maximum value of the initial velocity when all active sites are occupied, *Km* is the Michaelis constant and *[S]* is the substrate concentration. At low substrate concentrations (Figure 1), the occupation of active enzyme sites is low and the reaction rate is directly proportional to the number of sites occupied. That is, the behavior of the reaction is close to that of first-order kinetics, where the rate is proportional to the concentration of the substrate.

Figure 1 - Effect of substrate concentration on the initial rate of an enzyme catalyzed reaction in the presence of two different enzyme concentrations. Duplication of the enzyme concentration doubles the maximum initial rate, *Vmax*, but does not affect *Km*.



At high concentrations of substrate, all the active sites are occupied and the reaction becomes independent of its concentration, since there is no way to form more

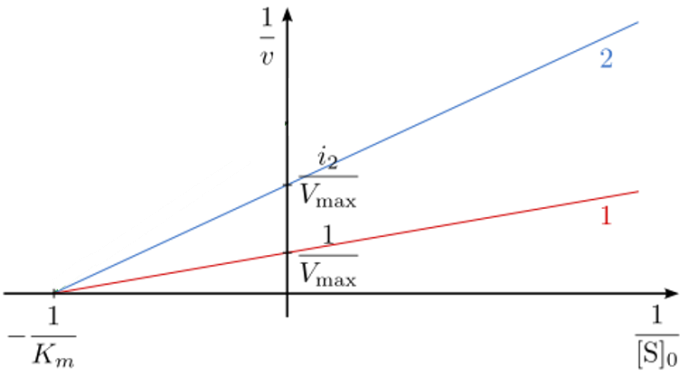
substrate-enzyme (ES) complexes, thus observing a zero order or saturation kinetics in relation to the substrate. Under these conditions, the rate of the reaction is only dependent on the conversion of the enzyme-substrate complex into product (P) and the diffusion of the products from the enzyme (WILSON and WALKER, 2010).

Although the Michaelis-Menten equation can be used to calculate *Vmax* and *Km*, its use is subject to difficulties in the experimental determination of *Vo* at high concentrations of substrate, in other words, in the extrapolation of the hyperbolic curve to obtain an exact value of *Vmax*. Linear transformations of the Michaelis-Menten equation are commonly used alternatives. The most popular of these transformations is the Lineweaver-Burk equation (Eq.2), that can be obtained by taking the reciprocal of both sides of the Michaelis-Menten equation (WILSON and WALKER, 2010).

$$\frac{1}{Vo} = \frac{Km}{Vm} * \frac{1}{[S]} + \frac{1}{Vmax} \qquad (2)$$

The graph of *1/Vo* versus *1/[S]* (Figure 2) results in a straight line, where the slope is *Km/Vmax*, the intercept on the *1/Vo* axis is equal to *1/Vmax* and the intercept on the *1/[S]* axis corresponds to the value of *-1/km* (WILSON and WALKER, 2010).

Figure 2 - Lineweaver-Burk chart. Duplication of the initial concentration of the enzyme does not affect the *Km* value.



Several factors affect the activity of an enzyme, of which we can highlight the pH, temperature and substrate concentration, the latter being the most important parameter to compare the activity and type of enzyme reaction (NAGAR; ARGIKAR; TWEEDIE, 2014).

Due to their characteristics, enzymes are active in a narrow pH range and are sensitive to changes in acidity or alkalinity in their environment. Compared to chemical reactions, they often act under relatively mild conditions in terms of temperature and acidity (WANDERLEY et al., 2011). The pH may influence the activity of an enzyme through distinct ways, leading to protein denaturation with consequent enzyme inactivation, altering the charge pattern of a given active or catalytic site, or altering the overall conformation of the protein so that the interaction of the site with the substrate is impaired (CORNISH-BOWDEN, 2012).

Is important to note, that the enzyme kinetics of the Michaelis-Menten model does not consider the presence of inhibitors. Naturally systems tend to be more complex and elaborate, and greater and more rigorous care must be taken. The Eq. 3 is a model that considers the inhibition by the substrate, being very common in the hydrolysis process, because when it is in high concentration there may be inhibition of the reaction. The reaction rate increases with the addition of substrate, reaching a maximum speed, and after that, the increase in concentration may inhibit the reaction (RIBEIRO, 1989).

$$V = \frac{Vmax * S}{Km + S + \frac{S^2}{Ki}} \qquad (3)$$

Where *V* is the rate of the reaction dependent on the substrate, *Vmax* is the maximum value of the initial velocity when all active sites are occupied, *Km* is the Michaelis constant, *[S]* is the concentration of the substrate and *Ki* corresponds to the inhibition constant of the enzyme by the substrate.

1.3 CONCENTRATION AND IMMOBILIZATION OF ENZYMES

Purification of enzymes is a process in which an enzyme of interest is obtained from a biological sample in order to optimize the catalytic yield for various industrial applications. The level of enzyme purification depends on the subsequent application to be used and the processes for separation are mainly based on the physical-chemical properties of the proteins (ZUÑIGA et al., 2003). Ion exchange chromatography (ionic charge), affinity chromatography (binding specificity), adsorption (polarity)

chromatography, ultrafiltration (molecular size) and salting in/out (solubility) are the main separation techniques for proteins and enzymes.

Once purified, the enzyme can be used directly in the processes involving enzyme reactions or being immobilized with recycling possibilities. The immobilization of enzymes consists in the confinement of the same in a solid support for the later reuse as biocatalyst. The advantages associated to this procedure include the possibility of continuous operation, greater stability as to pH and temperature, ease of reaction interruption and cost reduction among others (MENDES et al., 2011).

A variety of methods have been developed for immobilization of enzymes, but there is no overall application methodology. Thus, the characteristics of the target enzyme need to be studied to define the most appropriate procedure to guarantee operational simplicity and stability (MENDES et al., 2011).

1.4 SPENT BREWER'S YEAST FOR LARGE SCALE INVERTASE PRODUCTION

Currently, yeast biomass is an underutilized brewing residue. In addition, the use of this biomass can be considered an economical source for large scale invertase production, in order to meet the growing demand of the food industry, as well as for the development of biorefinery, using simple and efficient strategies that guarantee economic viability. Taking into account abundance, low cost and richness in nutrient and enzyme constituents, it is a good strategy to use this biomass for invertase extraction.

In view of the exposed characteristics, this work emerges as an attempt to elucidate and establish a methodology capable of extracting and immobilizing invertase from yeast from the beer industry, thus reducing the costs of obtaining this enzyme. The use of integrated methods such as yeast autolysis followed by ultrafiltration and immobilization of the enzyme in alginate microspheres could be viable strategies for obtaining an end product that can be used on an industrial scale.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

The present work aims to establish a methodology for obtainment, concentration and immobilization of invertase from residual brewer's yeast in sodium alginate and activated carbon beads.

3.2 SPECIFIC OBJECTIVES

- Characterize the reaction kinetics of the invertase obtained from residual yeast in relation to the optimum parameters of temperature and pH as well as determination of *Km* and *Ki* of the enzyme.
- Evaluate tangential ultrafiltration and lyophilization methods to concentrate the enzyme obtained.
- Immobilize the enzyme in alginate microspheres.
- Carry out hydrolysis of sucrose for the production of invert sugar using the enzyme free and immobilized.

4 MATERIAL AND METHODS

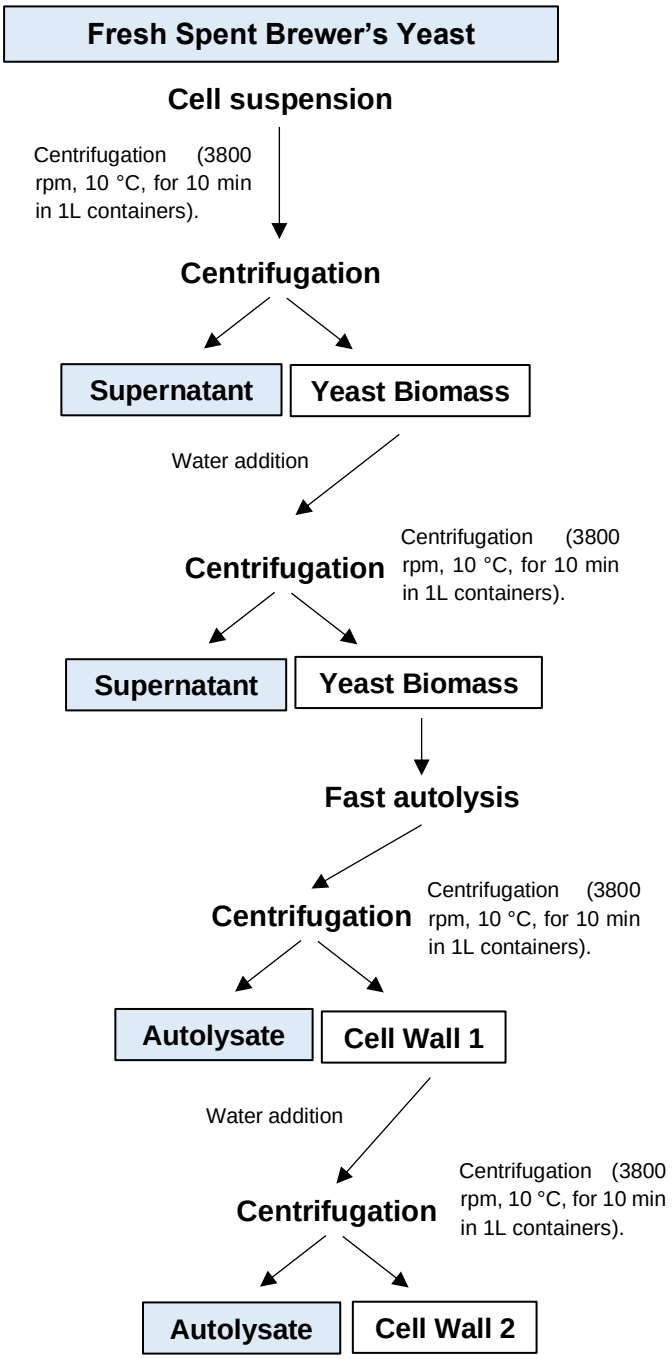
4.1 INVERTASE RECOVERY

Fresh yeast cells *Saccharomyces cerevisiae*, in the form of aqueous suspension, kindly provided by the brewery Malta Assis (Assis - SP) were used to obtain invertase. Approximately 1 liter of fresh cells were centrifuged at 3800 rpm (RCF = 4794 x G) for 10 min at 10 °C (Heraeus Cryofuge 6000i Centrifuge, Thermo Scientific, Osterode, Germany), thus obtaining the insoluble fraction (yeast mass) and supernatant 1 (rich in extracellular invertase). After centrifugation, the cell mass was resuspended in 500 mL of water and centrifuged again to perform a wash, thereby obtaining supernatant 2 and cell mass 2.

The intracellular invertase was extracted after a rapid autolysis process. A cell suspension consisting of 7.5% (w/v) cell mass 2 was subjected to autolysis at 60 °C for 60 minutes. After autolysis, the sample was centrifuged at 3800 rpm (RCF = 4794 x G) for 10 min at 10 ° C (Heraeus Cryofuge 6000i Centrifuge, Thermo Scientific, Osterode, Germany), thus obtaining cell wall 1 and rich autolysate 1 in intracellular invertase, which was used in the following steps. A new wash was performed with 300

mL of water to obtain autolysate 2 and cell wall 2. Figure 3 shows a summary flowchart of the methodology employed.

Figure 3 - Flowchart of the invertase extraction process from residual brewer's yeast.



4.2 INVERTASE ACTIVITY ASSAY

The activity of the enzyme invertase is determined by the concentration of reducing sugars produced after the test time. The quantification of reducing sugars

was performed according to Miller (1959), with adaptations, using 3,5-dinitrosalicylic acid (ADNS) as a color reagent. To determine the invertase activity, 0.1 mL of the enzyme-containing sample was added in 0.9 mL of 0.1 mol/L sucrose solution in 0.1 mol/L sodium acetate buffer pH 4.5. The mixture was incubated at 50 ° C for 10 minutes. Then, 0.5 mL of the ADNS reagent was added to paralyze the reaction. The mixture was brought to the boiling water bath for 5 minutes and cooled in an ice bath. The absorbance reading was performed at 540 nm in a spectrophotometer. Blank tests were performed with water instead of the sample. The reducing sugar content was determined by standard curve. A unit (U) of invertase activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing groups, measured as glucose per minute, under the reaction conditions used.

4.3 PROTEIN CONTENT QUANTIFICATION

The determination of the total protein was done using the classical Kjeldahl method, according to the methodology described by AOAC (2000), which basically comprises three steps: digestion of the sample in sulfuric acid with a catalyst, which results in the conversion of ammoniacal nitrogen; distillation of ammoniacal nitrogen into a boric acid capture solution; and quantification of ammonia by titration with standard hydrochloric acid solution.

4.4 DETERMINATION OF KM, TEMPERATURE AND PH OPTIMUM FOR INVERTASE ACTIVITY

In order to determine the optimum reaction temperature, the enzyme activity was performed under the same conditions previously mentioned, changing only the temperature values to 30, 40, 50, 55, 60, 65, 70 and 80 °C.

To determine the optimal pH conditions of the reaction, the following 0.1 mol/L buffers were used: glycine-HCl (for pH values in the range of 2.0 to 3), sodium acetate (for pH values in the range of 4.0 to 5.5), sodium phosphate (for pH values in the range of 6.0 to 8) and KCl-H_3BO_3 (for pH values in the range of 9.0 to 10.0). The determination of the enzyme activity was performed as previously described altering only the buffer solution and the pH during the reaction.

The determination of Km of the enzyme was performed by varying the initial concentration of sucrose between 2 and 500 g/L during the reaction. The kinetic model proposed by Michaelis-Menten with substrate inhibition (Eq. 3) was adjusted to the experimental results of enzyme activities during the reaction as a function of substrate concentration by means of nonlinear regression using MATLAB software R2017b (Mathworks®) so that the inhibition constant *Ki* is determined.

4.5 INVERTASE CONCENTRATION AND IMMOBILIZATION.

The enzyme extract obtained from the brewer's residual yeast was subjected to an ultrafiltration process using ultrafiltration membranes with 10 kDa cut molecular mass and 110 cm^2 area (UFP-10-E-3MA, GE Helthcare Bio-Sciences Corp., Westborough, USA) and a 30kDa and 110cm^2 area (UFF-30-E-3MA, GE Helthcare Bio-Sciences Corp., Westborough, USA), and UMP-1047R (200kDa) and UMP-153 microfiltration membranes (200kDa) (Microza®) in order to concentrate the enzymes in the retentate, drawing a comparison to define the one that presents the best result. The ultrafiltration parameters were operated at a temperature of 25 °C, feed flow 300 mL/min, and transmembrane pressure of 170 kPa. After the ultrafiltration, invertase activity analyzes were carried out in the permeate and the retentate to evaluate the efficiency of the ultrafiltration.

In addition to ultrafiltration, the efficiency of the lyophilization process for water withdrawal and enzyme concentration was evaluated. Approximately 100g of yeast cells on dry basis were autolysed as described in item 4.1. and all soluble fractions (supernatant 1 and 2, autolysate 1 and 2) were pre-frozen and then subjected to lyophilization (Liotop, L101, Liobras, São Carlos, Brazil) for 72 hours at 38 °C and pressure of 0.475μmHg. After lyophilization, a fraction of the lyophilized enzyme was resuspended in water and the total enzyme activity determined.

Then, the enzyme was subjected to the immobilization process by occlusion in 2% alginate and activated charcoal microspheres. For the preparation of the invertase support, 0.2 g of sodium alginate and 30mg of activated charcoal were dissolved in 5 ml of water and then 5 ml of a lyophilized enzyme preparation with 1000 E total activity was added. After homogenization, the solution was dripped using a pipette in 50 ml 0.3 M $CaCl_2$ solution to give spheres of the enzymes immobilized on sodium alginate. The beads formed were separated from the $CaCl_2$ solution by sieving and washed with

distilled water, all wash fractions being stored for the determination of enzyme activity losses during the immobilization process.

4.6 INVERTASE APPLICATION

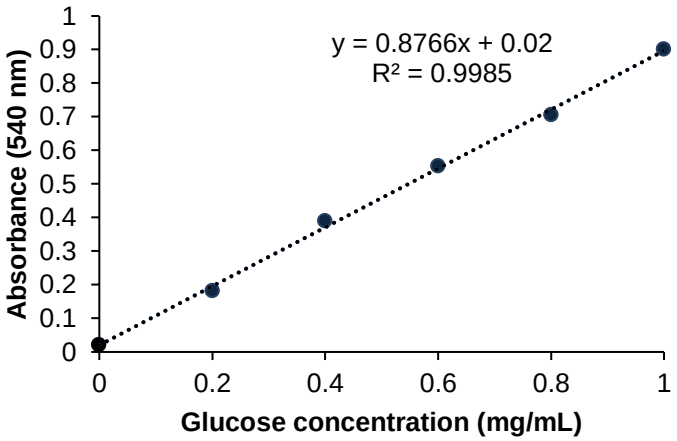
After obtaining and concentrating the enzyme, an application was made to compare the efficiency of the free and immobilized invertase. For this, 100 mL of 60 g/100 mL sucrose solution was prepared and then 5 mL of free enzyme with 100 U total activity was added. Another sample was also prepared under the same conditions however adding the enzyme immobilized on alginate. The hydrolysis reaction was processed at 55 °C for up to 32 hours, samples being withdrawn at time intervals for determination of the reducing sugar released over time.

5 RESULTS AND DISCUSSION

5.1 INVERTASE ACTIVITY OF THE ENZYME OBTAINED

Figure 4 shows the standard glucose curve obtained for quantification of reducing sugars by the ADNS method. This curve was used to determine the initial rates of sucrose hydrolysis reactions, according to item 4.2., at 50 °C using a 0.1 mol/L sucrose solution in acetate buffer, pH 4.5 for 10 minutes of reaction.

Figure 4 - Glucose standard curve.



As observed in Figure 4, the determination coefficient R^2 shows a value close to 1, indicating that 99.85% of the absorbance values can be explained by the variation of the glucose concentration.

5.2 INVERTASE EXTRACTION FROM RESIDUAL BREWER'S YEAST

Table 1 shows the enzyme activity for each fraction obtained during the invertase extraction process. For the extraction of the enzyme, a mass of 113.83 g of yeast on a dry basis was used.

Table 1 - Invertase activity in different fractions of the enzyme extraction process.

Fractions	**Volume (mL)**	**U/ml**	**U total**
Supernatant 1	367	41.73	15316.26
Supernatant 2	500	7.03	3519.14
Autolysate 1	555	23.02	12778.74
Autolysate 2	294	9.20	2705.23
		Total extracted (U)	**34319.37**

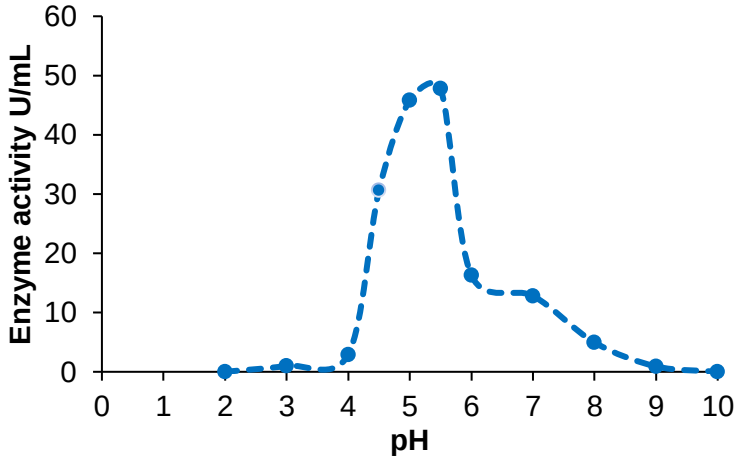
As shown in Table 1, the fraction of supernatant 1 had the highest yield in U. This result can be explained since most of the invertase produced by the yeast is in the extracellular portion. According to Hemming (1995), the invertase has two forms: one intracellular (20%) and the other extracellular (80%). Supernatants 1 and 2 correspond to the enzyme produced and released in the medium during fermentation of the brewer's wort. The invertase obtained in the fractions of Autolysate 1 and 2 correspond to the intracellular portion. From the total enzyme obtained in this work, a proportion of 45% of the enzyme was found in the intracellular portion and 55% of the enzyme in the extracellular portion.

Regarding the yield of enzyme extraction, Barbosa (2010) was able to extract approximately 33.9 U per gram of dry biological yeast (*Fleishmann* baking yeast) at 40 °C for 24 hours. In the present work it was possible to obtain approximately 301.5 U per gram of dry yeast from the brewing industry. Compared to Barbosa (2010), extraction yields were 8.87 times higher in a much shorter time period of only 60 minutes. This high yield demonstrates the promising potential of the ultra-fast autolysis, used in an innovative way in this work, as well as makes clear the advantage of residual brewer's yeast for invertase obtainment versus baker's residual yeast.

5.3 INFLUENCE OF PH ON NON-IMMOBILIZED INVERTASE ACTIVITY

The results of pH influence on the enzyme activity of non-immobilized invertase are shown in Figure 5. It is noted that the pH range at which the enzyme activity is maximal between 5 and 5.5 with the values of 45.86 U/ml and 47.77 U / ml respectively. This pH range found is in accordance with the results of Nugyen et al. (2005), which also obtained maximal activity at pH 5.5 using invertase produced by *A. niger* IMI 303386. The results of Yanase et al. (1995) are also in agreement with the values obtained here, which obtained maximum invertase activity at pH 5.5 using bacterial invertase produced by *Z. mobilis*.

Figure 5 - Invertase activity in different pH values.

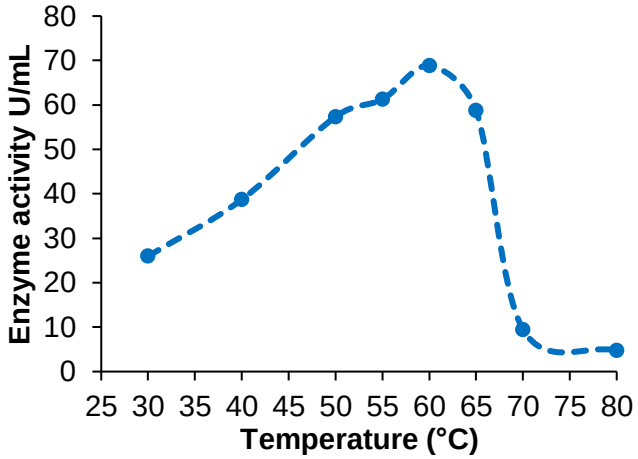


For the same species of yeast, *Saccharomyces cerevisiae*, Santos (2010) obtained maximum activity of invertase at pH 5, remaining good activity in the range of 4 to 5.5. Toralles (2014) also working with *S. cerevisiae* found an optimum activity range between 4 and 6, with maximum activity at pH 6. The pH plays an important role on the enzyme activity since it can affect the amino acid residues present in the enzyme which bind to the substrate and make it into a product (NOVAKI, 2010). Thus, knowing the optimum range of pH for the invertase activity is important in order to delineate and plan optimized conditions of operation in enzyme reactors.

5.4 INFLUENCE OF TEMPERATURE ON NON-IMMOBILIZED INVERTASE ACTIVITY

The results of temperature influence on the enzyme activity of non-immobilized invertase are shown in Figure 6.

Figure 6 - Invertase activity in different temperature values.



Observing Figure 6, it can be seen that the invertase activity increases gradually with increasing temperature, reaching a maximum value around 60 °C. The temperature has a direct influence on chemical reactions, because its increase leads to a greater agitation of the molecules of the reagents, increasing the effective collisions and consequently increasing the speed of the reaction (LESKOVAC, 2003). However, the increase in temperature should be studied with caution, since high temperatures can affect the tertiary structure of the protein, denaturing it and consequently impairing the stability between the enzyme-substrate complex (CABRAL; BARROS; GAMA, 2003). There was a marked drop in activity at temperatures above 65 °C, indicating a sensitivity of the enzyme at very high temperatures.

The temperature range of 55 to 60 °C found in this work is in accordance with the results of L'Hocine et al. (2000) and Guimarães et al. (2007), who also obtained an optimum activity in the 55 °C range using invertase produced by *A. niger* and in the 60 °C range using invertase produced by *A. ochraceus* respectively. For the same species of yeast, *Saccharomyces cerevisiae*, Santos (2010) also found close values, where the optimum activity was at 50 °C, and maintained a good activity in the range between 35 and 60 °C. Toralles (2014) in his experiments found optimum temperature around 50 ºC, with good activity in the range between 35 and 50 ºC.

5.5 INFLUENCE OF SUBSTRATE CONCENTRATION ON THE KINETICS OF NON-IMMOBILIZED INVERTASE

After determination of optimum pH (5.5) and temperature (60 °C) for invertase activity, the influence of substrate concentration on non-immobilized enzyme activity was studied (Figure 7). As seen in Figure 7, the reaction rate reaches a maximum value as the substrate concentration is increased and after this point, an addition of substrate inhibits the reaction. In order to mathematically model this behavior, the experimental results were adjusted to the substrate inhibition model according to Eq. 3 and the parameters Vmax, Km and Ki were determined by a non-linear regression. A comparison between the experimental results and those predicted by the model can be observed in Figure 8.

Figure 7 - Influence of sucrose concentration on non-immobilized invertase activity.

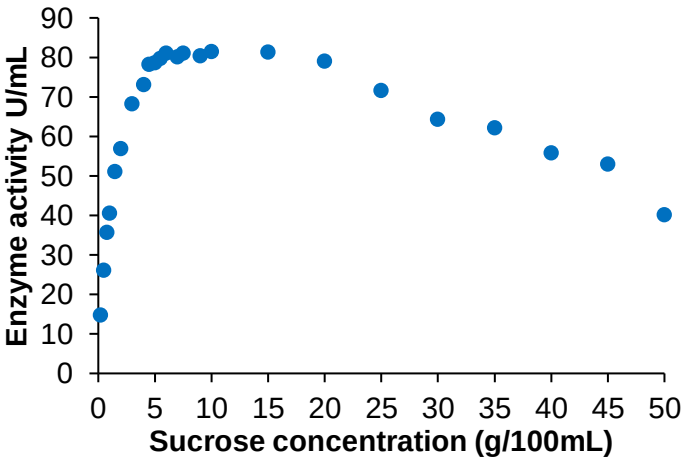
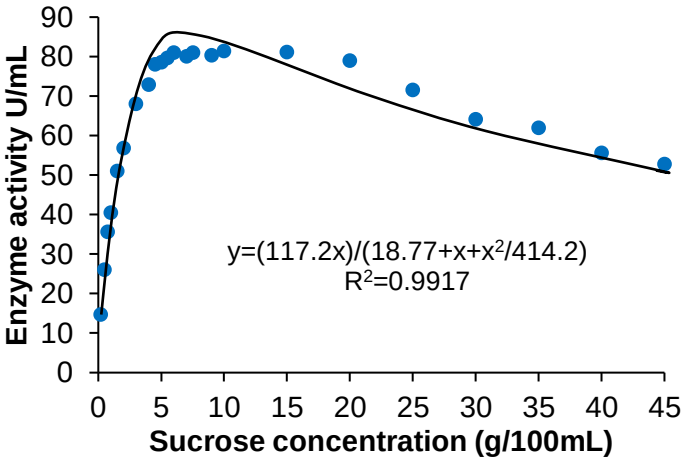


Figure 8 - Non-linear regression for the substrate inhibition model.



The adjustment of the model reached a coefficient of determination of 0.9917, indicating that 99.17% of the values of the enzyme activity obtained experimentally can be explained by the variation of sucrose concentration according to Equation 4.

$$V = \frac{117.2 * S}{18.77 + S + \frac{S^2}{414.2}} \quad (4)$$

Where: *Vmax* = 117.20 U/mL;

Km = 18.77 g_{sac}/L (54.88 mM_{sac});

Ki = 414.2 g_{sac}/L (1.20 M_{sac});

The Km value found is close to those found in the literature for the free enzyme. Ribeiro (1989) obtained a Km value equal to 21.20 g_{sac}/L (62.3 mM_{sac}). Isik et al. (2003), obtained a value of 20.17 g_{sac}/L (59 mM_{sac}) and Marquez (2007) obtained 15.47 g_{sac}/L (45.2 mM_{sac}).

It is important to note that the choice of the regression method to determine parameters such as Vmax and Km is very important because it interferes with the reliability of the results. Several authors have criticized linear regression methods because they have become obsolete over the years. According to Tseng and Hsu (1990), for example, the Lineweaver-Burke graphs distort the measurements performed at low substrate concentrations and this may give rise to not very accurate estimates of Vmax and Km. On the other hand, Km is a characteristic of the enzyme and its substrate and its value is independent of the amount of enzyme used for its experimental determination, but the same is not true for Vmax. There is no absolute value of Vmax and its value depends on the amount of enzyme used.

In addition to Vmax and Km, Ki is of great importance in the study of enzyme kinetics, since it allows to obtain information about the way in which the activity of the enzyme is affected by the operating conditions, being possible to plan and optimize the operational parameters of enzyme reactors. Invertase is an enzyme very sensitive to sucrose inhibition, so it is important to study the ideal concentration of substrate that does not present a risk of inhibition. The Ki value found in this work is slightly higher than that found by Marquez (2007), which obtained 363.27 g_{sac}/L (1.06 mM_{sac}). The Ki value indicates how potent an inhibitor is, and can be defined as the concentration of inhibitor in which, under saturating substrate conditions, the reaction rate is reduced to half the maximum reaction rate Vmax. The lower the Ki value for a given enzyme, the greater the inhibitory capacity of the substrate.

5.6 MICROFILTRATION AND ULTRAFILTRATION ENZYME CONCENTRATION ASSAY

The results of the invertase concentration by microfiltration and ultrafiltration are presented in Tables 2 and 3. For the microfiltration, the enzyme activity was analyzed before the procedure, in which an activity of 100% was defined, and after the process the retentate and the filtrate were also analyzed. For the UMP-1040R Membrane, it was observed that from the initial activity, a total of 66.10% of all the enzyme activity present in the sample was lost, and for the UMP-153 Membrane the reduction in the final result was larger still, with a reduction in activity of 90.17%.

Table 2 - Results of microfiltration evaluation in different membranes.

Fractions	**Membrane UMP-1040R-200 KDa**		**Membrane UMP-153-200KDa**	
	U total	**%**	**U total**	**%**
Sample	1668.77	100.00	1527.30	100.00
Filtrate	321.75	19.28	21.58	1.42
Retentate	243.94	14.62	128.48	8.41
Losses	1103.08	66.10	1377.24	90.17

Table 3 - Results of ultrafiltration evaluation in different membranes.

Fractions	**Membrane UFP-10-E-3MA**		**Membrane UFF-30-E-3MA**	
	U total	**%**	**U total**	**%**
Sample	578.06	100	578.06	100
Filtrate	2.35	0.41	10.81	1.870
Retentate	67.93	11.75	264.57	45.75
Losses	507.78	87.84	302.68	52.38

In the same way as mentioned above, the results were analyzed, but for the ultrafiltration membranes. For the Membrane UFP-10-E-3MA, the total activity loss was 87.84% and for the UFF-30-E-3MA it was estimated loss of 53.38%. Reductions are recurrent in all membranes tested, varying their reduction in enzyme activity. A relation between the size of the membranes and the reduction in activity can not be noticed for these results. Addezio (2014) reported reductions in invertase activity when using ultrafiltration membranes of different sizes (20 KDa, 30 KDa, 50 Kda and 100 Kda). The decrease of the activity occurs in the first moments of the inversion reaction, and stabilizes only after 6 hours of experiment. It relates this reduction of the activity to the

polymeric matrix of the membranes, because depending on the size of the membrane, an enzyme insertion may occur in this matrix, causing some type of structural stress, conformational or steric interferences, directly affecting the interaction between enzyme and substrate.

The reason for this drop in activity, according to Vitolo (2015), is that there may be electrostatic interaction between ionized groups of the enzyme and the polymer matrix, causing the fructosyl group to become positively charged. This positive group can be partially neutralized by the negative charges present in the matrix, reducing its affinity with water, and as a consequence making the fructose remain longer in the catalytic site of the invertase, and this reducing the volume of the reacting substrate, explaining the decrease of the enzyme activity.

5.7 ENZYME CONCENTRATION ASSAY BY LYOPHILIZATION

Table 4 presents the results of total enzyme activity in different fractions before and after lyophilization process for enzyme concentration.

Table 4 - Invertase activity before and after lyophilization step.

Fractions	**U total before lyophilization**	**U total after lyophilization**
Supernatant 1	15316.26	19713.74
Supernatant 2	3519.14	1640.66
Autolysate 1	12778.74	10627.65
Autolysate 2	2705.23	2335.56
Total	**34319.37**	**34317.60**

From the results of Table 4 it is possible to verify that the total invertase activity remained practically the same before and after the lyophilization process. Thus, it can be concluded that, unlike the ultrafiltration process, invertase lyophilization was effective in concentrating the enzyme and at the same time maintaining its biological activity preserved. The lyophilization process is widely used in the pharmaceutical and food industry ensuring stability, safety and ease of storage and transportation of the final product. In the context of this work, it was presented as an optimal strategy for invertase concentration after the rapid autolysis process.

In addition to the invertase activity, the total protein content was analyzed in each fraction by the Kjeldahl method, thus making it possible to evaluate the specific activity of the invertase as well as its degree of purification (Table 5).

Table 5 - Invertase specific activity in lyophilized fractions.

Fractions	Total mass (lyophilized) (g)	Total protein (g)	% of protein	Specific activity (U/mg protein)
Supernatant 1	19.48	1.61	8.25	12.27
Supernatant 2	4.43	0.47	10.60	3.49
Autolysate 1	10.91	5.13	47.08	2.07
Autolysate 2	0.77	0.41	53.59	5.67

It was observed that Supernatant 1 presented the highest value of specific activity and Autolysate 1 had the lowest value. The specific activity is directly related to the purity of an enzyme and represents the ratio of unit of enzyme activity per milligram of protein. The process of autolysis is responsible for solubilizing part of the intracellular content of the yeast, releasing to the extracellular medium mainly RNA, vitamins and proteins among others. Thus, the decrease in the specific activity in the fraction of the Autolysate 1 can be explained by the solubilization of proteins, which ends up reducing the degree of purity of the enzyme. The results presented in Table 5 help to better understand the influence of the rapid autolysis process on invertase production. In this work, no invertase purification step was performed, however, this study may be carried out in future works.

5.8 IMMOBILIZED ENZYME RESULTS

The ability of the alginate beads to retain the invertase molecules was assessed by the invertase activity before and after the immobilization procedure. Enzyme molecules that left the beads were therefore detected in the supernatant after dripping, $CaCl_2$ solution (wash 1 and 2). Figure 9 shows the beads formed after immobilization and table 6 shows the enzyme activity observed in the fractions obtained during the immobilization procedure.

Figure 9 - Visual aspect of spheres obtained after enzyme immobilization.

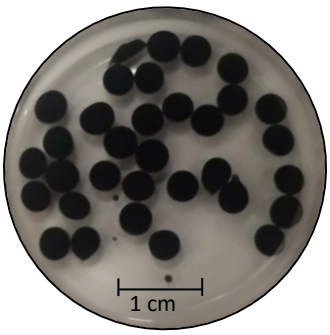


Table 6 - Enzyme activity during the immobilization of invertase in sodium alginate.

Samples	**U total**	**% of losses**
Enzyme before immobilization	1155.45	-
Wash 1	337.25	29.19
Wash 2	32.30	2.80
Total losses	**369.55**	**31.98**

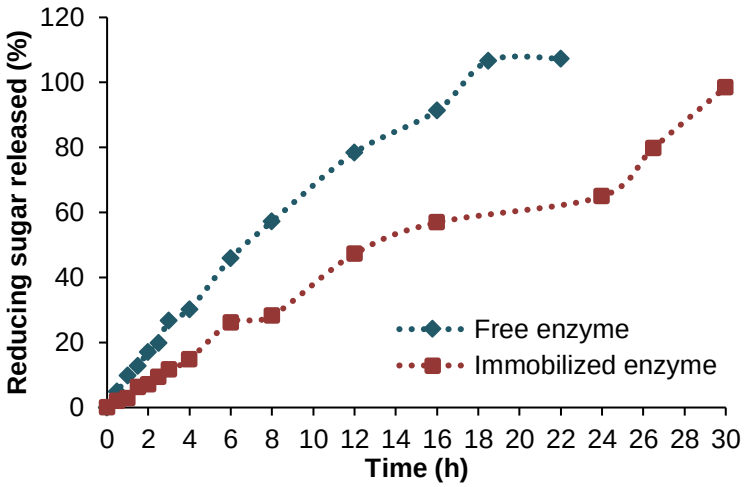
As observed in Table 6, there was a loss of 31.98% of the total activity of the enzyme that had been immobilized. Many factors influence the ability of the alginate beads to retain the enzyme such as the concentration of the alginate solution and the size of the beads. Arruda and Vitolo (1999) concluded that the origin of alginic acid and pH during immobilization have a direct influence, with losses of 34 to 50% in their trials. Buzato, Broggi and Celligoi (2000) in a study of immobilization of *S. crevisiae* invertase in calcium alginate and copper found that the immobilization of the enzyme in calcium alginate is loose, with less retention capacity, and that the immobilization of copper alginate is firm. However, although copper has a higher enzyme retention capacity, the manner in which it has been established has caused inactivation of the enzyme.

The loss of activity observed in this work corresponds to what is found in the literature and is even lower than some studies found, such as Ro and Kim (1991), which verified losses greater than 80% when invertase was encapsulated directly in calcium alginate. The smallest enzyme loss during the immobilization in the present work can be explained by the addition of activated charcoal, which may have positively influenced the retention of the enzyme within the beads. It is noteworthy that the losses can be reduced by reusing the same $CaCl_2$ solution for the realization of immobilization cycles. In this way, it is expected to reduce losses in subsequent immobilization using the same solution.

5.9 EVALUATION OF SUCROSE HYDROLYSIS BY FREE AND IMMOBILIZED ENZYME

Figure 10 shows the percentage of reducing sugar released in the medium over time from the hydrolysis of a 60% sucrose solution at 55 °C using free and immobilized enzyme.

Figure 10 - Hydrolysis kinetics of sucrose solution 60% at 55 °C, pH 5 using a total of 1115.45 U of free enzyme and 785.90 U of immobilized enzyme in alginate beads.



The total free and immobilized enzyme activity employed in the hydrolysis was 1115.45 and 785.90 U respectively. This difference in activity corresponds to loss of activity during the alginate immobilization process. As observed in Figure 10, it was possible to achieve 100% hydrolysis of the sucrose in a period of 17h and 30h for the reaction with free enzyme and immobilized respectively. The time difference for the total hydrolysis of the substrate is mainly due to the difference in the amount of total U used in each experiment and it is not possible to compare the two hydrolyses. However, future studies will be conducted in order to better study and compare the performance of the free and immobilized enzyme.

6 CONCLUSION

From the results found in this work it was possible to conclude that spent brewer's yeast presents great potential for obtaining invertase and other by-products from the ultra-fast autolysis process developed here. Microfiltration and ultrafiltration

procedures are not feasible to concentrate invertase. For this purpose, lyophilization presents excellent results and can be considered a method of choice. The immobilization in alginate beads with subsequent application presented results that justify the developed process as well as allow to open a range of applications and add value to spent brewer's yeast. It is expected for future studies to perform an optimization of the immobilization process as well as the application to obtain invert sugar using enzyme reactor. Finally, we highlight that the main advantage of this approach is the use of residual yeast as the source of the enzyme, i.e., it is not necessary to cultivate the microorganism to extract the enzyme. This strategy eliminates most of the costs associated with cell growth since the yeast used would be discarded or sold at a low cost. Better use of yeast residue from fermentation processes can open up a range of applications and add value to this product, making the industrial sectors more profitable.

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CHAPTER 6 DESIGN AND ECONOMIC ANALYSIS OF A BIOPROCESS PLANT FOR SPENT BREWER'S YEAST FRACTIONATION INTO HIGH ADDED VALUE BIOMOLECULES

ABSTRACT

In the previous chapters of this thesis, it was possible to study and identify approaches for yeast fractionation in different by-products. Thus, this chapter aims to aggregate all the produced knowledge to design and simulate a Spent Brewer's Yeast Processing Plant. The programs Intelligen SuperPro Designer v8.5, AutoCAD® 2018 and Office 2016 were used to design and simulate the plant as well as to perform an economic analysis. Different biotechnological downstream routes were integrated in a combined manner in order to aggregate scientific, technological and economic knowledge to manufacture products that meet a social or technological need, considering environmental and safety aspects in general. The results from economic analysis showed that the Total Capital Investment for the plant is estimated at R$ 46,732,000. In addition, the profitability analysis showed that the investment was viable with Gross Margin of 67.72%, Return On Investment of 88.78%, Payback time of 1.13 years and Net Present Value of R$ 268,593,474 at 7% interest rate. Finally, the sensitivity analysis in different economic scenarios showed that the Selling Price was the most sensitive economic variable.

Keywords: Bioprocess integration. Plant design and simulation. Economic analysis, Spent brewer's yeast. High added value products.

1 INTRODUCTION

Beer is one of the most widely consumed alcoholic drinks in the world and the third most popular drink overall after water and tea (NELSON, 2015). The beer market today is characterized by the search for modern technologies to meet the growing demand of consumers allied to sustainable practices that increase profits. Several technological advances in the last 20 years have provided the brewing industry large savings by lowering generation of wastes during the process (DOS SANTOS MATHIAS et al., 2014). However, certain residues inherent to beverage production hardly have their quantities reduced such as brewer spent grains, spent brewer's yeast and trub. These side products present a high content of organic substances and, therefore, a wide variety of potential applications in feed, food and industrial biotechnology (PODPORA et al., 2015). This dilemma situation is precisely one of the major challenges facing the brewery industry today: finding new ways to better reuse these materials and generate valuable by-products from them. Developing mechanisms and strategies to solve this problem is important because it leads to production optimization by increasing yields and reducing losses.

In the previous chapters of this thesis, it was possible to study and identify approaches for yeast fractionation in different by-products, exploring even the potential of using different residues that, although generated in different stages of the brewing process, are closely linked to produce new products. This is the case of the ribonuclease (enzyme), present in malt rootlets, and the RNA (substrate) present in high quantity in spent brewer's yeast. Through the experimental results, it was also possible to determine the best conditions of the process variables such as temperature, time, pH and concentration of substances that optimize the process steps. Thus, this chapter aims to aggregate all the produced knowledge to design and simulate a new industry called Spent Brewer's Yeast Processing Plant. It is believed that the basis for achieving success in this complex bioprocess engineering problem involves a careful understanding of all elements involved in the process and how they are related, as well as the conditions that optimize its production and scale-up.

2 OBJECTIVES

This work aims to design and simulate a bioprocess plant for spent brewer's yeast fractionation into several biomolecules: invertase, yeast extract rich in proteins and 5'-ribonucleotides. As specific objectives, this work aims to address and discuss aspects such the market analysis and a brief technological prospection of spent brewer's yeast. Other objectives include to plan and determine the annual production capacity, the process flowchart, the process mass balance, the architectural design of the project plant, the biosafety aspects, the human resources required and, finally, to perform an economic investment analysis.

3 MATERIAL AND METHODS

3.1 BIOPROCESS PLANT DESIGN AND SIMULATION

The programs AutoCAD® 2018 and Intelligen SuperPro Designer v8.5 were used to design and simulate the plant as well as to perform an economic analysis. For the conceptual project, a market analysis and a brief technological prospecting were carried out on search engines such as Google, INPI and Espacenet in order to verify the state of the art as well as the potential market that the proposed technology is inserted in. From the results of this study, the plant's installation site, the annual production volume and suppliers were defined. The main raw material was defined as the spent brewer's yeast from two breweries: Malta Cervejaria (Assis - SP) and Casa de Conti (Cândido Mota - SP). The malt rootlet supplier was defined as the company Maltaria Agrária (Guarapuava - PR). The downstream routes were designed considering the optimized operating conditions (pH, temperature, time, concentration, etc.) described in the previous chapters of this thesis.

The production flowchart, the mass balances in each unit operation, the sizing of equipment and materials, the necessary human resources, the number of employees and salaries per function, the production schedule by cycles and finally the economic analysis were designed and simulated using the SuperPro Designer software.

3.2 ECONOMIC ANALYSIS

In this work, the fundamental economic parameters of an investment were analyzed following common approaches according to Couper (2003) in order to determine four major economic aspects of an investment. These are the Total Capital Investment, the Annual Operating Cost, the Annual Income, and the Annual Profits. The Brazilian Real (BRL) was used as the reference currency.

3.2.1 Total Capital Investment

The Total Capital Investment (TCI) refers to the fixed costs that are associated with a process and was calculated as the sum of Direct Fixed Capital (DFC), Working Capital and Startup Cost over all sections of the plant (Eq.1).

$$TCI = DFC + Working\ Capital + Startup\ Cost \quad (1)$$

The DFC refers to the fixed assets of an investment, such as plant and equipment. It was estimated at the process section level as the sum of direct, indirect and miscellaneous costs that are associated with the plant's capital investment. In this work, the following parameter were considered in the Total Plant Direct Costs (TPDC): Total Equipment Purchase Cost (PC), Installation Cost, Process Piping Cost, Instrumentation Cost, Insulation Cost, Buildings Cost, Yard Improvement and Auxiliary Facilities Cost. As Total Plant Indirect Costs (TPIC), Engineering and Construction Cost were considered. Furthermore, additional costs such as the Contractor's Fee and Contingency were considered in Other Costs (OC). For this preliminary economic analysis, these costs were estimated by multiplying PC by a suitable factor according to Couper (2003). The following equations (Eq. 2 to 21) were used to calculate each parameter.

$$PC = Listed\ Equipment\ Purchase\ Cost + Unlisted\ Equipment\ Purchase\ Cost \quad (2)$$

$$Unlisted\ Equipment\ Purchase\ Cost = 0.20 * PC \quad (3)$$

$$Piping\ Cost\ (A) = 0.35 * PC \quad (4)$$

$$Instrumentation\ Cost\ (B) = 0.40 * PC \quad (5)$$

$$Insulation\ Cost\ (C) = 0.03 * PC \quad (6)$$

$$Electrical\ Facilities\ Cost\ (D) = 0.10 * PC \quad (7)$$

$$Building\ Cost\ (E) = 0.45 * PC \quad (8)$$

$$Yard\ Improvments\ Cost\ (F) = 0.15 * PC \quad (9)$$

$$Auxiliary\ Facilities\ Cost\ (G) = 0.40 * PC \quad (10)$$

$$Installation\ = Instalation\ of\ Listed\ Equip. + Installation\ of\ Unlisted\ Equip. \quad (11)$$

$$Unlisted\ Equip. Installation\ Cost = 0.5 * Unlisted\ Equip. Purchase\ Cost \quad (12)$$

$$TPDC = PC + Installation + A + B + C + D + E + F + G. \quad (13)$$

$$Engineering\ Cost\ (H) = 0.25 * DC \quad (14)$$

$$Construction\ Cost\ (I) = 0.35 * DC \quad (15)$$

$$TPIC = H + I \quad (16)$$

$$Total\ Plant\ Cost\ (TPC) = TPDC + TPIC \quad (17)$$

$$Contractors' Fee\ (J) = 0.05 * (TPC) \quad (18)$$

$$Contingency\ (K)\ = 0.10 * (TPC) \quad (19)$$

$$Other\ Cost\ (OC) = J + K \quad (20)$$

$$DFC = TPC + OC \quad (21)$$

The PC corresponds to the vendor's selling price of major equipment and was estimated through direct quotation from supplier companies in 2020. The more speculative a process is, the more likely it is that key elements could be overlooked during the project's early stages. Thus, the Unlisted Equipment Purchase Cost refers to overlooked equipment and its estimate is important because makes the economic analysis more precise. The Process Piping Cost incorporates the cost of process fluid piping that connects the equipment, as well as connections to the main utility headers and vents. Included are valves, piping supports, insulation, and other items associated with equipment piping. The Instrumentation Cost includes the costs of transmitters and controllers with all required wiring and tubing for installation, field and control room terminal panels, alarms, on-stream analyzers, control computers and control room display graphics. The Insulation Cost includes the cost of insulation and painting. The Electrical Facilities Cost includes battery limits substations and transmission lines, motor switch gear and control centers, emergency power supplies, wiring and conduit, bus bars, and area lighting. The Buildings Cost includes the cost of process towers,

concrete slabs, stairways, control rooms and other battery limits buildings (e.g., change rooms, kitchen, furnished offices, warehouses, etc.). It also incorporates the costs for non-electric building services as well as for a variety of safety-related items. The Yard Improvement Cost refers to the costs of roads, fences, fire hydrants, parking spaces, and others. The Auxiliary Facilities Cost includes the cost of satellite process-oriented service facilities that are vital to the proper operation of the battery limits plant. The Installation Cost refers to the in-place erection of equipment at the new plant site and includes the cost of foundations, slabs, supports, and local equipment services. The Engineering Cost includes the preparation of design books that document the whole process (e.g., the design of equipment, specification sheets for equipment, instruments, auxiliaries, the design of control logic and computer software, and the preparation of drawings) and other engineering-related costs. The Construction Cost includes the costs associated with the organization of the total construction effort. They do not include the cost of construction labor. This was already incorporated in direct cost items that involve construction. The Contractor's Fee refers to the contractor's profit. Finally, the Contingency cost attempts to compensate for missing elements and to account for unexpected problems during construction, such as strikes, delays, and unusually high price fluctuations. Based on the above definitions, the total DFC of the investment was calculated as the sum of TPC and OC (Eq. 21).

The Working Capital represents tied-up funds required to operate the business and includes the investment in raw materials and consumables. In this work, it was estimated as the sum of major operational costs, the costs for labor, raw materials and utilities, covered for 30 days operating period. The cost of each item was calculated by multiplying the specified number of days (30) by the corresponding daily cost of that item.

The Startup Cost includes pre-opening, one-time expenditures incurred to prepare a new plant for operation. In this work, this cost was estimated as 5% of DFC.

3.2.2 Annual Operating Cost

The Annual Operating Cost (AOC) of the Spent Brewer’s Yeast Processing Plant was estimated as the sum of the following cost items: Raw Materials, Consumables, Labor-Dependent, Utilities, Facility-Dependent and Transportation. They were estimated according to Couper (2003).

The Raw Materials Cost correspond the total cost of all bulk materials (pure components and stock mixtures) and discrete entities that were utilized as raw materials in the process. The annual cost of each material was calculated by multiplying the corresponding unit cost (i.e., purchasing price) by the corresponding annual amount that was utilized in the process.

The Consumables Cost includes the costs of periodically replaced materials, such chromatography resins and other materials which may be required for the operation of process equipment. The annual cost of a consumable type utilized by an equipment unit was calculated by multiplying the corresponding Unit Cost (expressed as purchase cost per consumable amount) by the corresponding Annual Amount consumed (Eq. 22).

$$Annual\ Cost = Unit\ Cost * Annual\ Amount \quad (22)$$

The Annual Amount consumed was calculated by multiplying the consumable amount per use by the Annual Number of Replacements (Eq. 23).

$$Annual\ Amount = Amount\ Per\ Use * Annual\ Replacement \quad (23)$$

The consumable Amount Per Use was calculated by multiplying the Consumption Rate (expressed as consumable amount per consumption basis) by the Consumption Basis (number of equipment unit or equipment size) (Eq. 24).

$$Amount\ Per\ Use = Consumption\ Rate * Consumption\ Basis \quad (24)$$

Finally, the Annual Number of Replacements was calculated by multiplying the consumable life (or Replacement Frequency) expressed per operating basis (operating cycles or hours) by the equipment's Annual Operating Basis (Eq. 25).

$$Annual\ Replacement = Repacement\ Frequency * Annual\ Operating\ Basis \quad (25)$$

The Labor-Dependent Cost includes all labor-dependent operating costs and was calculated for each section as the sum of the labor costs of the different labor types (i.e., operator, supervisor) that may be required for that section. The Utilities Cost was estimated by the sum of the total cost of heating/cooling utilities and power utilized

in every process operation. The mass flow rate of a heat transfer agent and the power consumption of a power type was calculated as part of the simulation by the program SuperPro Designer.

The Facility-Dependent Cost accounts for additional costs related to the use of a facility. This is typically calculated as the sum of the costs associated with equipment maintenance, depreciation of the fixed capital cost, and miscellaneous costs such as insurance, local (property) taxes and possibly other overhead-type of factory expenses. In this work the estimate of this parameter was simplified to 6% of the DFC.

The Transportation Cost accounts for the cost of long-distance transportation of raw materials and products by truck. The annual Transportation Cost was estimated based on the number of shipments per year, the fixed cost, the quantity per shipment and the shipping distance.

Finally, the Unit Production Cost (R$/kg) was calculated by dividing the AOC specific for each product by its annual mass flow rate basis.

3.2.3 Annual Income

The Annual Income (or Total Annual Revenues) of the plant was calculated as the sum of the revenues of all streams denoted as revenue, i.e. 5'-CMP/5'-UMP, 5'-GMP/5'-AMP, Invertase and Yeast Extract.

3.2.4 Annual Profits

A profitability analysis was performed in order to determine the Annual Gross Profit, the Annual Net Profit of the investment, as well as key economic indicators like the Gross Margin, the Return On Investment, the Payback Time, the Net Present Value and the Internal Rate Of Return. All economic parameters were determined according to Couper (2003).

The Annual Gross Profit of the designed plant was calculated by subtracting the AOC and Depreciation from the Total Annual Revenues (Eq. 26).

$$Annual\ Gross\ Profit = Total\ Annual\ Revenues - AOC - Depreciation \quad (26)$$

Depreciation is an income tax deduction that represents a fixed capital loss which is mostly due to equipment wear out and obsolescence. It may be considered as a time-dependent operating cost spread over a predefined depreciation period. It was defined following the criteria established by the Brazilian government, through the Federal Revenue Service, art. 305 of RIR/99. There are three legal methods for depreciation in Brazil. The chosen one was the Sum-Of-The-Years-Digit method because it enables to assign decreasing depreciation rates, which guarantees the payment of lower income taxes in the early years of the project (Couper, 2003). Others legal methods are the Straight-Line Method and the Units Produced Method. The Total Plant Cost were considered for depreciation over ten years period.

According to the Brazilian tax system, companies are subject to Annual Income Taxes of 15% with an additional of 10% on the portion of the profit that exceeds R$ 20,000.00/month. In addition to this tax, they are subject to other federal taxes such CSLL, PIS and COFINS described in the Table 1.

Table 1 - Brazilian taxes with their respective aliquots.

Name	Tax	Type	Aliquots
“Imposto de Renda Pessoa Jurídica”	IRPJ	Federal	15% on Gross Profit plus 10% on the portion of the profit that exceeds R$ 20,000.00/month.
“Contribuição Social sobre o Lucro Líquido”	CSLL	Federal	9% on Gross Profits
“Programas de Integração Social	PIS	Federal	2,32% on Total Revenues
Contribuição para Financiamento da Seguridade Social”	COFINS	Federal	7.6% on Total Revenues

Source: Law n° 9.249 (IRPJ), Law n° 7.689 (CSLL), Lei n° 13.097 (PIS/COFINS).

In this work, the total tax burden was defined as 43% of the Annual Gross Profit for simplification purposes. Thus, the Annual Net Profit, by default, was calculated as the Annual Gross Profit minus the Annual Income Taxes plus the Annual Depreciation (Eq. 27).

$$Net\ Profit = Gross\ Profit - Income\ Taxes + Depreciation \qquad (27)$$

The Gross Margin was calculated dividing the Annual Gross Profit by the Total Annual Revenues (Eq. 28). This indicator is a measure of profit that directly tells about what percentage of the Annual Revenues is Gross Profit.

$$Gross\ Margin = \frac{Annual\ Gross\ Profit}{Total\ Annual\ Revenues} * 100 \quad (28)$$

The Return On Investment (ROI) was calculated by dividing the Annual Net Profit by the TCI (Eq. 29). This indicator is used to evaluate the viability of an investment or to compare the profitability of a number of different investments.

$$ROI = \frac{Annual\ Net\ Profit}{TCI} * 100 \quad (29)$$

The Payback Time is a measure of the time needed for the TCI to be exactly balanced by the cumulative Net Profits (Couper, 2003). It was calculated by dividing the TCI by the Annual Net Profit (Eq. 30). The shorter the Payback Time, the more attractive the project appears to be.

$$Payback\ Time = \frac{TCI}{Annual\ Net\ Profit} \quad (30)$$

A Cash Flow analysis was also performed to determine the Net Profits and Net Cash Flow for each year over the project's lifetime. In this work, the project lifetime, the construction period and the startup period were designed to be 15 years, 24 months and 4 months, respectively. Besides, the distribution of the project's DFC was designed into the first two years of the project according to the respective percentages per year 40% and 60%. For each year before the start of operation (i.e., during construction and startup), the Net Cash Flow consisted of the amount of money borrowed minus capital expenses for that year. For each operating year during the expected lifetime of the project, the Net Cash Flow consisted of the Net Profit.

As a profitability measure, the Net Present Value (NPV) was calculated for three different interest rates, 7% (low), 9% (medium) and 11% (high) according to Eq. 31.

$$NPV = \sum_{k=1}^{N} \frac{NCF_k}{(1+i)^k} \quad (31)$$

where “i” is the interest rate, “NCF_K” is the Net Cash Flow in year k, and “N” is the project lifetime, in number of years. The interest rate is a percentage charged on the total amount that a bank, for example, pays its savers for keeping money in an account. This indicator was used to evaluate the viability of the investment. It represents the total value of future Net Cash Flows during the life time of a project discounted to reflect the time value of money at the beginning of a project (i.e., at time zero). If an investment does not have a positive NPV, or if there are other opportunities with a higher NPV, then the investment should not be undertaken (Couper, 2003).

Finally, the Internal Rate of Return (IRR), which is also known as Discounted Cash Rate Of Return (DCRR) was calculated based on cash flows before and after income taxes. The Cash Flow after income taxes corresponded to the Net Cash Flow. The Cash Flow before income taxes was calculated as the Net Cash Flow plus the income taxes. The method is analogous to the NPV method, but instead of asking what is the NPV for a prescribed interest rate, it was sought a value of the interest rate which makes the NPV of all the cash flows just equal to zero (Eq. 32)

$$0 = NPV = \sum_{k=1}^{N} \frac{NCF_k}{(1+i)^k} \tag{32}$$

3.2.5 Sensitivity Analysis

In view of possible fluctuations of the consumer and supplier market, such as the unit selling price and the cost of inputs, the Sensitivity Analysis is of great relevance for evaluate an investment project. In the Sensitivity Analysis, different variables were tested to understand the individual effect that each of them produces at the end of the process. For this purpose, the Strauss Graph Method was used (Couper, 2003), varying the values of unit Selling Price, TCI and Raw Material Cost to assess the interference in the values obtained from NPV. Finally, the Break-Even Point was calculated according to Couper (2003). The Break-Even Point determines the amount of sales needed to achieve a Net Income of zero. This number shows the point when the company's revenue equals total fixed costs plus variable costs.

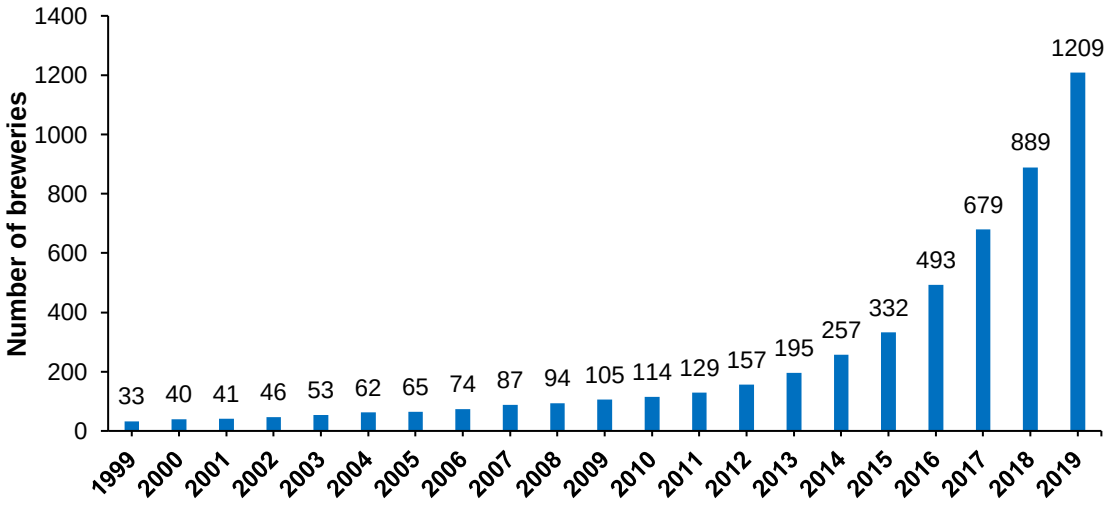
4 RESULTS AND DISCUSSION

4.1 MARKET ANALYSIS

4.1.1 The Brazilian brewing market and the generation of spent brewer's yeast

According to the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), a federal agency that regulates and inspects beverage producing establishments in the country, Brazil reached 1,209 registered breweries in 2019 distributed among 26 federation units (MAPA, 2020). About 320 new factories started their production last year, which means almost one new brewery per day and 36% growth compared to 2018 (Figure 1). Through its multiplier effect, the brewing sector generates about 2.7 million jobs; for each job directly created in a beer factory installed in Brazil, 52 new indirect jobs are created. Thus, this sector accounts for 2% of the Brazilian GDP, with revenues of more than R$ 100 billion per year and corresponds to about 15% of the manufacturing industry. The data are in the 2019 Beer Yearbook, which brings statistics from the Brazilian brewing sector (MAPA, 2020).

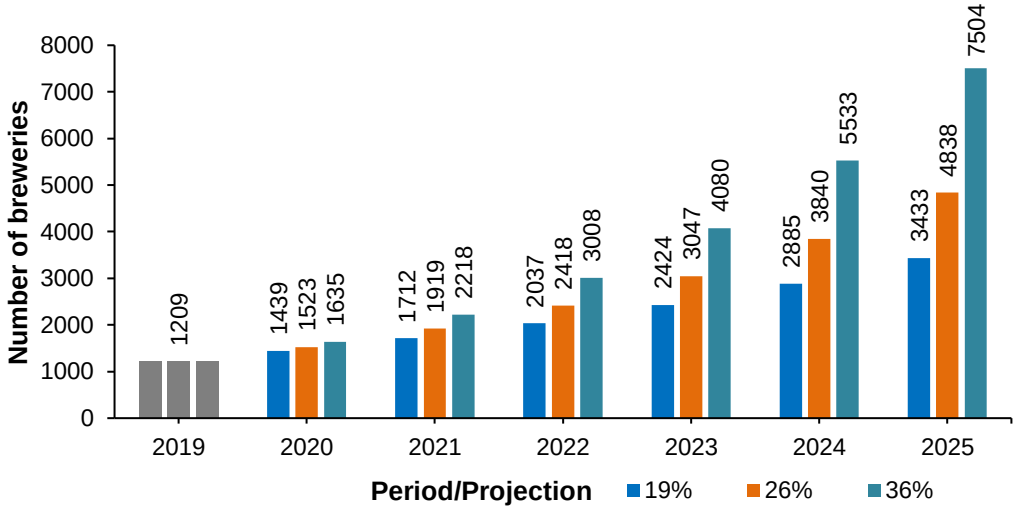
Figure 1 - Number of breweries registered in the Brazilian Ministry of Agriculture, Livestock and Supply in the last 20 years (MAPA, 2020).



As seen in Figure 1, the large growth in the number of brewery registrations has been sustained for decades and there is no slowdown in this movement. In this sense, growth can be projected for the coming years based on the last periods. The average growth rate in the last 20, 10 and 5 years corresponds to 19%, 26% and 36%,

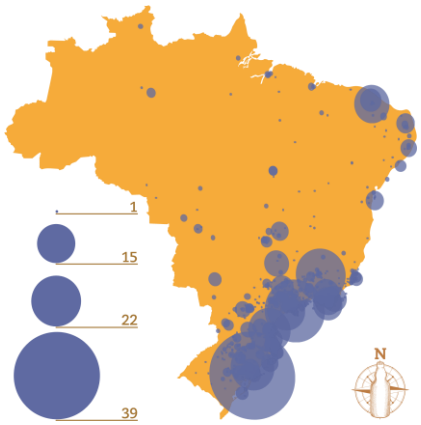
respectively. Thus, it is possible to make projections for the next 5 years for each of these growth rates (Figure 2) (MAPA, 2020).

Figure 2 - Brewery growth projection for the 2020-2025 period (MAPA, 2020).



In the most moderate scenario, if the rate of growth of 19% is maintained until 2025, the country would reach the mark of 3,433 breweries. As observed, intermediate growth comes close to 5,000 breweries and the most accelerated growth at 7,504. Another important point to be considered in the brewing market is its geographic distribution. Figure 3 shows the distribution map of breweries in Brazil in order to highlight the places with a high concentration of beer producers (MAPA, 2020).

Figure 3 - Quantity and distribution map of breweries in Brazil (MAPA, 2020).



As noted, the distribution is heterogeneous with the south and southeast regions showing a strong beer tradition. A punctual discreet number of breweries is observed in other areas such as the Midwest and Northeast. Knowing this distribution profile is important because the proposed Spent Yeast Processing Plant, objective of this work,

must be located in regions with a high concentration of beer industries in order to reduce the costs of transporting raw materials.

Finally, it is worth mentioning that the average beer production in Brazil is around 14 billion liters per year. If all this development and brewing sector growth is cause for celebrations in the economy field; on the other hand, the wastes resulting from this process is something of concern as it can be a source for environment contamination (LOUSADA JUNIOR, 2005). The manufacture of beer inevitably involves generation of various residues and by-products. The most common by-products are spent grains, spent hops and surplus yeast. According to Aliyu and Bala (2011) and Ferreira et al. (2010), for every 100 liters of beer produced there is the generation of 20 kg of spent grains and around 0.17 to 0.23 kg of spent yeast cell on a dry basis. This represents around 28 thousand tons of spent brewer's yeast produced annually in Brazil if considering the total beer production of 14 billion liters per year. As seen, these brewery by-products are available in large quantities, but their use is still limited, being basically sold to local dairy farmers to be used as cattle feed, or simply as a land fill. They represent however large potential, in particular yeast, for the use in biotechnological processes, due to their complex compositions (MUSSATTO, 2009).

4.1.2 Economic development of the plant's installation site

The chosen region for the spent yeast processing plant installation was Assis-SP, located in the Paranapanema Valley, in the central-west region of the state of São Paulo. In this region there is the Intermunicipal Consortium of Paranapanema Valley (CIVAP), which covers 24 municipalities including Assis, has a population of approximately 376,162 inhabitants, and occupies a territorial extension of 11,895,025 km^2, including the hydrographic basins of the Pari and Capivari River (CIVAP, 2018). The strategic geographical position of the city allows the integration of the southeast, central-west and south of the country, establishing a road-rail junction for the flow of agricultural, industrial and regional commercial production, mainly through the Raposo Tavares Highway (SP- 270) and the railroads administered by Ferroban company.

Assis is the largest municipality that integrates CIVAP, both in population and in participation in the total GDP, accounting for 20.14% of all wealth produced in the region, in addition to 30.93% of the total jobs generated in the same area. Besides, the city shows an expressive growth in the industrial, commerce and services sector. The

analysis by industrial subsectors showed that the majority of formal employment is in the food industry (33.2%), followed by the beverage industry (21.1%) (CIVAP, 2018). Regarding the existence of local policies and actions to attract investment, 12 municipalities of CIVAP have an Industrial District, especially Assis, which has three districts. In addition, 10 municipalities including Assis have the General Law on Micro and Small Enterprises (Complementary Federal Law No. 123/2006, regulated in 2013), which provides differentiated and favored treatment to microenterprises, with SEBRAE responsible for assisting municipalities interested in implement that law.

Another strategic point for choosing this location is the presence of two breweries: Cervejaria Malta, located in Assis-SP with beer annual production capacity around 100 million liters, and Casa de Conti, located in Cândido Mota-SP with beer annual production capacity around 180 million liters (RIB, 2017). Based on total beer production, it is estimated that the two breweries can supply around 560 tons of spent brewer's yeast per year. Thus, the installation of the yeast processing plant in this site can create new business opportunities in addition to strengthening the productive chains in the region.

4.1.3 Brief technological prospecting of spent brewer's yeast

The technological prospecting of spent brewer's yeast was carried out through an online search for patents related to the theme in the following databases: National Institute of Industrial Property (INPI) and European Patent Office (Espacenet), which has the worldwide collection of submissions from more than 90 countries, including data from the World Intellectual Property Organization (WPO). The following keywords were used to prospect in these databases: "*spent*", "*surplus*", "*residue*", "*brewer's*", "*brewery*" and "*yeast*" in the international database and the respective words, in Portuguese, at the national bank. Several combinations of keywords were established and used to prospect patents that contain such words in the title/summary field.

Table 2 shows a large difference in the number of patents at the national level, in comparison with the international one. There is no patent in the Brazilian patent bank, INPI, that contains the words "*spent brewer's yeast*" and only one patent containing the words "*yeast residue*" and "*brewery*" in the title/summary field. This result shows how this field needs innovation and can be explored. In general, there is still a lack of patents involving spent yeast; the number is insignificant when compared

to the total number of patents related to the term "*beer*" found on Espacenet, 154506 patents.

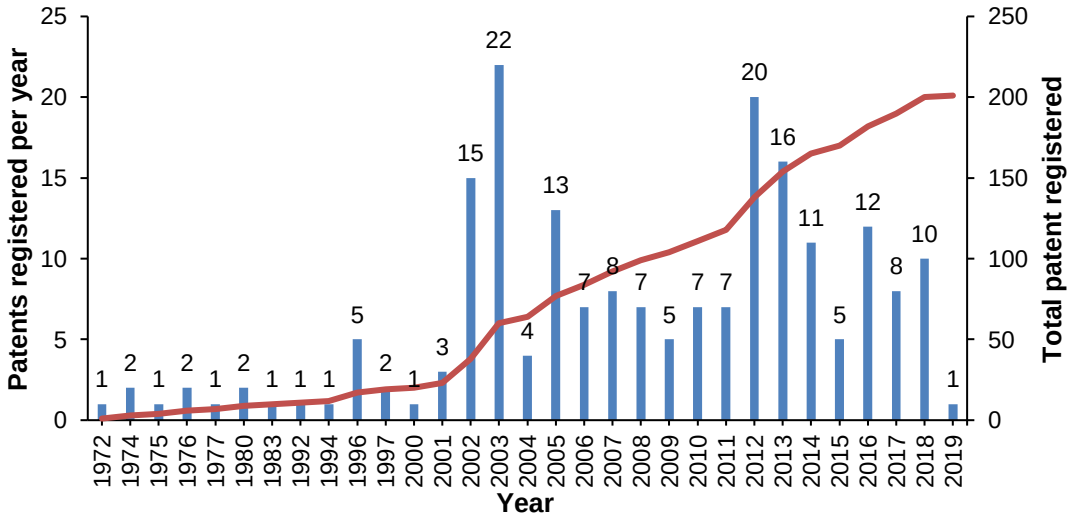
Table 2 - Number of patents containing the following keywords on INPI and Espacenet.

Keyword	INPI	Espacenet
"*spent yeast*"	6	653
"*spent brewer's yeast*"	0	201
"*spent yeast*" and "*brewery*"	0	94
"*surplus yeast*"	0	170
"*surplus brewer's yeast*"	0	18
"*surplus yeast*" and "*brewery*"	0	53
"yeast residue"	23	774
"*brewer's yeast residue*"	0	20
"*yeast residue*" and "*brewery*"	1	102

Source: Authors (2020).

Figure 4 shows the patents registered in Espacenet database containing the keywords "spent brewer's yeast" in the title/summary over the years.

Figure 4 - Patents registered in Espacenet database containing the keywords "spent brewer's yeast" in the title/summary. Source: Authors (2020).



Between 1972 and 2001, it can be seen that the international production was very small with an average of 0.79 patents per year. From 2001 on, there is an increase in the production of patents especially at two different periods, 2002-2003 and 2012-2013. In those same years, the United Nations Conference on Environment and Development took place, being called Rio +10 in 2002 and Rio +20 in 2012, in which the integration of the idea of social economic development were highlighted and delimited by the perspective of environmental sustainability. These conferences, an attempt by the UN to advance the discussions on global environmental problems, end up promoting discussion about the development of new technologies that take the

environment factor as an element to be considered in issues related to economic development. This fact may be related the increase in technological production in this period. Future trends are moving towards more and more companies and governments encouraging development based on three pillars: social, economic and environmental. Thus, new approaches on waste management, as proposed in this work, are justified in order to guarantee the integrity of the planet, nature and society over the generations.

4.1.4 Target market profile

The target market expected for the products from the Spent Yeast Processing Plant typically consists of food and pharmaceutical companies. For invertase, the main market may consist of industries that produce inverted sugar, among others in the food and pharmaceutical, chemical, beverage, candy industries, cookies, bakery and others. The main possible target companies include Dulcini, Raízen, Tereos, ProAgri, RCumin Alimentos Eireli and Cristalina.

For yeast extract and 5'-ribonucleotides, the main market may consist of food companies with applications such as natural flavor enhancer used to compose savory, condiments, meat products, and bakery products. Due to its high nutritional value, it can also be used in special food formulations and culture medium for the growth of microorganisms. Potential target industries include Pepsico, Nestlé, General Mills, Temabi, Milho de Ouro, Ki Mania Salgadinhos, Seniram, Coinmil Comércio e Indústria de Corn, Keleck and Tec-Snacks Produtos e Equipamentos Alimentícios Ltda.

For RNA, the main market may consist of start-ups that produce and manufacture products based on RNA interference (RNAi). RNAi can control crop-munching insects better than chemical sprays. This called RNAi technology interrupts target organism’s ability to produce key proteins and has important applications in several fields as species-specific insecticidal. Potential target companies include GreenLight Biosciences (US), Renaissance BioScience (CN), AgroRNA (KR), Alnylam Pharmaceuticals, Arbutus Biopharma (Tekmira), Arrowhead, Dicerna Pharmaceuticals, Mirna Therapeutics, Quark Pharmaceuticals, RXi Pharmaceuticals, Silence Therapeutics, Benitec Biopharma, miRagen Therapeutics, Sylentis, Gradalis, Sirnaomics, Silenseed.

4.1.5 Definition of annual production capacity

The production capacity refers to the maximum number of production units that an organization is able to make with its available resources (HO and FANG, 2013). Thus, the estimation of production capacity depends on the market to be reached, the availability of raw materials and competition. The annual availability of spent yeast provided by the two breweries is around 560 tons per year. According to the results from the previous chapters of this thesis, it was possible to obtain a yield of yeast extract, RNA and invertase of 31%, 9% and 3.2% respectively from this raw material. Based on this estimation, Table 3 shows the annual production capacity estimate for the proposed bioprocess plant.

Table 3 - Annual production capacity estimate.

Products	**Annual capacity production***
Yeast extract	176 tons/year
5'-ribonucleotides	52 tons/year
Invertase (350U/g)	18.4 tons/year

*The spent brewer's yeast processing capacity is estimated at 560 tons per year.

An important point in planning production capacity is forecasting demand and sales. Without demand forecasting, the business takes the risk of operating in a market that potentially does not need its product. In 2018, The Global RNAi for Therapeutic market size was 990 million US$ and it is expected to reach 5,200 million US$ by the end of 2025, with an annual growth rate of 23.1% during 2019-2025 (MARKET WATCH, 2020). The global yeast and yeast extract market is projected to grow at 9.11% during the same forecast period (RESEARCH AND Markets, 2020). Once there is a growing trend in the market, it is estimated that there will be an increasing demand over the years and that the proposed production will be absorbed by the market.

4.2 SPENT YEAST PROCESSING PLANT DESIGN AND SIMULATION

4.2.1 Process Flow Chart

The qualitative flowchart and the combined flowchart containing the mass balance of the entire process are shown in Figures 5, 6 and Appendix A.

Figure 5 - Proposed yeast fractionation process flow chart.

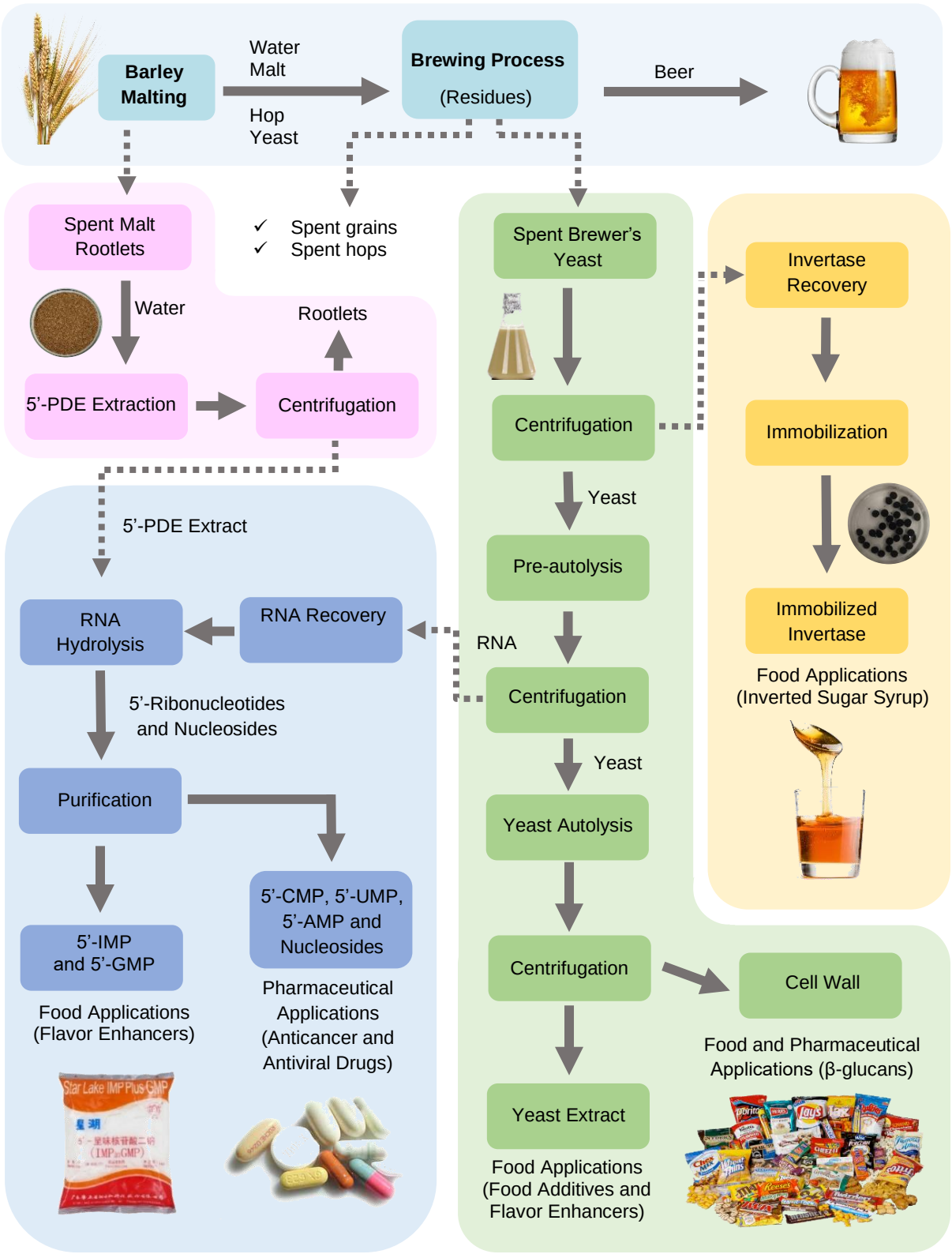
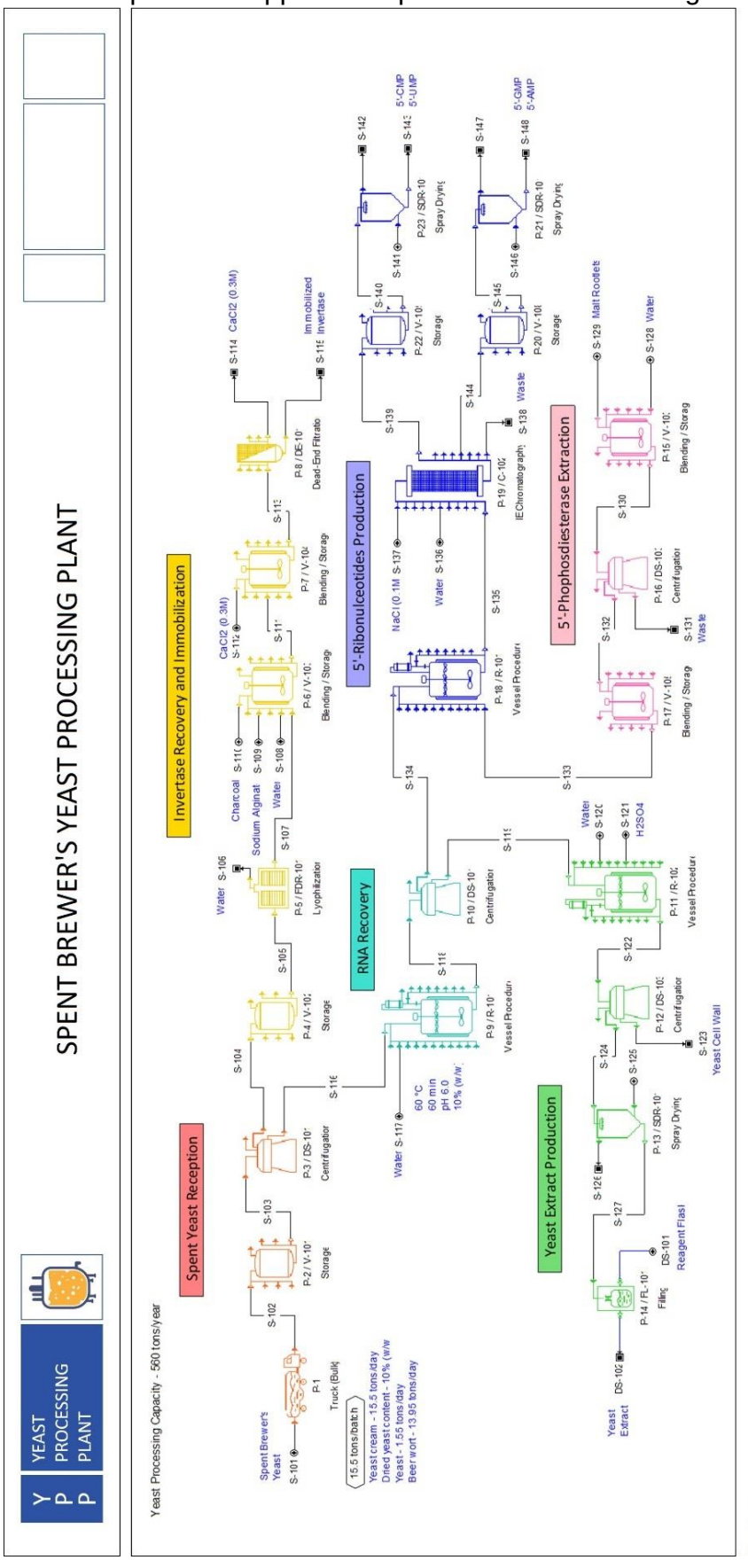


Figure 6 - Proposed yeast fractionation process flow containing the mass balance of the entire process. Appendix A presents the same image in high resolution.



Mass Balance

Time Ref: Batch	S 101	S 102	S 103	S 104	S 105	S 106	S 107	S 108	S 109	S 110	S 111	S 112	S 113	S 114	S 115	S 116	S 117	S 118	S 119	S 120	S 121	S 122	S 123	S 124	S 125	S 126	S 127	S 128	S 129	S 130	S 131	S 132	S 133	S 134	S 135	S 136	S 137	S 138	S 139	S 140	S 141	S 142	S 143	S 144	S 145	S 146	S 147	S 148	DS 101	DS 102
Type:	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	Emi.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	Rec.	R.Mat.	Rec.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.M.	R.Mat.	R.Mat.	R.Mat.	Emi.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	R.Mat.	Emi.	Rec.	R.Mat.	R.Mat.	R.Mat.	Emi.	Rec.	R.Mat.	Rec.
Total Flow (kg)	15500	15500	15500	7749	7749	7292	457	457	18	3	935	3738	4673	3743	930	7751	7756	15501	7751	7740	10	15502	3426	12076	67782	69339	519	1148	144	1292	417	875	875	7750	8625	26400	4924	35024	2438	2438	1933	14320	51	2487	2487	11926	14311	102	42	561
Temperature °C	25	25	25	27	27	12	12	25	25	25	23	25	25	25	25	27	25	60	62	25	25	55	30	30	25	70	70	25	25	25	20	25	25	25	25	62	69	25	25	25	25	25	70	70	25	25	25	25	70	70	25	54

Content (kg)

5'-AMP	60	60	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	60	8	0	0	8	7	1	0	0	1	0	0	0	0	0	0	52	52	0	0	0	0	0	0	0	0	52	52	0	52	0	1
5'-CMP	20	20	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	20	3	0	0	3	2	0	0	0	0	0	0	0	0	0	0	17	17	0	0	17	0	0	17	0	0	0	0	0	0	0	0	0
5'-GMP	31	31	31	0	0	0	0	0	0	0	0	0	0	0	0	31	0	31	4	0	0	4	4	0	0	0	0	0	0	0	0	0	0	26	26	0	0	0	0	0	0	0	0	26	26	0	0	26	0	0
5'-UMP	29	29	29	0	0	0	0	0	0	0	0	0	0	0	0	29	0	29	4	0	0	4	4	0	0	0	0	0	0	0	0	0	0	25	25	0	0	25	0	0	25	0	0	0	0	0	0	0	0	0
Ash	124	124	124	0	0	0	0	0	0	0	0	0	0	0	0	124	0	124	62	0	0	62	31	0	0	0	31	0	0	0	0	0	0	62	62	0	0	62	0	0	0	0	0	0	0	0	0	0	0	31
CaChloride	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carbohydrates	972	972	972	380	380	0	380	0	0	0	380	0	380	0	380	543	0	543	271	0	0	271	136	136	0	0	136	0	0	0	0	0	0	271	271	0	0	271	0	0	0	0	0	0	0	0	0	0	0	136
Carbon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fibers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Flask	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteins	922	922	922	54	54	0	54	0	0	0	54	0	54	0	54	868	0	868	679	0	0	679	339	339	0	0	339	0	49	49	35	14	14	189	203	0	0	203	0	0	0	0	0	0	0	0	0	0	42	42
Sodium Alginate	0	0	0	0	0	0	0	0	18	0	18	0	18	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sodium Chloride	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	288	260	7	7	0	0	7	21	21	0	21	0	0
Sodium Hydroxid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sulfuric Acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Water	13392	13392	13392	7315	7315	7292	23	457	0	0	480	3614	4093	3632	461	6077	7750	13827	6720	7740	0	14460	2892	11568	0	11556	12	1148	0	1148	287	861	861	7107	7968	26400	4637	34228	2389	2389	0	2387	2	2388	2388	0	2385	2	0	12

The main focus of the proposed flowcharts is on the downstream steps for recovery and purification of bioproducts of commercial interest. The flowchart of Figure 6 was divided into sections, which comprise the “Spent Yeast Reception”, “Invertase Recovery and Immobilization”, “RNA Recovery”, “Yeast Extract Production”, “5’-Phosphodiesterase Extraction” and “5’-Ribonucleotides Production”. The nomenclature of procedures and equipment in each section follows the structure ‘P’ ‘N’/‘E’-‘N’, which stands for ‘Procedure’ + ‘Number’/‘Equipment-Prefix’ + ‘Number’. In order to make best use of equipment resources that may be sitting idle, the process flowchart was optimized, when possible, to have more than one processing steps, that are part of the same batch or different batches, executed in the same hosting equipment. In these cases, procedures sharing equipment have the same ‘Equipment-Prefix’ + ‘Number’. Clearly, such reuse saves on extra equipment but at the same time imposes a constraint on the times that each of those procedures may be carried out since their engagement times cannot overlap.

The first step take place at the “Spent Yeast Reception” section, which corresponds to residual yeast coming from Cervejaria Malta and Casa de Conti. For the mass balance simulation, the spent brewer’s yeast was decomposed into yeast (10% w/w) and beer wort (90% w/w). Beer wort (rich in invertase) was composed of 96% water, 3.5% carbohydrates and 0.5% protein. The process begins with yeast arriving at the plant by road transport P-1 and being stored in the tank P-2/V-101 (capacity of 17,114 L) until the moment of use. The tank desired features basically consist of a cylinder with a large diameter in relation to height, straight stainless-steel side walls and an inclined roof. The fractionation starts with the centrifugation of the yeast cream in P-3/DS-101 (processing capacity of 4,500 L/h), in which the beer wort and the cells are separated. The liquid fraction (beer wort) is stored in the tank P-4/V-102 (capacity of 8,637 L) for invertase recovery and the solid fraction (yeast cells) goes to the bioreactor P-9/R-101 for RNA recovery.

The beer wort is lyophilized in P-5/FDR-101 (capacity 600 kg/cycle) for 72 hours in order to remove part of the water and promote invertase concentration. Then, the enzyme is immobilized in two stages in tanks P-6/V-103 and P-7/V-104 with a capacity of 1,424 L and 5,110 L respectively. In the first tank, sodium alginate, activated carbon and water are added to reach the respective final concentrations 2%, 0.3% and 50%. The system is stirred for 1h to homogenize the mixture and then transferred by dripping to the second tank, which contains $CaCl_2$ 0.3M solution (final concentration). The

alginate, when in contact with the divalent metallic cation, forms a gel with characteristics such as uniformity and sufficient matrix porosity to trap the enzyme inside but allowing the substrate and product to diffuse. After 30 minutes, the entire mixture pass through the P-8/DE-101 filter (flux of 250 L/m^2-h and filter area of 30 m^2) and the immobilized enzyme and the calcium chloride solution are separated. The calcium chloride can be reused in the next batch.

In the bioreactor P-9/R-101 (capacity of 17,342 L), water is added to resuspend the yeast to a final concentration of 10% (w/w). This device is supposed to be made up of a conical cylindrical stainless-steel tank with a thermal insulation layer, temperature and pH sensors, an agitator and a clean in place system (CIP), that allows easy sanitation of the equipment. In this step, the system is heated to 60 °C for 60 min with agitation of 30 rpm so that the RNA is released into the extracellular medium. The solution is centrifuged in P-10/DS-101 with a protocol similar to the process described in P-3/DS-101. Thus, the RNA-rich liquid fraction is obtained and goes to the bioreactor P-18/R-101 (capacity of 17,342 L) and the solid fraction composed of yeast cells goes to the bioreactor P-11/R-102 (capacity of 17,396 L) to produce the yeast extract.

Water is added in P-11/R-102 to resuspend the yeast to a concentration of 10% (w/w) and H_2SO_4 is added until it reaches 0.065% in order to perform an acid shock at the beginning of the autolysis. The process takes place at 60 °C for 12h under constant agitation (40 rpm) and then it is centrifuged in P-12/DS-103 with a protocol and equipment similar to the process P-3/DS-101. At the end of the centrifugation, the solid fraction (cell wall and debris) and the soluble fraction (the yeast extract) are obtained. With the aid of a spray dryer P-13/SDR-101 (capacity of 4,622 kg/h) the liquid portion is dried producing the yeast extract which goes for packaging in 1 kg plastic bottles, P-14/FL-101 process (capacity of 207 entities/h).

In another section, malt rootlets and water are mixed with proportion of 1:8 in the tank P-15/V-103 (capacity of 1,424 L), for 12h at 20 °C for extraction of the 5'-phosphodiesterase enzyme. After extraction, the mixture is centrifuged in P-16/DS-103 (capacity of 1,500 L/h) where the enzymatic extract (liquid portion) and the rootlets (solid portion) are obtained. The enzyme extract is stored in the tank P-17/V-105 (capacity of 1000 L) at 20 °C until the time of use in P-18/R-101. The hydrolysis of RNA for the production of 5'-ribonucleotides takes place in the bioreactor P-18/R-101 at 69 °C for 3h and with the addition of the 5'-phosphodiesterase extract at 10% (w/w) concentration.

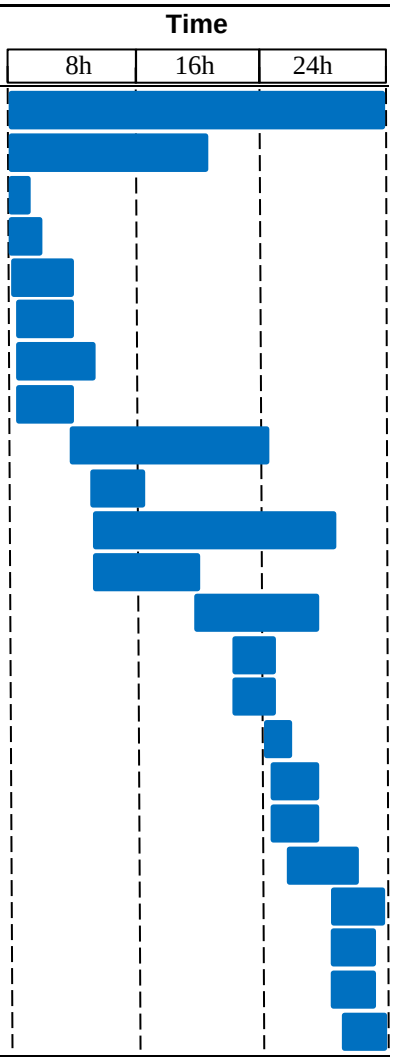
After the reaction, the solution proceeds to ion exchange chromatography for the separation of 5’-ribonucleotides fractions in P19/C-101. The column, with a bed volume of 600 L, is filled with strong anion exchange resin containing quaternary ammonium groups. Deionized water pH 7 (A) and 0.5 M NaCl solution (B) is used as mobile phase during the elution. The chromatographic run is performed at room temperature with a flow velocity of 100 cm/hr. The column is equilibrated for 10 minutes with solution (A), then the hydrolyzed is injected into the system in order to saturate the adsorptive capacity of the column. Thereafter, the unbound molecules are removed from the system in the wash step, in which just water is used. Finally, during the elution step (4 bed-volume), the ionic force of mobile phase is increased linearly from 0 to 100% of the solution (B), aiming to separate 5’-ribonucleotides in two fractions according to the force with they are adsorbed on the resin. The elution fractions are collected in tanks P-20/V-108 and P-22/V-109, both with a capacity of 3,000 L. In tank P-20/V-108, the first elution fraction, rich in 5'-AMP and 5'-GMP, is stored. In the tank P-22/V-109, the fraction rich in 5'-CMP and 5'-UMP is stored. Finally, the fractions are dried in a spray dryer (P-21/SDR-101 and P-23/SDR-01) with a process and equipment similar to P-13/SDR-101.

The labeling of chemical products in Brazil is governed by NBR 14725, prepared by ABNT. Thus, all the company's products must be packaged and labeled according to this standard, which provides information on safety, health and the environment in relation to chemicals. The legislation requires that a series of information be informed, on the label, such as the batch number, product validity, product name, chemical composition, warning word, precautionary and danger phrases, company name, address, among others. After labeling according to legal standards, the products (immobilized invertase, yeast extract and 5'-riboncleotides) are ready to be sold to the consumer.

Finally, the Table 4 presents an overview of the entire schedule for a single batch as a Gantt chart. This report is important because it helps managers to readily visualize possible problematic part of the schedule and allow him/her to propose a solution. In this simulation, the plant was designed to perform 340 cycles (batches) per year.

Table 4 - Production schedule by cycles.

Task	D.T. (h)	S.T. (h)	E.T. (h)	Description
Complete Recipe	23.4	0	23.4	
P-15 in V-103	12.12	0	12.12	Blending / Storage
P-16 in DS-103	1	0	1	Centrifugation
P-17 in V-105	1.71	0	1.71	Blending / Storage
P-2 in V-101	3.65	0	3.65	Storage
P-3 in DS-101	3.4	0.25	3.65	Centrifugation
P-9 in R-101	4.9	0.25	5.15	Vessel Procedure
P-4 in V-102	3.4	0.25	3.65	Storage
P-5 in FDR-101	12.31	3.65	15.96	Lyophilization
P-10 in DS-101	3	5.15	8.15	Centrifugation
P-11 in R-102	15.02	5.15	20.17	Vessel Procedure
P-18 in R-101	6.43	5.15	11.58	Vessel Procedure
P-19 in C-101	7.65	11.58	19.23	IE Chromatography
P-22 in V-109	2.5	13.9	16.4	Storage
P-23 in SDR-101	2.5	13.9	16.4	Spray Drying
P-6 in V-103	1.47	15.96	17.43	Blending / Storage
P-20 in V-108	2.5	16.4	18.9	Storage
P-21 in SDR-101	2.5	16.4	18.9	Spray Drying
P-7 in V-104	4.17	17.43	21.6	Blending / Storage
P-12 in DS-103	3	20.17	23.17	Centrifugation
P-13 in SDR-101	2.5	20.17	22.67	Spray Drying
P-14 in FL-101	2.67	20.17	22.83	Filling
P-8 in DE-101	2.3	21.1	23.4	Dead-End Filtration

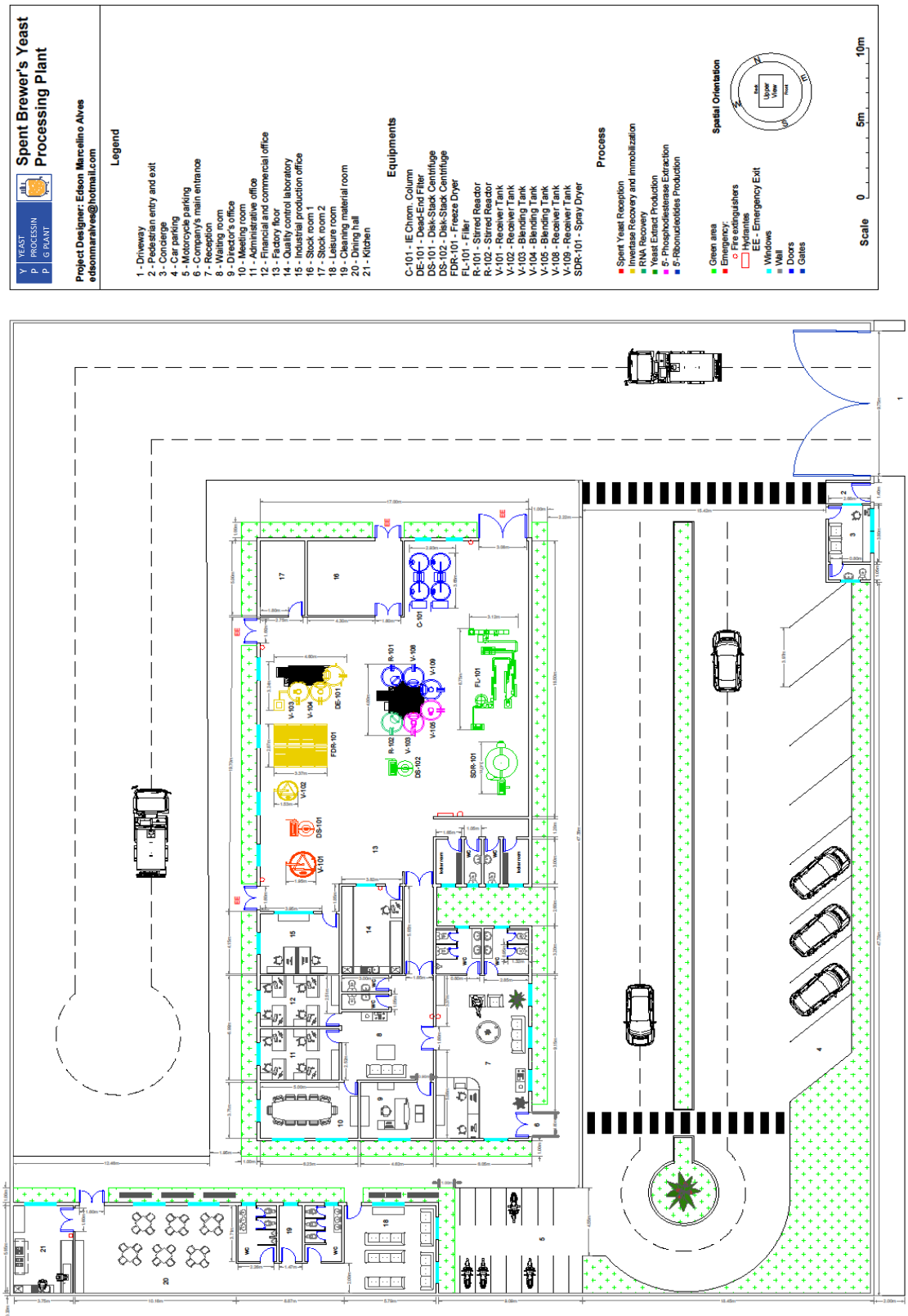


D.T. - Duration Time. S.T. - Start Time. E.T. - End Time

4.2.3 Architectural design

The Spent Yeast Processing Plant was designed using AutoCAD® 2018 software and is presented in Figure 7 and Appendix B. The distribution of equipment was determined according to the sections presented in the flowchart of Figure 6. The general layout of the architectural project consisted in two regions, an administrative area and an operational area. In addition, specific considerations of finishing, cleaning, locker room, security, signage and ventilation and plant's operational area were addressed. The finishing consists of using stainless steel and epoxy on work surfaces, such as countertops and equipment surfaces, as it provides ease cleaning, impermeability to water and resistance to chemicals used in the production process. It also consists of the impermeability of the floor, which must also have a texture that provides its complete cleaning.

Figure 7 - Proposed Spent Yeast Processing Plant. Appendix B presents the same image in high resolution.



The joints between the walls and floors must be sealed. The walls, which in this project were determined to be 20 cm thick for the exterior and 15 cm for the interior,

must be painted with light colored epoxy and their junction with the ceiling must be finished with non-porous and moisture resistant material. The roof must be constructed of easily removable material instead of masonry, facilitating access to perform hydraulic or electrical maintenance services. The tiles chosen for the roof must be impermeable and placed at an angle to promote the proper drainage of rainwater, as well as contain a gutter system attached to the ends (HIRATA; HIRATA; MANCINI FILHO, 2012).

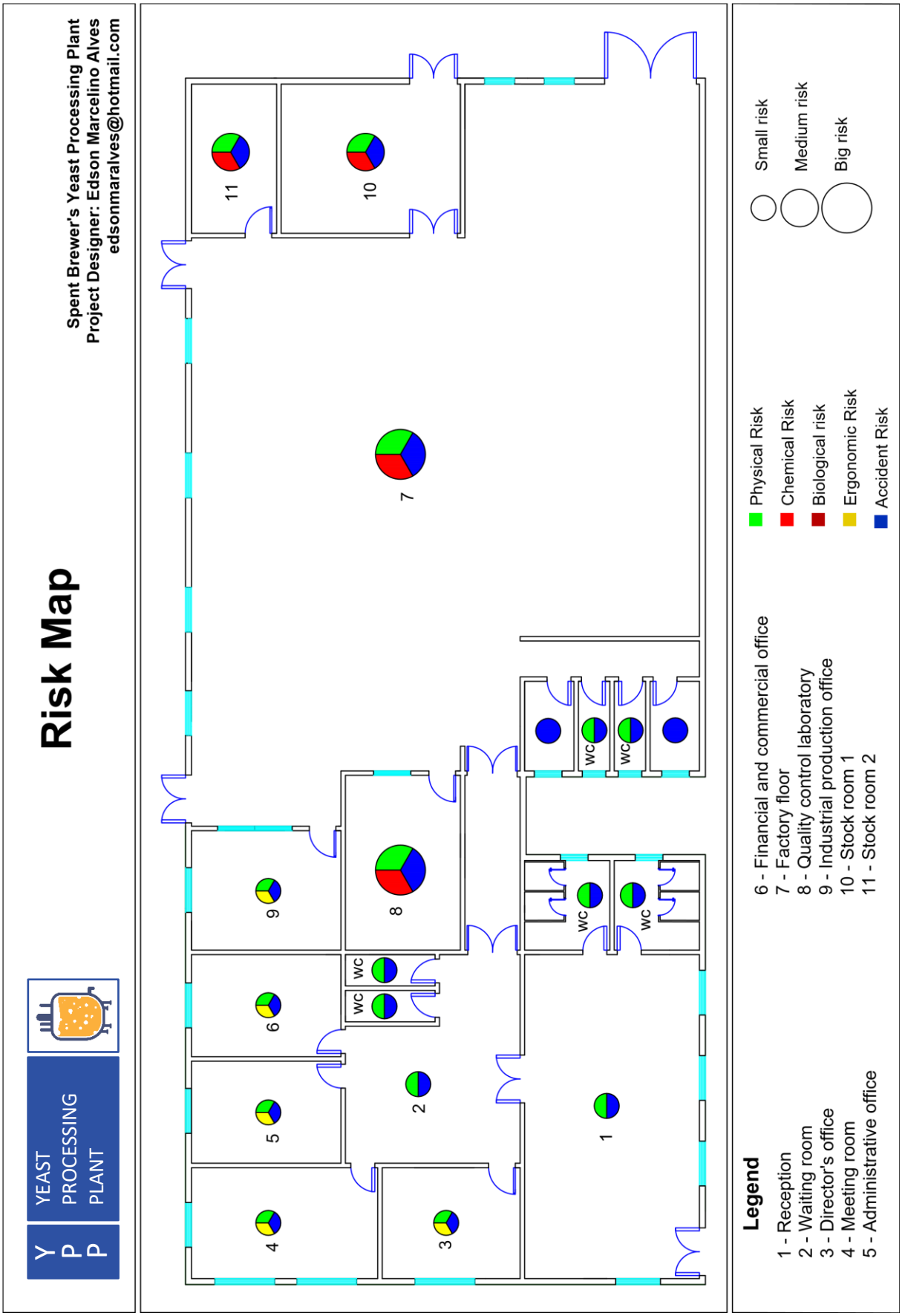
Cleaning includes the utensils used for personal hygiene, present in the quality control laboratory, clothing and bathrooms, being easily accessible and adequate to the risk of exposure to microorganisms and chemicals. It is suggested to install a simple stainless-steel sink for washing hands in the laboratory and a porcelain sink for bathrooms. The use of uniforms by the company's operating personnel is advised. For strictly areas involving production stages, the use of a lab coat over personal clothing is essential. It is also necessary to use Individual Protective Equipment (PPE) and, in order to facilitate their exchanges, there must be changing rooms close to the area of operation (HIRATA; HIRATA; MANCINI FILHO, 2012).

Access to the plant's operational area should be restricted to employees in the sector, with all locations well signposted with the name of the section and their possible risk. At the main entrance, visitors are allowed, as long as they are properly identified and have scheduled their visits in advance. The entry of people outside the work area must be minimal, in order to provide protection against contamination to people and products. Every emergency exit must be signposted on the interior wall, above the door. Fire extinguishers must be signposted with signs providing the date of the next exchange. According to Hirata, Hirata and Mancini Filho (2012), good manufacturing practices recommend the use of cascading airflow, that is, from the production area to the outside. The biosafety standards for laboratory areas of this industry do not require the installation of filters to perform air exhaustion.

4.2.4 General aspects of biosafety

The Figure 8 shows the risk map of the Spent Yeast Processing Plant.

Figure 8 - Spent Yeast Processing Plant Risk Map.



In this context of biosafety, the risks present in the industry must be correctly identified, evaluated and controlled, as they can harm the progress of production, in addition to personal physical and psychological integrity. The risks addressed in this work refer to physical, biological, chemical, ergonomic and accident risks.

The proposed biotechnological process of yeast processing comprises a series of steps which can be simple or complex. Some of them have the potential to generate substances that pose in risk human, animal and environmental health. These substances can be raw materials, microorganisms, crude or purified final product, by-products and residues. By applying the necessary biosafety recommendations, the probability of the occurrence of an adverse effect (risk), as well as its severity, can be reduced or until approaching zero. According to Hirata, Hirata and Mancini Filho (2012), in order to avoid the occurrence of accidents, some factors have to be taken into account: adequate work instructions; quality control and efficient supervision; proper layout and maintenance; control and treatment of wastes; personal hygiene; working hours, auditing and training. Thus, it is important to have an organized team management, whose mission is to impose appropriate policies, rules and regulations related to risks.

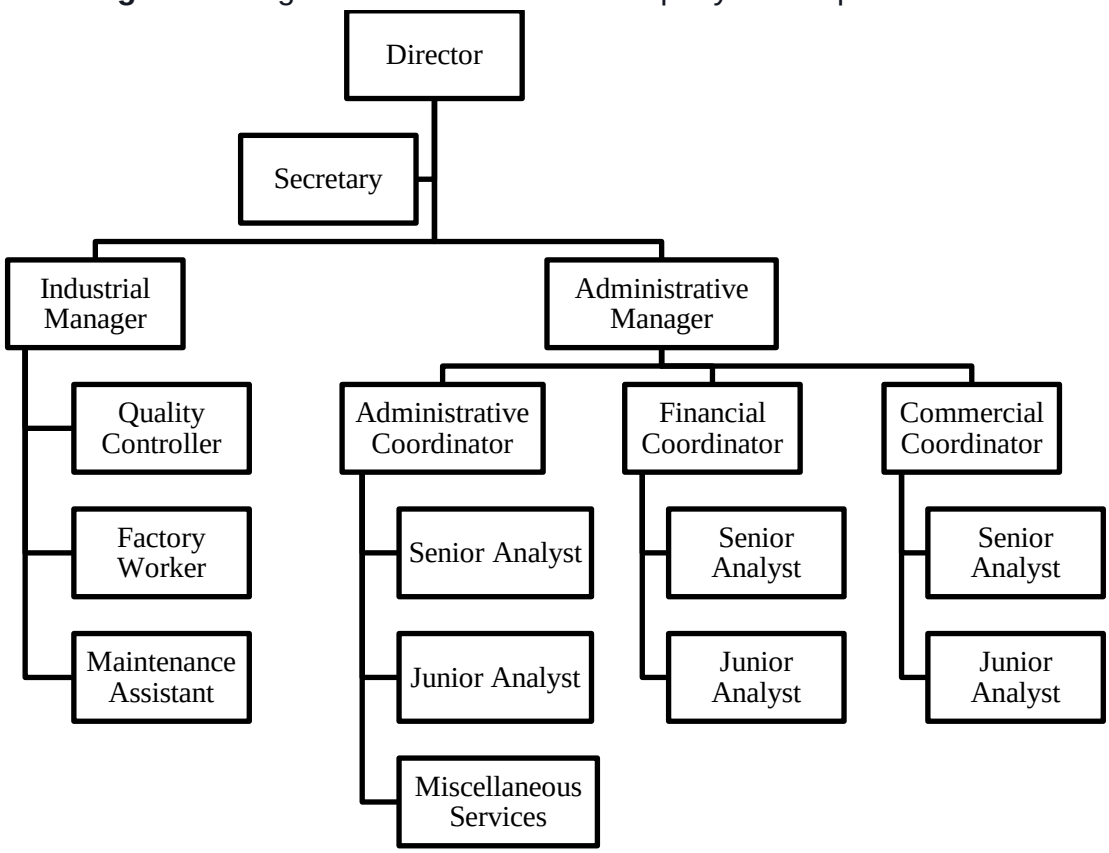
4.2.5 Human Resources and company organizational structure

Figure 9 represents the proposed organizational chart of work positions, which were grouped into four major departments: Industrial (1), led by the industrial manager; Administrative (2), Financial (3) and Commercial (4), which are under the leadership of the administrative manager. In addition, all departments are under the supervision of the CEO. Table 5 summarizes the main sub-areas, which are under the responsibility of each department.

Table 5 - Summary of departments responsibilities: Industrial, Administrative, Financial and Commercial.

Department	Occupation
Industrial	Utilities, maintenance, production, filling, quality and projects.
Administrative	Supplies, logistics, human resources, security, lobby and environment.
Financial	Accounting, payment and charge
Commercial	Marketing / advertising, sales and customer service.

Figure 9 - Organization chart of the company's work positions.



4.3 ECONOMIC ANALYSIS

4.3.1 Direct Fixed Capital

The Direct Fixed Capital includes the assets and capital investments, such as property, plant, and equipment that are needed to start up and conduct business. These assets are considered fixed as they are not consumed or destroyed during the actual production of a good or service but have a reusable value. The Table 6 presents

a detailed list of all equipment used in the proposed Spent Yeast Processing Plant as well as their estimated prices for the year 2020.

Table 6 - Major equipment specification and costs estimate summary.

Name	Type	Units	Size (capacity)	Purchase Cost (R$/Unit)	Total Cost (R$)
C-101	IE Chrom. Column	2	600.62 L	300,000	600,000
DE-101	Dead-End Filter	1	20.00 m^2	10,000	10,000
DS-101	Disk-Stack Centrifuge	1	5,202.74 L/h	100,000	100,000
DS-102	Disk-Stack Centrifuge	1	5,218.96 L/h	100,000	100,000
FDR-101	Freeze Dryer	8	911.48 kg	600,000	4,800,000
FL-101	Filler	1	207.8 units/h	25,000	25,000
R-101	Stirred Reactor	1	17,342.47 L	50,000	50,000
R-102	Stirred Reactor	1	17,396.53 L	50,000	50,000
V-101	Receiver Tank	1	17,114.74	35,000	35,000
V-102	Receiver Tank	1	8,637.36 L	20,000	20,000
V-103	Blending Tank	1	1,424.13	8,000	8,000
V-104	Blending Tank	1	5,110.25 L	15,000	15,000
V-105	Blending Tank	1	976.15 L	5,000	5,000
V-108	Receiver Tank	1	2,754.57 L	10,000	10,000
V-109	Receiver Tank	1	2,713.29 L	10,000	10,000
SDR-101	Spray Dryer	1	46,225.93 L	250,000	250,000
	Unlisted Equipment				1,522,000
			Total Equipment Purchase Cost		**7,610,000**

The equipment cost estimate was made through direct quotes from supplier companies. According to the Table 6, the total purchase costs of freeze dryers for the invertase concentration correspond to the major part (63%) of the total equipment purchase costs. Lowering these costs with other alternatives is difficult because, as noted in Chapter 5 of this thesis, other methods of invertase concentration such as microfiltration and ultrafiltration were already tested as alternatives but they were not feasible to concentrate this enzyme. Table 7 presents an estimate of Direct Fixed Capital calculated according to PC and Eq. 1 to 21.

Table 7 - Direct Fixed Capital estimate summary.

Total Plant Direct Cost (TPDC)		**R$**
Total Equipment Purchase Cost		7,610,000.00
Installation Cost		1,814,000.00
Process Piping Cost		2,664,000.00
Instrumentation Cost		3,044,000.00
Insulation Cost		228,000.00
Electrical Cost		761,000.00
Buildings Cost		3,425,000.00
Yard Improvement Cost		1,142,000.00
Auxiliary Facilities Cost		3,044,000.00
	TPDC	**23,731,000.00**
Total Plant Indirect Cost (TPIC)		**R$**
Engineering Cost		5,933,000.00
Construction Cost		8,306,000.00
	TPIC	**14,239,000.00**
Total Plant Cost (TPC = TPDC+TPIC)	**TPC**	**37,970,000.00**
Other Costs (OC)		**R$**
Contractor's Fee		1,898,000.00
Contingency		3,797,000.00
	OC	**5,695,000.00**
Direct Fixed Capital Cost (DFC = TPC+OC)	**DFC**	**43,665,000.00**

As shown in Table 7, the estimative for *DFC* was about R$ 43,665,000.00, in which 86.95% corresponds to the TPC. Estimating this cost is important because it makes up the Total Capital Investment.

4.3.2 Annual Operating Costs

On the other side of the Fixed Capital Investments is that one which circulates, or which is consumed by a company in the process of production. This includes Raw Materials, Labor, Operating Expenses, and Others. Detailed cost breakdowns of the annual materials cost are shown in the Table 8. It presents the Unit Cost, Annual Amount, and Annual Cost of individual Raw Materials, Consumables and Utilities.

In a company, the employees' payroll is a fundamental document for business management. From an accounting point of view, it is crucial, because salaries and social charges (taxes) considerably affect the company's profit. The job positions and their monthly remunerations designed for the Spent Yeast Processing Plant are shown in Table 9. The wages for each function were estimated from data collected by

consultancy companies in Human Resources, which developed analyzes of the labor market in 2020.

Table 8 - Materials cost summary.

Raw Material	Unit Cost	Annual Amount	Annual Cost (R$)	%
Spent Brewer's Yeast	0.16 R$/kg	5,270,000 kg	8,43,200	12.330
Water	20.26 R$/$m^3$	14,732 m^3	298,471	4.36
Sodium Alginate	736 R$/kg	6,212 kg	4,571,885	66.84
Charcoal	50 R$/kg	932 kg	46,580	0.68
$CaCl_2$ solution (0.3 M)	0.018 R$/kg	1,270,920 kg	22,87	0.33
Sulfuric Acid	2.20 R$/kg	3,505 kg	7,712	0.11
Malt Rootlets	360 R$/ton	54 ton	19,429	0.28
NaCl (1 M)	0.088 R$/kg	1,674,284 kg	146,768	2.15
Reagent Flask	5 R$/entity	176,615 entity	883,074	12.91
		Total	**6,839,995**	**100.00**
Consumable				
DEF Membrane	564.00 R$/$m^2$	1 m^2	564	0.00
IE Chrom. Resin	1500.00 R$/L	10,210 L	15,315,740	100.00
		Total	**15,316,303**	**100.00**
Utility				
Std Power	0.49 R$/kW-h	355,369 kW-h	174,131	46.80
Steam	12.00/m^3	16,482 m^3	197,785	53.15
Chilled Water	0.40/m^3	7,522 m^3	3,009	0.05
		Total	**374,925**	**100**

The cost of labor is the sum of all wages paid to employees, as well as the cost of employee benefits and payroll taxes paid by an employer. The payroll is regulated by the legislation of each country, i.e. it follows rules implying rights and duties. In Brazil, companies that follow the Real and Presumed Profit Regime must calculate their payroll considering the following taxes shown in Table 10.

The Table 10 reveals that for an employee with a base salary of R$ 1,045.00, the company will have to pay R$ 712.48 in fees (68.18% of R$ 1,045.00). In other words, the total cost will be R$ 1,757.48 per month. Following the same calculations, the total Annual Labor-Dependent cost for the company proposed here would be R$ 2,460,444.00. Brazil is the country that pays the highest rates of labor taxes in the world (UHY, 2020) and, as observed, payroll expenses may be higher than imagined. In fact, accessing the Labor-dependent cost is important because it gives tools that entrepreneur needs in order to know the real cost of his employees. Thus, it is possible to find ways to reduce expenses, schedule investments and calculate the ideal number

of employees needed for a business. Furthermore, it ensures that the company is able to face growth with the best cost-benefit ratio, avoiding the existence of an “overpopulation” of employees or a shortage of labor.

Table 9 - Employee wages by function performed.

Department	Quantity	Salary	R$/month
Directorship			
Director	1	R$ 20.900,00	R$ 20.900,00
Secretary	1	R$ 3.135,00	R$ 3.135,00
Subtotal	**2**		**R$ 24.035,00**
Industrial			
Industrial Manager	1	R$ 8.360,00	R$ 8.360,00
Quality Controller	2	R$ 2.612,50	R$ 5.225,00
Factory worker	8	R$ 2.090,00	R$16.720,00
Maintenance assistant	2	R$ 2.090,00	R$ 4.180,00
Subtotal	**13**		**R$ 34.485,00**
Management			
Administrative Manager	1	R$ 8.3600,00	R$ 8.360,00
Administrative Coordinator	1	R$ 5.225,00	R$ 5.225,00
Senior Analyst	1	R$ 4.140,00	R$ 4.140,00
Junior Analyst.	1	R$ 3.135,00	R$ 3.135,00
Watchman	2	R$ 2.090,00	R$ 4.180,00
Storekeeper	1	R$ 2.567,50	R$ 1.576,50
Receptionist	1	R$ 2.090,00	R$ 2.090,00
Cleaner	2	R$ 1.567,50	R$ 3.125,00
Subtotal	**10**		**R$ 32.101,50**
Financial			
Financial Coordinator	1	R$ 5.225,00	R$ 5.225,00
Senior Analyst	1	R$ 4.140,00	R$ 4.140,00
Junior Analyst	1	R$ 3.135,00	R$ 3.135,00
Subtotal	**3**		**R$ 15.665,00**
Commercial			
Commercial Coordinator	1	R$ 5.225,00	R$ 5.225,00
Senior Analyst	1	R$ 4.140,00	R$ 4.140,00
Junior Analyst	1	R$ 3.135,00	R$ 3.135,00
Subtotal	**3**		**R$ 15.665,00**
Total	**31**		**R$ 121.915,50**

Base minimum salary R$ 1.045 (2020).

Table 10 - Brazilian payroll taxes for Real and Presumed Profit Regime companies.

Social and Labor Charges	**(%)**
“INSS”	20,00 %
“Seguro Acidente de Trabalho”	3,00 %
“Salário Educação”	2,50 %
“INCRA/SENAI/SESI/SEBRAE”	3,30 %
“FGTS”	8,00 %
“FGTS/Provisão de Multa para Rescisão”	4,00 %
Total Social Security	**40,80 %**
“13º Salário”	8,33 %
“Férias”	11,11 %
“Previdenciário sobre 13º / Férias / DSR”	7,93 %
Total Taxes	**68,18 %**

The Tables 11 and 12 provide cost breakdowns per cost item and per section, respectively, of the total Annual Operating Cost over all process sections of the proposed Spent Yeast Processing Plant.

Table 11 - Annual Operating Cost, summary per cost item.

Cost Item	**R$/year**	**%**
Raw Materials	6,839,995	24.67
Labor-Dependent	2,460,444	8.88
Facility-Dependent	2,619,900	9.45
Consumables	15,316,303	55.25
Utilities	374,925	1.35
Transportation	110,500	0.40
Total	**27,722,067**	**100.00**

Table 12 - Annual Operating Cost, summary per section.

Section	**R$/year**	**%**
Spent Yeast Reception	1,420,519	5.12
Invertase Recovery	7,189,046	25.93
RNA Recovery	514,305	1.86
Yeast Extract Production	1,677,435	6.05
5’-PDE Extraction	457,525	1.65
5’-Ribonucleotides Production	16,463,262	59.39
Total	**27,722,092**	**100.00**

In both tables, it is possible to readily identify the cost-sensitive sections of the process, i.e. the economic hot-spots. For instance, Table 11 reveals that the cost of C*onsumables* is the largest cost in this process, accounting for roughly 55% of the total Annual Operating Cost over all items. This cost accounts for the expensive

chromatography resins that need to be replaced periodically. Furthermore, the Table 12 reveals that the ‘5’-Ribonucleotides Production’ section is the most expensive part of the process, accounting for roughly 59% of the Annual Operating Cost. Thus, it would be wise to allocate resources to optimize this section, as opposed to using those same resources elsewhere where optimization would have little effect on the overall project cost.

The above analysis shows how the economic reports can be used as a tool to optimize a process through ‘what-if’ scenarios by examining individual cost items. Would it make economic sense to use a less expensive chromatography resin if it required more cycles to be run and more buffer solution to be used? It depends on how many more cycles are needed, and how much cheaper the new resin is. Would a radically modified purification scheme be better than the current scheme? It depends on what equipment, reagents, etc. would be required for the modified scheme, and what would be the impact on the overall product yield and costs.

On the other hand, it is easy to identify from Table 12 that the cheapest section corresponds to the “Extraction of 5'-phosphodiesterase”. The process for this enzyme extraction is very simple and, the way that was designed here, deserves to be highlighted once it has numerous economic advantages compared to other techniques such as its production via solid or submerged fermentation using microorganism.

4.3.3 Annual Income

Table 13 shows the annual mass production as well as the Unit Cost of each good produced. From this last value and based on similar products available on the market it is possible to estimate the Selling Price.

Table 13 - Unit Production Cost, Selling Price and Revenues estimate summary.

Products	Amount (kg/year)	Unit Production Cost (R$/kg)	Selling Price (R$/kg)	Revenue (R$/year)	%
5'-CMP/5'UMP	17,493.88	339.40	678.80 R$/kg	11,874,848	12.16
5'GMP/5'AMP	34,519.83	339.40	678.80 R$/kg	23,432,063	24.00
Invertase (350U/g)	316,050.3	24.25	121.25 R$/kg	38,321,103	39.24
Yeast extract	176,614.8	13.60	136.00 R$/kg	24,019,613	24.60
			Total	97,647,627	100.00

The company Sigma Aldrich sells 5'-ribonucleotides with 99% purity at the prices around 350.00 R$/g. As 5'-ribonucleotide compositions are produced here containing 46% purity on average and considering its Unit Cost, it was opted for an estimated sale price of R$ 0.67/g (around two times its Unit Cost) to carry out the economic analysis. A survey of the free invertase enzyme price practiced in the market resulted in values in the range of 20.00 to 100.00 R$/$10^6$ U. The Selling Price for this product was set at 346.42 R$/$10^6$ U (or 121.25 R$/kg, five times its Unit Cost), much more expensive, due to the fact that the enzyme is in immobilized form and can be used more than once. Some works reinforce the high stability of the immobilized enzyme in relation to repeated use capability. In a study carried out by Milovanović, Božić and Vujčić (2006), for example, yeast cell wall invertase was immobilized within calcium alginate beads. After 40 consecutive cycles, each of 1 h duration, the immobilized enzyme retained 90% of its original activity.

Finally, the price of yeast extract was estimated at R$ 136.00 R$/kg (ten times its Unit Cost) to carry out economic analysis. The average price found in the market for this product is around 600.00 to 1000.00 R$/kg.

From these estimates, it was possible to simulate the Annual Income at R$ 97,647,627. In addition, invertase is the product that most contributes to the company's revenue due to its greater annual production volume, around 58% of the company's annual mass production.

4.3.4 Profitability Analysis

The Profitability Analysis is the study of a company's profit-generating capacity and seeks to verify whether the survival and development intended by the company is possible. In this work specifically, the Profitability Analysis was responsible for anticipating sales and potential profits of the hypothetical company that market products derived from spent brewer’s yeast. A traditional way to measure whether the company is growing or not is to assess its Cash Flow. If the available resources are not being sufficient to cover the expenses for the execution of the operational activities, it is time to rethink how the money is entering the cash and where it is being destined. In order to discuss such issues, the Table 14 shows the expected cash flow for the yeast fractionation plant.

Table 14 - Cash flow analysis (thousand R$).

Year	DFC	WC	SC	SR	AOC	Dep.	Gross Profit	Taxes (43%)	Net Profit	NCF	CCF
0	-17,466	0	0	0	0	0	0	0	0	-17,466	-17,466
1	-26,199	0	0	0	0	0	0	0	0	-26,199	-43,665
2	0	-884	-2,183	65,098	20,175	6,904	38,020	16,348	28,575	25,508	-18,157
3	0	0	0	97,648	27,722	6,213	63,712	27,396	42,529	42,529	24,373
4	0	0	0	97,648	27,722	5,523	64,402	27,693	42,232	42,232	66,605
5	0	0	0	97,648	27,722	4,833	65,093	27,990	41,936	41,936	108,541
6	0	0	0	97,648	27,722	4,142	65,783	28,287	41,639	41,639	150,179
7	0	0	0	97,648	27,722	3,452	66,474	28,584	41,342	41,342	191,521
8	0	0	0	97,648	27,722	2,762	67,164	28,881	41,045	41,045	232,566
9	0	0	0	97,648	27,722	2,071	67,854	29,177	40,748	40,748	273,314
10	0	0	0	97,648	27,722	1,381	68,545	29,474	40,451	40,451	313,766
11	0	0	0	97,648	27,722	690	69,235	29,771	40,154	40,154	353,920
12	0	0	0	97,648	27,722	0	69,926	30,068	39,858	39,858	393,778
13	0	0	0	97,648	27,722	0	69,926	30,068	39,858	39,858	433,635
14	5,694	884	0	97,648	27,722	0	69,926	30,068	39,858	46,435	480,070

DRF-Direct Fixed Capital, **WC-**Work Capital, **SC-**Startup Cost, **SR-**Sales Revenue, **AOC-**Annual Operating Cost, **Dep-**Depreciation, **NCF**-Net Cash Flow, **CCF**-Cumulative Cash Flow.

According to Couper (2003), it is possible to mention three cash flow functions: it allows to project payments and receipts; to design adjustments to stem losses and overcome crises and to project investments in business growth and expansion. Besides, if there is a mismatch between the deadline to pay suppliers and receive from customers or if there is fixed capital, for example, the cash flow will reveal and, from there, the attentive manager is able to define a better management strategy.

As noted in Table 14, the company starts to have positive Net Cash Flow from year 2 on. It reaches its maximum value at year 3 and start to decrease over the following years. This behavior is due to the choice of depreciation method. The Sum-Of-The-Years-Digit Method was applied with decreasing depreciation rates. Since depreciation can be considered an operating cost, expenses will be virtually higher in the first few years. Consequently, the income tax on profits will be lower, but after taxes, the depreciation amounts return to the cash flow. This strategy designed here is excellent for the payment of less taxes in the initial years of the company since statistics show that, in Brazil, 6 out of 10 companies, do not survive after five years of activity (IBGE, 2019). Thus, the initial moments of the company are crucial for its consolidation in the market. The depreciation planning has a direct impact on the cash flow and can allow the company to have more cash on hand in the initial years and, consequently, greater chances of success.

Another way to assess the profitability of an investment is through economic indicators. Therefore, the Table 15 provides an overview of the Economic Evaluation of the proposed plant, including the major economic aspects of the investment as well as some key economic indicators.

Table 15 - Executive summary.

Parameter	
Direct Fixed Capital	R$ 43,665,000
Working Capital	R$ 883,552
Startup Cost	R$ 2,183,270
Total Capital Investment	R$ 46,731,822
Total Revenues	97,647,627 R$/year
Annual Operating Cost (AOC)	27,722,092 R$/year
Depreciation (Linear)	3,797,108 R$/year
Gross Profit (Total Revenues - AOC - Depreciation)	66,128,427 R$/year
Taxes (43%)	28,435,224 R$/year
Net Profit (Gross Profit - Taxes + Depreciation)	41,490,311 R$/year
Gross Margin	67.72 %
Return On Investment (ROI)	88.78 %
Payback Time	1.13 years
IRR (Before Taxes)	95.08%
IRR (After Taxes)	64.63%
NPV (at 7.0% Interest)	R$ 268,593,474
NPV (at 9.0% Interest)	R$ 230,501,306
NPV (at 11.0% Interest)	R$ 198,768,520

The Table 15 shows that Total Investment Capital is in the order of R$ 46,7 million. Gross Margin's value for this investment project is 67.72%. This value represents the portion of each dollar of revenue that the company retains as gross profit. In fact, that means the company retains R$ 0.67 from each R$ 1.00 of revenue generated. In other words, it is the sales revenue a company retains after incurring the direct costs associated with producing the goods it sells, and the services it provides. The higher the Gross Margin, the more capital a company retains on each dollar of sales, which can be used to pay other costs or satisfy debt obligations. Because AOC have already been taken into account, those remaining funds may consequently be channeled toward paying debts, general and administrative expenses, interest fees, and dividend distributions to shareholders. The administrator can use Gross Margin to measure how the production costs relate to the revenues. For example, if the company's Gross Margin is falling, it may strive to slash labor costs or source cheaper

suppliers of materials. Alternatively, it may decide to increase prices, as a revenue increasing measure. Gross Margins can also be used to measure the company efficiency or to compare two companies of different market capitalizations.

Among the economic indicators, ROI stands out for being able to identify financial returns, both potential and past. The ROI metric shows, through a rate of return, how much an investor has gained (or lost) in relation to the amount invested in a given investment, while giving an analysis of what happened and a perspective on the future of the same. As in this project the value of ROI was 88.78%, this means that for every R$ 1.00 invested, the investment returns R$ 0.88. Thus, ROI relates to the net profit of investments in a relative way, giving a better measure of profitability by situation. With this, investors are able to know and compare exactly how is their performance in each of their applications, encouraging them over time to be better.

When making any type of investment it is necessary to go through a period of expenses, in which the money comes out of the cash. But, if everything is well planned, there will come a time when the revenues will recover the invested capital. And it is this moment, presented in a period of time, that the Payback method shows. According to table 14, the Payback time is 1.13 years, that is, the investment is recovered in less than one year. Thus, this index works as an indicator that shows how long the loan or investment will take to return to the investor or the company. "Will the investment pay off?" "How long will it take for the project to pay off?" "How do the investor know how much money he needs to have?" "Is this the best alternative to invest at the moment?" The main advantage of payback is that it provides information that help to answer such questions and give a more solid idea of the company's future cash flow. This measure becomes even more important in times of economic crisis. Therefore, as it is a concept it is essential when deciding the attractiveness of an investment, this indicator ends up becoming an important factor within the feasibility analysis of any initiative.

Finally, Table 15 reveals NPV (at 7% interest) and IRR after taxes equals to R$ R$ 268,593,474 and 64.63% respectively. The NPV represents the profitability of the investment, taking into account the total amount invested and the project lifetime, as well as the correction of money over time. This is important because money in the present is worth more than the same amount in the future due to inflation and to earnings from alternative investments that could be made during the intervening time.

In other words, one dollar earned in the future won't be worth as much as one earned in the present. The interest rate element of the NPV formula is a way to account for this.

To better understand this value, assume that an investor could choose to receive an amount of money today, the TCI (R$ 43,660,000) for example, or the TCI plus 7% (R$ 43,716,200) after one year. If the payer was reliable, that extra 7% may be worth the wait, but only if there wasn't anything else the investors could do with the R$ 43,660,000 that would earn more than 7%. An investor might be willing to wait a year to earn an extra 7%, but that may not be acceptable for all investors. In this case, the 7% is the interest rate which will vary depending on the investor. In fact, if an investor knew they could earn 9% from another relatively safe investment over the next year, then in this case, the investor's interest rate would be 9%. As observed in Table 14, when the interest rate increases, the NPV falls. This phenomenon occurs due to the inversely proportional relationship between the respective indices, since the higher the interest rate of a safe investment, the lower the attractiveness of any other investment with greater risks.

A company may determine the interest rate using the expected return of other projects with a similar level of risk or the cost of borrowing money needed to finance the project. Thus, by choosing a certain interest rate and applying the NPV method, it is possible to verify the profitability of investments. As a rule, a positive NPV indicates that the projected earnings generated by a project or investment, in present values, exceeds the anticipated costs, also in present values. It is assumed that an investment with a positive NPV will be profitable, and an investment with a negative NPV will result in a net loss. If the NPV is zero, the investment is indifferent. An important drawback of using an NPV analysis is that it makes assumptions about future events that may not be reliable.

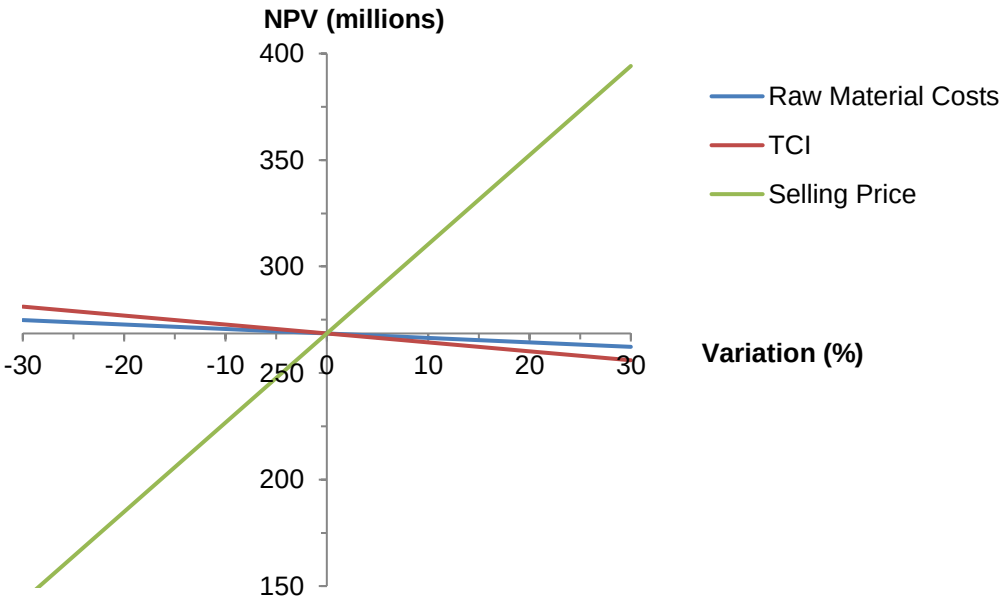
Finally, the IRR is the interest rate at which the net present value of an investment is equal to zero. In other words, it is the compound annual return an investor expects to earn over the life of an investment. Thus, the investment in the Yeast Processing Plant should be rejected only if it is possible to find some other safe investment with 64.63% interest rate.

4.3.5 Sensitivity Analysis

In addition to the profitability analysis, it is very important to take into account the financial sensitivity analysis in an economic analysis of an investment, once it is very difficult to predict the future accurately. Sensitivity analysis has the role of precisely making the investor aware of the behavior of his investment in different scenarios. In this analysis, optimistic and pessimistic estimates are made about a group of variables that have an impact on the NPV or ROI. The sensitivity parameters can be accessed through the break-even point and the Strauss graph. Based on these assumptions, points of improvement in activities can be identified even before there is any negative impact on the expected results.

Figure 9 shows the Strauss Graph in which scenarios were projected for variations in Unit Selling Price, TCI and Raw Material Costs in order to assess their influence on NPV.

Figure 9 - Strauss Graph projected for variations in Unit Selling Price, TCI and Raw Material Costs.

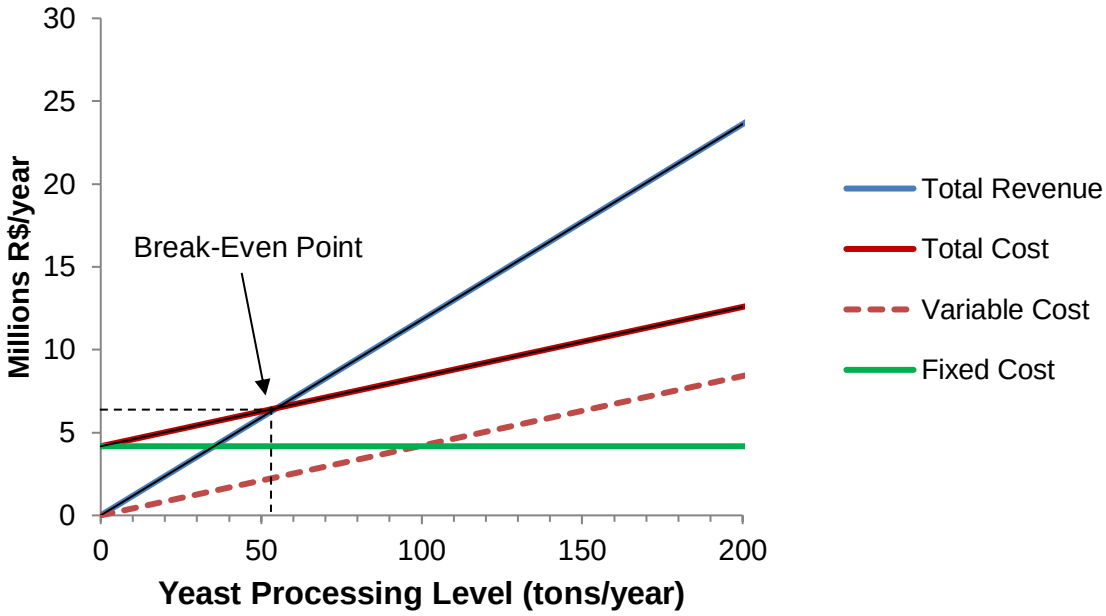


As noted, the selling price is the most sensitive variable. Its line has the largest slope (m = 4.1854), that is, a small variation on the x axis (increase or decrease in the sale price) results in a large variation on the y axis (NPV). Variations in the TCI or in the Raw Material Cost have little influence on the NPV, since these variables presented angular coefficients close to zero (m = -0.4195 and m = -0.2094 respectively). In adverse market situations, this chart is important because it allows the manager to

assess in advance what are the best strategies to be taken and what are their impacts on the company's economy.

Finally, the Figure 10 presents the Break-Even Point graph, a form commonly used to determine the company's production capacity in which revenue equals company expenses.

Figure 10 - Break-Even Point graph.



According to figure 10, the Break-Even Point of the Spent Yeast Processing Plant is 54.88 ton/year. This is the level of yeast processing that the company needs to work on in order to have no losses or profits. Above this level, the company would present profits, while values below this point would mean financial losses for the business.

5. CONCLUSION

The design and simulation developed in this work reached satisfactory and positive results, showing that it is possible to integrate different biotechnological downstream routes in order to process wastes from environmental problem into source of income. In addition, in view of growth trends for the yeast extract and ribonucleotide market, as well as the search for innovation in the industrial sectors, the implementation of a bioprocess plant for spent yeast processing into compounds of high added value brings a new perspective to the brewing sector. From the technology

proposed here, these companies can increase income by selling by-products other than beer. Thus, they can diversify their portfolio and product range, becoming stronger and more competitive, besides contributing to the development of technologies focused on gain and productivity without neglecting environmental responsibility.

The results showed that the installation of the Spent Yeast Processing Plant in Assis-SP is pertinent. In addition, Through the economic analysis it was possible to find characteristics of the company and also to design situations that it may experience further ahead. The projection of the cash flow and the determination of the financial indexes demonstrated that, despite the high initial investment, the project is viable and brings positive results quickly. Finally, it is concluded that the fractionation plant would be a good investment, not neglecting the need to optimize the factors that directly or indirectly affect the economic viability of the project.

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