UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
Department of Biochemical and Pharmaceutical Technology
Fermentation Technology

Production of L-asparaginase of pharmaceutical interest from yeasts isolated from the Antarctic continent

Ignacio Sánchez Moguel

Thesis to obtain the degree of DOCTOR
Thesis director: Prof. Dr. Adalberto Pessoa Junior

SÃO PAULO

2018

UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
Department of Biochemical and Pharmaceutical Technology
Fermentation Technology

Production of L-asparaginase of pharmaceutical interest from yeasts isolated from the Antarctic continent

Ignacio Sánchez Moguel

Original Version

Thesis to obtain the degree of DOCTOR
Thesis director: Prof. Dr. Adalberto Pessoa Junior

SÃO PAULO

2018

Ficha Catalográfica
Elaborada pela Divisão de Biblioteca e
Documentação do Conjunto das Químicas da USP.

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

Sánchez Moguel, Ignacio
S211p Produção de L-asparginase de interesse farmacêutico a partir de cultivo de leveduras isoladas do continente Antárctico / Ignacio Sánchez Moguel. -- São Paulo, 2018.
120p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Tecnologia Bioquímico-Farmacêutica.
Orientador: Pessoa Junior, Adalberto

1. Tecnologia químico-farmacêutica 2. Enzima : purificação : bioquímica I. T. II. Pessoa Junior, Adalberto, orientador.

660 CDD

SÁNCHEZ-MOGUEL, I. Production of L-asparaginase of pharmaceutical interes from yeasts isolated from Antarctic continent. 2018. 120 p. Thesis (Ph.D). –Faculty of Pharmaceutical Sciences, University of São Paulo, 2017.

Examining Board

_____

Prof. Dr. Adalberto Pessoa Junior

(Thesis director/President)

_____

1º examiner

_____

2º examiner

_____

3º examiner

_____

4º examiner

São Paulo_____ de_____ de 2018.

In memory of my father, Macario

To my mother, Mónica

AKNOWLEGDMENTS

Thank you,

To life for this experience, for having brought me here, for having taken a great professional step, but even more personally, where I got great friendships, and life lessons.

To my family, Judith, Ariadna, Alonso, David, Saiyd, Zayra, Jacob, André, Rodolfo, Simón, to all of you even when we were away, you were present all this way, my dreams became yours, you always They supported me in everything. I love you brothers.

To my father, Macario for guiding me in this walk of constant questions and search for answers.

To my mother, for all your love and care.

To my teachers Adalberto, Solange for everything I learned from them and for being an essential part of my professional and personal preparation.

To all the people who made this long road, a more enjoyable, more cheerful, more vibrant, of great experiences, of many laughs and that we share great moments and we share the same dreams, we learn together and grew professionally and personally. Thank you for teaching me that borders do not exist.

To all my friends

To Mexico, to CONACYT for the scholarship granted for the realization of this doctorate.

To Brazil, for this great experience

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

AHA	β-aspartohydroxamic acid
ALL	Acute lymphoblastic leukemia
ANVISA	National Health Surveillance Agency
AsnSynt	Asparagine Synthetase enzyme
ASNase	L-asparaginase
CCD	Central composited design
CD	Circular dichroism
cdw	Cell dry weight
DEAE	Diethylaminoethyl cellulose
ΔH	Entalpy
DHA	Docohexanoic acid
DoE	Design of experiments
EcASNase	*Escherichia coli* Asparaginase
FAME	Fatty acid methylesters
GC	Gas chromatography
GDH	Glutarate dehydronase enzyme
GHA	γ-glutamohydroxamic acid
GLNase	L-glutaminase
GOT	Glutamic oxaloacetic transaminase enzyme
GRAS	General Recognized As Safe
hASNase	Human asparaginase
K_{av}	Partition coefficient
k_{cat}	Turn over constant
K_La	Oxygen transference coeficient
$K_{0.5}$	Constant
K_M	Michaelis –Menten constant
LDH	Lactate dehydrogenase enzyme
LsASNase	Leucospordium scotti L- Asparaginase
MUFA	Monounsaturated Fatty Acids
MW	Molecular weight
β-NADH	Nicotin Adenosin des

$\mathbf{n_H}$	Hill coefficient
OD	Optic density
$\boldsymbol{P}$	Product concentration
$\boldsymbol{P_f}$	Final product concentration
$\boldsymbol{P_0}$	Initial product concentration
PB	Plackett –Burman design
PBS	Phospate buffer solution
PEG	Polyethylene glicol
PEG-ASNase	Pegylated L-asparaginase
PMSF	Phenyl metil sulfonyl F
PUFAs	Polyunsaturated fatty acids
Q	Volumetric productivity
rDNA	Ribosomal DNA
$\mathbf{R_f}$	Migration distance
S	Substrate concentration
$\mathbf{S_0}$	Initial substrate concentration
$\mathbf{S_f}$	Final substrate concentration
ScASNase	*Scharomyces cerevisiae* Asparaginase
SCOs	Single cell oils
SD	Standard deviation
SDS-PAGE	SDS- PolyAcrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
TCA	Trichloroacetic acid
TGA	Triacylglycerol
TLC	Thin Layer Chromatography
$\mathbf{V_e}$	Elution volumen
$\mathbf{V_0}$	Void volumen
$\mathbf{V_C}$	Column volume
$\mathbf{V_{max}}$	Máximum velocity
X	Cell mass concentration
$\mathbf{X_0}$	Initial cell mass concentration
$\mathbf{X_f}$	Final cell mass concentration
YPD	Yeast peptone dextrose

$\boldsymbol{Y_{X/S}}$	Conversion factor of substrate to cells
$\boldsymbol{Y_{P/S}}$	Conversion factor of substrate to products

ABSTRACT

Production of L-asparaginase of pharmaceutical interest from yeasts isolated from the Antarctic continent

The L-asparaginase (ASNase) obtained from yeasts species has been poorly studied and a new yeast ASNase could be an alternative to minimize the side effect in the treatment of lymphoblastic leukemia. The Antarctic ecosystems have a great potential to obtain novel enzymes produced from psychrophilic and psychrotolerant microorganisms. Yeasts isolated from samples collected in the Antarctic Peninsula by the PROANTAR expedition team were tested for the production of ASNase and L-glutaminase (GLNase). From this screening, the strain *Leucosporidium scottii* L115 presented the highest ASNase activity (6.24 U g^{-1} of dried cell weight (dcw)) with a combination of low GLNase activity (0.41 U g^{-1} dcw). The ASNase belonging to *L. scottii* L115 (LsASNase) was purified 227 fold with a specific activity of 137.01 U mg^{-1} at 37 ºC, and with 0.93 U mg^{-1} for GLNase. Moreover, the maximum activity was observed at pH 7.5 at 55 ºC. The enzyme is a multimer presenting a single band of 54.5 kDa of molecular weight in reduced conditions and 462 kDa by size exclusion chromatography. The LsASNase is a glycosylated enzyme that presented a band lower at 25 kDa when was treated with PGNase F. The enzymatic kinetic reveals an allosteric regulation of the enzyme and the kinetic parameters were determined at 37º C, pH 7.0 as $K_{0.5}$ = 233 μM, k_{cat} = 54.7 s^{-1} and n_H = 1.52 demonstrating a positive cooperativity by the enzyme and the substrate. The ASNase production by *L. scottii* L115 was improved by applying DoE for the culture medium development. The PB and CDD designs were used to optimize the ASNase production providing the nutrient values of 6.15 g L^{-1} of proline, 28.34 g L^{-1} sucrose, and 15.61 g L^{-1} of glycerol for a maximal production. The synthetic medium containing the optimized quantities was added with the salts: KCl, 0.52 g L^{-1}; $MgSO_4.7H_2O$, 0.52 g L^{-1}; $CuNO_3.3H_2O$, 0.001 g L^{-1}; $ZnSO_4.7H_2O$, 0.001 g L^{-1}; $FeSO_4.7H_2O$, 0.001 g L^{-1}.The optimized medium produces a 23.75 ULh^{-1} of ASNase in shake flask culture. Furthermore, *L. scottii* is characterized as an oleaginous yeast that accumulates lipids with a suitable fatty acid profile. The production of ASNase and lipids were scaled up in the 1 L bioreactor to evaluate the initial cell concentration, carbon source, and oxygen transfer rate (k_La).The experiments were performed at 15ºC in the bioreactor BIOSTAT®Q plus (Sartorius Stedim, Germany) in batch mode, using 0.5 L of the optimized medium culture in phosphate buffer 50 mM pH 7.0. The initial cell concentration was evaluated at 1%, 3%, and 5% (v/v). Sucrose and glycerol were tested alone to examine if the combination of both is mandatory to produce ASNase. All these assays were carried in duplicate. The k_La was assessed through a CCD design in the range of 1.42 – 123.0 h^{-1}. The performance in bioreactor showed the productivity of 36.95 $ULh^{-1}$of ASNase under the optimized conditions (growth temperature 15º C, X_0: 5 g L^{-1}, pH 7.0, 48 h, k_La 89-92 h^{-1}). The cultivation of *L. scottii* L115 at 15ºC in sucrose and glycerol as carbon sources generate an interesting lipid profile, where it presents monounsaturated and polyunsaturated lipids.

Keywords: L-asparginase; *Leucosporidium scottii*, psychrotolerant yeast, purification; characterization, purification, production, DoE, bioreactor, lipid accumulation.

Resumo:

Produção de L-asparaginase de interesse farmacêutico a partir de leveduras isoladas do continente Antártico

A L-asparaginase (ASNase) obtida a partir de espécies de leveduras tem sido pouco estudada e uma nova ASNase de levedura pode ser uma alternativa para minimizar os efeitos adversos no tratamento da leucemia linfoblástica. Os ecossistemas Antárticos têm um grande potencial para obter novas enzimas produzidas a partir de microorganismos psicrofílicos e psicotrolerantes. As leveduras isoladas de amostras coletadas na Península Antártica pela equipe de expedição do PROANTAR foram testadas para a produção de ASNase e L-glutaminase (GLNase). A partir desta triagem, a cepa *Leucosporidium scottii* L115 apresentou a maior atividade de ASNase (6,24 U g^{-1} dcw) com uma combinação de baixa atividade de GLNase (0,41 U g-1 dcw). A ASNase pertencente a *L. scottii* L115 (LsASNase) foi purificada 227 vezes com uma atividade específica de 137,01 U mg-1 a 37 ºC e com 0,93 U mg^{-1} de GLNase. A atividade máxima foi observada a pH 7,5 a 55 ºC. A enzima é um multímero que apresenta uma banda única de 54,5 kDa de peso molecular em condições redutoras e 462 kDa por cromatografia de exclusão molecular. A LsASNase é uma enzima glicosilada que apresentou uma banda menor a 25 kDa quando tratada com PGNase F. A cinética enzimática revela uma regulação alostérica da enzima e os parâmetros cinéticos foram determinados a 37º C, pH 7,0 como $K_{0,5}$ = 233 μM, kcat = 54,7 s^{-1} e n_H = 1,52 demonstrando uma cooperatividade positiva pela enzima e o substrato. A produção de ASNase por *L. scottii* L115 foi melhorada aplicando DoE para o desenvolvimento do meio de cultura. Os desenhos experimentais de PB e CDD forma usados para otimizar a produção de ASNase e forneceram os valores de nutrientes de 6,15 gL^{-1} de prolina, 28,34 gL^{-1} de sacarose e 15,61 gL^{-1} de glicerol para uma produção máxima. O meio sintético contendo as quantidades otimizadas foi adicionado com os sais: : KCl, 0.52 g L^{-1}; $MgSO_4.7H_2O$, 0.52 g L^{-1}; $CuNO_3.3H_2O$, 0.001 g L^{-1}; $ZnSO_4.7H_2O$, 0.001 g L^{-1}; $FeSO_4.7H_2O$, 0.001 g L^{-1}.O meio otimizado produz 23.75 ULh^{-1} de ASNase em cultivo em frasco agitado. Além disso, *L. scottii* é caracterizada como uma levedura oleaginosa que acumula lipídios com um perfil adequado de ácidos graxos. A produção de ASNase e lipídios foi ampliada no biorreator de 1 L para avaliar a concentração celular inicial, fonte de carbono e taxa de transferência de oxigênio (k_La). Os experimentos foram realizados a 15ºC no biorreator BIOSTAT®Q plus (Sartorius Stedim) em modo batelada, utilizando 0,5 L da cultura de meio otimizado em tampão fosfato 50 mM pH 7,0. A concentração celular inicial foi avaliada em 1%, 3% e 5% (v / v). Sacarose e glicerol foram testados isoladamente para examinar se a combinação de ambos é obrigatória para produzir ASNase. Todos esses ensaios foram realizados em duplicado. O k_La foi avaliado através de um planejamento CCD na faixa de 1,42-123,0 h^{-1}. O desempenho no biorreator mostrou a produtividade de 36,95 ULh^{-1} de ASNase sob condições otimizadas (temperatura de crescimento 15º C, X_0: 5 g L^{-1}, pH 7,0, 48 h, kLa 89-92 h^{-1}). O cultivo de *L. scottii* L115 a 15ºC em sacarose e glicerol como fontes de carbono gera um perfil lipídico interessante, onde apresenta lipídios monoinsaturados e poliinsaturados.

Palavras-chave: L-asparginase; Leucosporidium scottii, levedura psicotrolerizante, purificação; caracterização, purificação, produção, DoE, biorreator, acumulação de lipídios.

1 CHAPTER I. LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The bacterial type II enzyme L-asparaginase (ASNase) is an antitumor agent used in the treatment of acute lymphoblastic leukemia (ALL). In December 2012 there was a shortage of ASNase by the interruption in the production of the biopharmaceutical drug, mobilizing the Brazilian health agencies to search a biosimilar drug. This shortage of ASNase could have compromised the therapy of 3300 patients treated every year in Brazil, by the lack of a drug without a substitute in the Brazilian market. The eventual absence of ASNase forced suppliers to seek the drug in alternative markets such as India, Europe, and China (CANAL SAÚDE / FIOCRUZ, 2014).

In the current year, the use of a Chinese ASNase formulation again attracted the attention of media, researchers and the National Health Surveillance Agency (ANVISA) in the country due to the high content of impurities in the imported drug used in the treatment of LLA patients. Currently, ASNase formulations are produced around the world but a few have the quality specifications of the reference drug. These events have placed the ASNase in the focus of Brazilian researchers and government, interested in the national production of the enzyme. Although the ASNase has approximately 40 years of use for the treatment of ALL, there are still deficiencies and problems to be solved. The adverse effects, the short half-life, the low levels of production at the industrial level are the main topics concerning to be solved for the clinical use of the enzyme (ZUO et al, 2015).

Approximately in 60% of the patients treated with ASNase is reported the formation of antibodies anti-ASNase, producing immunogenic reactions or neutralizing the enzyme affecting the half-life of the drug. Also, the toxicity of ASNase is attributable to the Glutaminase (GLNase) activity of the enzyme producing several side effects such as leucopenia, neuralgia immunosuppression, acute pancreatitis, thromboembolism and, hyperglycemia (OHNUMA et al, 1970; PANOSYAN et al, 2004). Being a serendipitous biopharmaceutical drug born before the era of genetic engineering, the re-study and production improvement of the enzyme during the next years could solve the drawbacks of the ASNase therapy.

The microorganisms have been considered as the most important source of ASNase, a wide range such as bacteria, fungi, yeasts, actinomycetes, and algae have proved to be proficient sources of this enzyme and, are easily cultivated in bioreactors, with controlled conditions such as pH, temperature, aeration, medium composition and other parameters, leading to high reproducibility titters of the enzyme. The bioprospection for new microorganisms producing ASNase as potential candidates for the formulation of new drugs is needed to improve the production, activity, and specificity of the enzyme (SAVITRI and AZMI, 2003).

The Brazilian biomes are an important hotspot for biodiversity, occurring also in the microbial life of Brazilian soils, such as those from Amazonia, Catinga, Atlantic rainforest, Pantanal, and Pampa (ANDREOTE et al 2017). Moreover, trough the Brazilian Antarctic Program (PROANTAR), Brazil participates in the scientific research in the Antarctic realizing scientific explorations and bioprospection of this little-explored biome with an incredible biotechnological potential. The Antarctic cold environments were successfully colonized by numerous microorganisms, including yeasts, fungus and bacteria. Organisms from these habitats may provide unique biomolecules for industry and medicine. These organisms have no temperature regulation and their internal temperature is close, if not identical, to that of the environment. Therefore, they have developed several adaptations through structural changes at the level of membranes, proteins and constitutive enzymes that allow them to compensate the effects of low temperatures (GERDAY, 2000).

The enzymes are essential for the adaptation of an organism to a cold environment. This fundamental aspect is closely associated with a strong biotechnological interest in the unique properties of these enzymes produced by microorganisms capable of growing at temperatures close to 0 ° C. Most psychrotolerant and psychrophilic enzymes are characterized by a change in the apparent optimum temperature of activity, a high rate of reaction (up to 10 times higher in comparison with the k_{cat} of homologous mesophilic enzymes) and many cold adapted proteins have flexible point regions, around the active site. The high flexibility of the enzyme is reflected in reduction of ΔH, high k_{cat} and in most cases a higher K_M (SIDDIQUI, 2006).

The yeasts present some characteristics that make them attractive for the production of biopharmaceuticals, because they are organisms well studied, most are listed as GRAS (General Recognized As Safe) organisms and have been used in industrial applications, grow relatively fast in simple media,and there are on the market fermentation equipment and technologies that operate on industrial scales (WALSH 2007) .

In this context, due to the possibility of finding in yeasts species producers of ASNase in combination with the property of the psychro-tolerant and psychrophile microorganisms to generate enzymes with improved kinetic properties that could have different characteristics of the current ASNases. The present study aims the screening of ASNase production in yeast isolated from samples collected in the Antarctic Peninsula, during the expeditions realized by the PROANTAR program. The search and studied the new ASNases enzymes could allow the generation of a new biopharmaceutical that could be used for the treatment of ALL.

1.2 L-ASPARAGINASE AS A BIOPHARMACEUTICAL DRUG.

1.2.1 Anti-tumor action of L-Asparaginase

The LLA and other tumor cells are unable to produce the enzyme Asparagine Synthetase (AsnSynt) responsible for the *de novo* synthesis of L-asparagine in normal cells. This dependence of exogenous L-asparagine, to survive turns it essential for malignant cells and not for normal cells, this metabolic deficiency makes possible to attack cancer cells with specificity. The presence of ASNase in the blood stream, limit the intake of L-asparagine for tumor cells necessary for the synthesis of proteins triggering a metabolic imbalance in the cell resulted in the cell death via apoptosis (AVRAMIS and TIVARI, 2006; COVINI et al, 2012). For this reason, the use of ASNase represents a potent anti-leukemic agent, especially when used in combination with other chemotherapeutic agents such as vincristine, methotrexate, cytarabine, daunorobicin and doxorubicin (VAN DEN BERG, 2011).

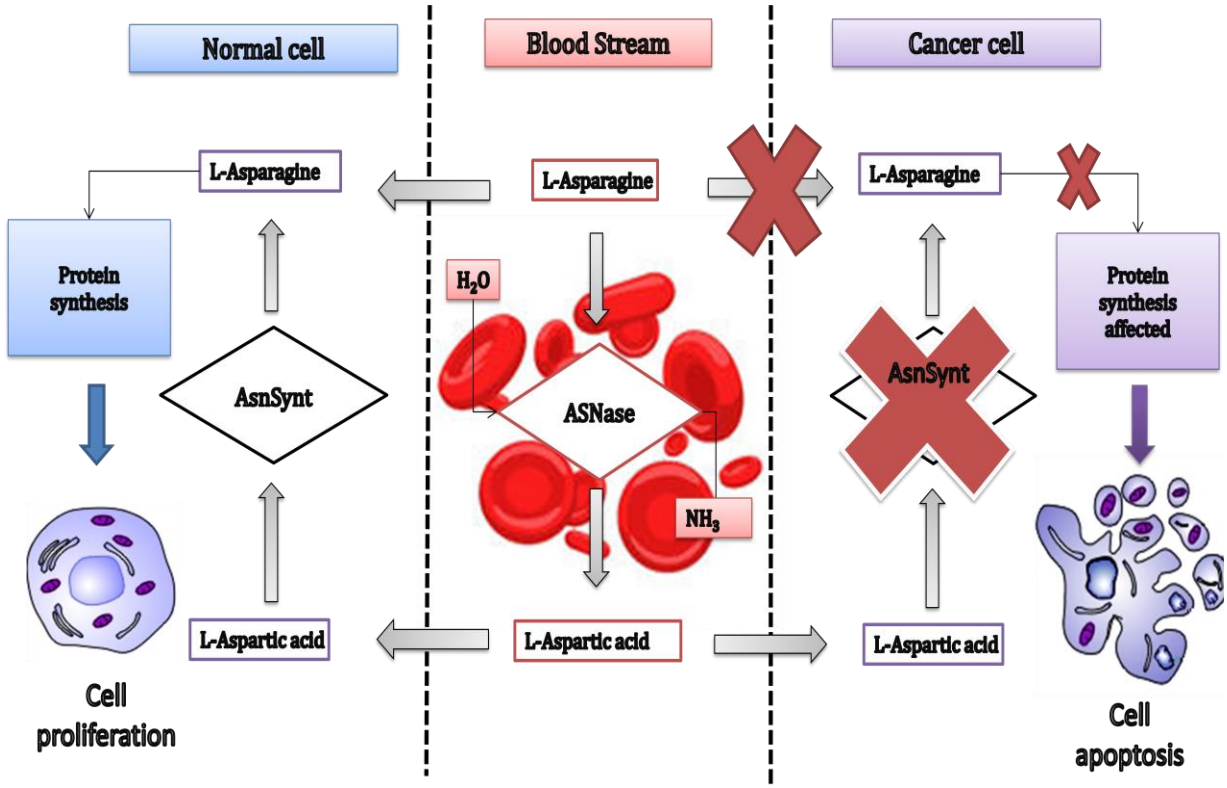


Figure 1. Therapeutic action of L-asparaginase (Modified from Van der Berg et al 2011).

The history of ASNase begins in 1953 when Kidd of Cornell Medical College discovers that guinea pig serum reverses induced leukemia in rats, latter in 1961 Broome and Cornell identified the ASNase as the inhibitory agent present in guinea pig serum, the presences of ASNase in the blood of guinea pigs was previously reported by Clementi in 1922, who suggested that ASNase was an adaptation of the herbivore animals to a diet rich in L-asparagine. Due to the large amounts of serum required for the inhibition of tumors, alternative sources of the enzyme were sought. In 1964 Mashbur and Wriston reported the production of ASNase by *Escherichia coli,* this discovery allowed the supply of quantities needed to conduct of ASNase clinical studies (COONEY and HANDSCHUMACHER, 1970; LIVINGSTON and KRAKOW, 1970). Finally in 1978 after many clinical trials, the FDA approved ASNase as a drug for the treatment of leukemia. And, in 2011 the ASNase from *E. chrysanthemi* was approved for its use in patients with hypersensitivity to *E. coli* ASNase (GERVAIS et al 2013).

The bacterial type II ASNase are the only currently type of enzyme uses for therapeutic treatment of leukemic lymphoid and other cancers. There are three dosage forms commercially available, the ASNase from *Escherichia coli*, (Elspar®, Kidrolase® Leunase®, Medac™, Crastinin™), some of these formulations there are no longer available, the enzyme isolated from *Erwinia chrisantemy* (Erwinase®) and the pegylated *E. coli* enzyme (Oncospar®) that presents fewer hypersensitivity reactions that native form (PIETERS et al, 2012). The enzyme as a drug from both sources has identical mechanism of action, although, the pharmacokinetic properties of the two enzymes differ, for example, Erwinia's ASNase is considered less toxic and is often employed when there are allergic reactions to the E. *coli* ASNase. However, it has a shorter half-life that *E. coli* enzyme, suggesting the need to discover new ASNases that are serologically different and have similar therapeutic effects (NARTA et al, 2007). Since then the search for ASNases has been based on the kinetic properties and the physicochemical and biochemical conditions (such as optimum pH, optimum temperature, substrate specificity, inhibition patterns, toxicity) and this varies in each microorganism, these differences stimulate the demand for the search the best ASNase for a better treatment of ALL, that is, an enzyme with the highest activity, lower adverse effects and a higher half-life. This requires the screening of samples from several potential sources for the isolation of microorganisms that have the desired capacity to produce the enzyme (VERMAN et al, 2007).

Table 1. Characteristics of Biopharmaceutical L- Asparaginases.

Enzyme	Specific activity (U/mg)	Asn K_M (μM)	Gln K_M (mM)	Side activity of GLNase	Molecular weight (kDa)	Pi	Half life
***E. coli* Native**	280-400	15	6.25	3-5 %	140	5.0	8-30 h
***E. coli* PEG**	28-40*	**	**	**	--	**	5.2-7.0 days
***E. chysantemi* Native**	650-700	18	1.1	9%	138	8.7	8-22 h

* Modification of ASNase with activated PEG_2 results in a loss up to 90% of its activity.

** The PEG-ASNase from *E. coli* showed the same pH, temperature dependencies of activity and kinetic parameters as the native ASNase (WADA et al 1990).

1.2.2 **Adverse effects of L-Asparaginase treatment**

Although the bacterial type II ASNase has been used for more than 40 years, there are some intrinsic problems to overcome. Hypersensitivity reactions and inactivation by antibodies production against the enzyme are reported in a 60% of patients with ALL. Alternatively, in some patients the presence of anti-ASNase antibodies may not lead to signs and symptoms; this is known as silent inactivation or subclinical hypersensitivity (PANOSYAN, et al 2004). Hypersensitivity to ASNase can manifests as an overt allergic reaction, with symptoms including anaphylaxis, pain, edema, urticaria, erythema, rash, and pruritis (PIETERS et al., 2011). Side effects like thrombosis, hepatic dysfunctions, acute pancreatitis, brain dysfunctional syndrome, coagulopathies, and glycemia are attributed to the GLNase side activity presented by the ASNase (OHNUMNA et al., 1970; OLLENSCHLAGER et al., 1988, LIU et al., 2016).

Table 2. Adverse reactions produced by the L-Asparaginase treatment.

Adverse reaction	% of patients affected by the ASNase treatment (evaluated/affected)
• Immediate	
1. Nausea, vomiting, and chills	(27/38) 71
• Delayed	
1. Hepatic: Increase in bilirubin, GOT, LDH, alkaline phosphatase, decrease in albumin, cholesterol, fibrogen, and other hepatic coagulation factor- fatty metamorphosis.	(32/33) 97
2. Loss of weight	(24/30) 80
3. Azotemia	(25/38) 68
4. Neurological: Headache, drowsiness, depression, disorientation, confusion.	(13/39) 33
5. Pancreatic: Abdominal pains, increase in amylase or lipase, hyperglycemia, hypoinsulinemia, malabsorption syndrome	(6/39) 15
6. Inmunological: Positive skin test, anaphylactoid reaction	(5/39) 13

* Modified from Ohnumna et al 1970.

Asparagine and glutamine differ structurally in only one methyl group, and hence L-asparaginases have the dual substrate specificity resulting in a decrease in the concentrations of both amino acids in the body. Therefore, the production of an ASNase with a lower affinity by L-glutamine and regimens with fewer doses need to be reconsidered in order to avoid these difficulties. Hence, present-day researchers are mainly focused on minimizing or completely eliminating the L-glutaminase activity of the enzyme L-asparaginase (RAMYA et al., 2012; KIN CHAN et al., 2014).

But, the need to have a GLNase activity together with ASNase to achieve a therapeutic effect has generated controversy since in the last decade there has been suggested that GLNase activity generally increases the efficiency of ASNase and this activity is required to produce an antitumor effect (ANISHKIN et al., 2015). Clinical studies of an ASNase with high GLNase activity obtained from *Acinetobacter glutaminasificans* shows considerable toxicity, suggesting that the decrease in glutamine produces greater toxicity rather than the improvement in antitumor activity.

On the other hand, there is a hypothesis that an improvement in the treatment with ASNase can occur if it diminishes the GLNase activity. A "GLNase free" ASNase isolated from *Wolinella succinogenes* has been studied by the US National Cancer Institute Rapid Access to Intervention Development. Unexpectedly this ASNase showed GLNase activity and was toxic to the patients (NGUYEN et al, 2016). Whereas this enzyme was not actually free of GLNase activity the question of whether the reduction of GLNase activity improves the therapeutic index is still unresponsive (CHAN. et al 2014).

1.3 ASPARAGINASES TYPES AND STRUCTURAL ASPECTS

1.3.1 Mechanism of enzymatic action and structural aspects of L-asparaginase

L-Asparaginases (EC. 3.5.1.1) are enzymes that catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia, the proposed mechanism of enzymatic action of the ASNase is analogous to that of serine proteases. In the first step, a nucleophilic attack on the amide carbon atom of the L-asparagine substrate, launched by the hydroxyl group of a catalytic residue, Thr12 or Thr89, leads via a tetrahedral transition state to a β-acyl-enzyme intermediate, which in the second step undergoes another nucleophilic attack from the water molecule, with conversion of the second tetrahedral species into the final product. Structural and functional studies revealed that the so-called catalytic triad composed of three polar amino acids, namely Thr-Lys-Asp is essential for enzyme activity (BOREK, et al, 2014).

Although the active site residues of L-asparginases have been identified in the early crystallographic studies, and the structural as well as kinetic experiments resulted in a definition of two basic requirements for suitable active site ligand as its size and chemical composition, details of specificity determinants for both the enzyme and ligand molecules are just beginning to emerge. This knowledge is instrumental for providing more complete understanding of the biological properties of ASNase and well as for guiding ist possible modifications that would allow creation of more efficient therapeutic molecules. (AGHAIÝPOUR et al 2001).

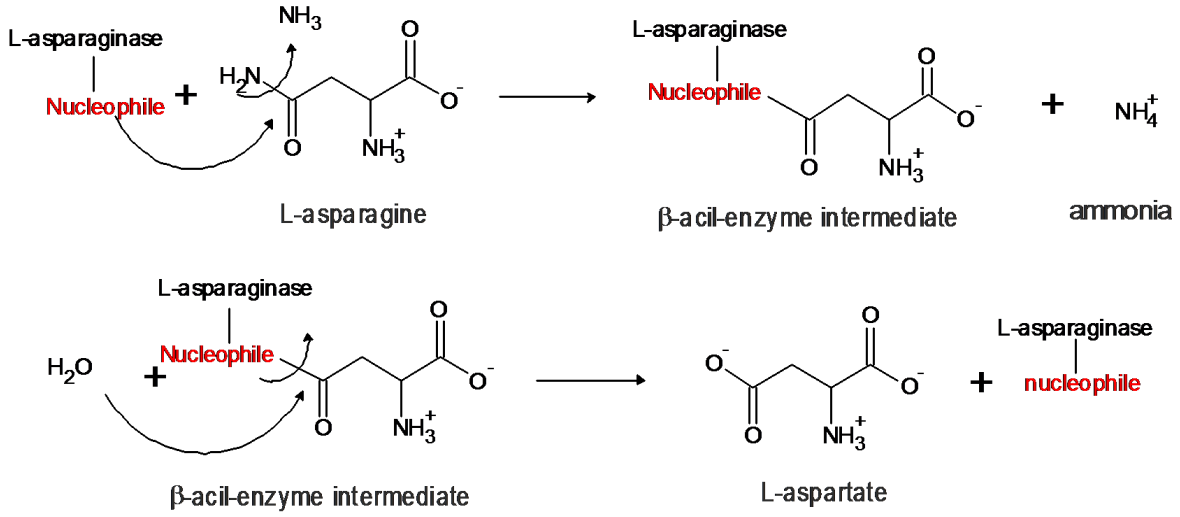


Figure 2. Mechanism of L-asparginase enzymes (Sanson and Jaskolsky, 2004).

1.3.2 **Asparaginases classification.**

From an amino acid sequence analysis, the ASNases can be classified as bacterial type, plant type and enzymes similar to *Rhizobium etli* ASNase (Figure 2) (BOREK and JASKÓLSKI, 2001). Bacterial ASNases can be further subdivided into two types: type I, which are expressed constitutively and display enzymatic activity towards both L-asparaginase and L-glutaminase, and type II, induced by anaerobic condition, which have a higher specific activity towards L-asparagine. The bacterial-type enzymes frequently exhibit other activities as well, and this family may be significantly larger than the collection of sequences deposited as asparaginases. In particular, enzymes such as glutamin-(asparagin)-ases (EC 3.5.1.38), lysophospholipases (EC 3.1.1.5), and the α-subunit of Glu-tRNA amidotransferase (EC 6.3.5.-) can also be considered part of the bacterial asparaginase family (BOREK et al, 2004).

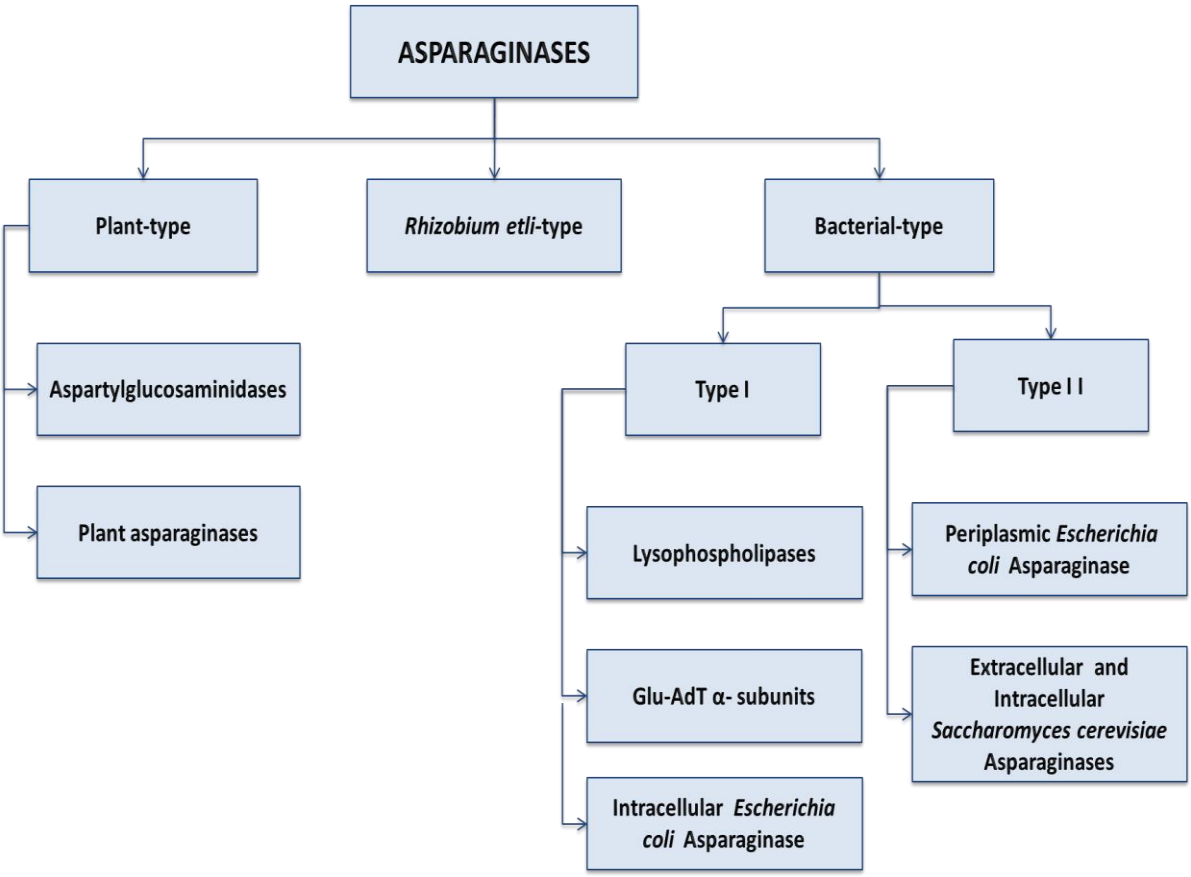


Figure 3. General classification of Asparaginases (Borek and Jalskolski 2001).

The bacterial ASNase are the more studied and the best characterized of this enzyme family, they are composed of four identical subunits, each monomer consist of about 330 amino acid residues, each monomer is formed by two easy identifiable α/β domains, a larger N-terminal and a smaller C-terminal domain, connected by a loop consisting of about 20 residues. It has been suggested that the flexible loop has an important role in the catalytic reaction. In an open conformation, it assists in substrate recognition, once the substrate is bound, the loop undergoes a series of conformational changes allowing several residues to interact with the substrate molecule and to determine its proper orientation with respect to the rigid part of the active site. Therefore, loop flexibility is considered to be fundamentally involved with the enzymatic activity of the type II ASNases. Conversely, is observed that in the structure of type I ASNases this region is stabilized by the formation of a β-hairpin that augmented its rigidity, decreasing the affinity for the substrate (SANCHES et al 2007).

The monomers are able to associate tightly with each other forming intimate dimers characterized by an extensive interface between the subunits that are held together by several interactions, mainly van der Waals and electrostatic interactions. Four independent ASNase catalytic sites are located at the intersubunit interface of the intimate dimers and, the association of the two dimers results in the tetrameric biological unit with 222-symmetry and a molecular mass in the range of 140-150 kDa, which is kept together by molecular interactions similar to those found in the homodimers, more accurately the ASNase is described as dimers of intimate dimers (KOTZIA et al 2007; JASKOLSKI et al 2011).

1.4 **FONTS OF ASPARAGINASES**

The interest for studied new fonts of asparaginases is promoted basically by the applications anti-carcerigenics properties of these enzymes. The ASNase is present in a wide variety of organisms including animals, microbes, plants and the serum of some rodents. Although, ASNase has been found in some species of plants and animals, due to the difficulty in extracting and purifying other sources such as micro-organisms are preferred (N. EL-AHMADY EL-NAGGAR et al 2014). Microorganisms are the best sources of ASNases because they can be grown easily and extraction and purification can be carried out on a large scale and relatively easily. A wide variety of bacteria, fungi, yeasts, actinomycetes, algae have been reported as producers of ASNase. Even though not all enzymes of microorganisms have anti-tumor properties, the variation of this activity has been related to their affinity for the substrate and the rate of elimination of particular types of enzymes (EL-GONEMY 2014). According to the literature, the production of ASNase can occur under different conditions by different microorganisms (SAVITRI and AZMI, 2003).

1.5 GENERAL OBJETIVES

Current reviews listed the producer yeasts of ASNases, but none goes beyond that a few ASNases of yeasts reports in past decades, even so little it is known about the properties of yeast ASNases as biopharmaceutical, it is expected that the ASNases of yeasts will exhibit different biochemical and serological characteristics but offering similar or better therapeutics effects derived from microorganisms that not been studied yet for this purpose.

In this context of the possibility of finding in yeasts species producers of ASNase in combination with the property of the psychro-tolerant and psychrophile microorganisms to generate enzymes with improved kinetic properties that could have different characteristics of the current ASNases. The present study aims the screening, characterization and production of ASNase from yeast isolated from samples collected in the Antarctic Peninsula, during the expeditions realized by the PROANTAR program. The search and studied the new ASNases enzymes could allow the generation of a new biopharmaceutical that could be used for the treatment of ALL.

1.5.1 **SPECIFIC OBJETIVES**

The aims of the present study were to

- Screening the production of L-Asparaginase by psychrotolerant yeast isolated from Antarctic Peninsula and select the best producing strain.

- Purify and characterize the: L-Asparaginase of *Leucosporidium scottii* to determine the optimum pH, optimum temperature and determined kinetic parameters.

- Apply statistical DOE methods to optimize the medium composition for the production of L-Asparaginase by *L. scottii* by submerged fermentation in shake flask.

- Study in bioreactor the effect of different conditions of agitation and aeration in the enzyme production and lipid accumulation in *Leucosporidium scottii* L115.

CHAPTER II. SCREENING AND CHARACTERIZATION OF L-ASPARAGINASE PRODUCED BY A PSYCHROTOLERANT YEAST *Leucosporidium scottii* L115

1.6 INTRODUCTION

1.6.1 Psychrotolerant and psychophiles microorganisms

Cold environments were successfully colonized by numerous organisms, particularly bacteria, single-celled algae, fungi, and yeasts. Ecological niches little explored like the Antarctic continent has received attention due to the biotechnological potential of the complex metabolic diversity and unique and particular biomolecules that can present the microorganisms that inhabit these ecosystems (SHIVAJI and PRASAD 2009). The Antarctic continent is characterized by its extreme environmental conditions, presenting the coldest and driest climates known on the planet, very low temperatures (average temperatures below 0 ° C), frequent freeze-thaw cycles, low precipitation and low availability of nutrients, as well as high salinity and high UV radiation alternated with prolonged periods of obscurity, these characteristics represent a state of constant stress for the microorganisms that inhabit there (ONOFRI et al. 2007; MARGESIN and MITEVA 2011). The ability of microorganisms to survive and grow in cold environments is the result of a range of molecular and physiological adaptations. Considering that these organisms do not have temperature regulation, their internal temperature is close, if not identical, to that of the environment. Therefore, they have developed several adaptations in the form of structural changes at, for example, their membranes, proteins and constitutive enzymes, to compensate for the drastic effects caused by the low temperatures (GERDAY et al 2000).

1.6.1 Psychrophyilic and psychrotolerant enzymes

The enzymes are an essential target for adapting an organism to a cold environment. Therefore, the easiest strategy to maintain a permanent activity at low temperature is the production of enzymes adapted to cold environments capable of increasing its catalytic efficiency. The cold-adapted enzymes tend to have an increase in the structural flexibility resulting in a reduction of the activation energy and consequently increasing the catalytic efficiency (k_{cat}/K_M ratio). In general, the specific activity of psychrophilic enzymes is higher at temperatures of 0 to 30 ° C than those isolated from mesophiles (FELLER and GERDAY, 1997; HOYOUX et al., 2004)

Most psychrophilic enzymes are characterized by a change in the apparent optimum temperature of activity, a high rate of reaction (up to 10 times higher in comparison with the k_{cat} of homologous mesophilic enzymes), and many cold-adapted proteins have flexible point regions around the active site. The high flexibility of the enzyme is reflected in the reduction of ΔH, high k_{cat} and in most cases a higher K_M (SIDDIQUI, 2006). Loops are considered a diverse class of secondary structures comprising turns, random coils, and stands which connect the main secondary structures (α-helices and β-strands), and these regions belong to the most flexible parts of enzyme structures and sequential changes in these regions are frequently related to the evolution of the enzymes (NESTL and HAUER, 2014).

From a structural point, these proteins have a higher content of α-helix relative to the β-sheets, which is considered an important factor to maintain the flexibility even at low temperatures. At low temperature, the low kinetic energy of the molecules involved in the reaction is compensated by the flexibility in the structures of the enzymes. This flexibility is a function of the combination of structural characteristics, such as reduction in the hydrophobicity of the catalytic core, decrease of electrostatic and ionic interactions, increased loading of surface residues that promote the interaction of solvents, additional loops on the surface, substitution of proline residues by glycines in these surface loops, decreased arginine/lysine ratio, less interactions between subunits and interdomains, and less aromatic interactions. The final effect of these modifications is that the active site and adjacent regions remain flexible. This increase in conformational flexibility is accompanied by an increase in thermolability (CAVICCHIOLI et al 2002).

For extracellular enzymes that work at substrate saturation concentrations, the adaptation mainly consists of increasing k_{cat}. In the contrary, for intracellular enzymes which may be at conditions where there are low substrate concentrations, the adaptation is observed in a decreased K_M, which means a higher affinity for the substrate. A compilation of available data indicates that the enzymes isolated from psychrophilic and psychrotolerant microorganisms optimize their catalytic efficiency (k_{cat} / K_M) mainly by increasing the k_{cat}, in some cases the K_M reduction is achieved and in the best panorama the modification of both parameters. Thus, an appropriate physiological adaptation at enzyme level is a key aspect for microorganism survival, being these biocatalysts of great biotechnological interest for the industry (GERDAY et al., 2000; D´AMICO et al., 2002; CAVICCHIOLI et al., 2011).

1.6.2 **Yeast Isolated from Antartic ecosystems**

About 90% of the yeasts isolated from Antarctica and other cold environments are from the basidiomycetous origin (DE GARCÍA et al., 2006; SHIVAJI and PRASAD, 2009; CARRASCO et al., 2012; BUZZINI et al., 2012, DUARTE et al., 2013). The basidiomycetous yeasts are been poorly explored for the industrial applications but they are capable to produce valuable metabolites such as enzymes, terpenoids, and carotenoids among others biomolecules used in pharmaceutical and chemical industries (JOHNSON, 2013). The Table 3 shows some enzymes isolated from yeasts isolated from Antarctic samples.

Table 3. Enzymes reported produces by psychrotolerant and psychorphile yeasts.

Enzyme	Yeast	Reference
Serine protease	*Leucosporidium antarticum*	Turkiewicz et al 2003
Subtilase	*Leucosporidum antarcticum*	Pazgier et al 2003
Lipase A e B	*Pseudozyma Antarctica (Candida antarctica)*	Michiyo 1986
Pectinase	*Mrakia frigida*	Margesin et al 2005
α-Amilase	*Candida Antarctica*	De Mot and Verachtert, 1987
Invertase, α-glucosidase	*Leucosporidium antarcticum*	Turkiewicz et al 2005
Acid β-galactosidase	*Guehomyces pullulans*	Nakagawa et al 2006
Xylanase	*Crytococcus adelaie*	Petrescu et al 2000
Xylanase (termolabile)	*Crytococcus adelaie*	Gomes et al 2000
Polygalacturonases	*Cryptococcus aquaticus, Cryptococcus macerans, Cystofilobasidium capitatum, Cystofilobasidium lari-marini*	Birgisson et al 2003
β-galactosidase	*Guehomyces pullulans 17-1*	Song et al 2010
Acid protease	*Rhodotorula mucilaginosa* L7	Lario et al 2015
Aspartic protease	*Sporobolomyces roseus*	Krysiak et al 2016
Pectinases	*Cystofilobasidium infirmominiatum, Cryptococcus adeliensis, Guehomyces pullulans*	Cavello et al 2016

The lipase B isolated from *Candida Antarctica* is used in a very large number of organic synthesis applications related to food, pharmaceutical, and cosmetics industries, generating diverse patents, demonstrating the potential of yeasts species that inhabit cold environments to produces novel molecules with industrial (JOSHEP et al 2008). The yeasts isolated from Antarctic ecosystems are mainly species belonging to the genus *Cryptococcus, Rhodotorula, Leucosporidiella, Sporobolomyces, Leucosporidium, Candida, Mrakia,* and *Meyerozyma*.

1.6.3 **Asparaginases of eukaryotic microorganisms**

In comparison to the bacterial enzymes, the ASNases from eukaryotic microorganisms have been studied and reported in a smaller number of species (Table 4). Eukaryotic microorganisms like filamentous fungi of the genus *Aspergillus, Penicillium*, and *Fusarium*, are commonly reported in the literature as producers of ASNase and GLNase. It has been observed that eukaryotic microorganisms like yeasts and filamentous fungi are commonly reported in scientific literature as producers of ASNases with less possible adverse effects.

Table 4. Eukaryotic microorganisms reported as producers of ASNase.

Microorganism	***Species***	**Reference**
Yeasts	*Hansenula sp. , Cryptococcus sp., Candida utilis, Rhodotorula rosa*	ARIMA et al 1972
	Hansenula sp. Cryptococcus sp., Rhodotorula sp., Sporobolomyces sp.	IMADA et al (1973)
	Saccharomyces cerevisiae	DUNLOP and RON (1978)
	Pichia polymorpha	FODA et al (1980)
	Candida utilis	KIL et al (1995)
	Rhodosporidium toruloides	RAMAKRISNAN and JOSEPH (1996)
Fungus	*Aspergillus sp., Penicillium sp.*	ARIMA et al 1972
	Fusarium sp., Hypomyces sp., Nectria sp., Penicullium sp.	IMADA et al 1973
	Aspergillus nodulans	DRAINAS et al 1977
	Aspergillus terreus, Aspergillus tamarii	SARQUIS et al (1994)
Algae	*Chlamydomonas sp*	PAUL 1982
	Spirulina máxima	ABD EL BAKY and EL BAROTY (2016)

1.6.4 **Yeast Asparaginases**

There are few studies on the production and characterization of anti-ALL ASNases by yeasts. By far, the most studied asparaginase of yeast is the ASNase II from Saccharomyces *cerevisiae*. The production of ASNases in *Saccharomyces cerevisiae* was studied by Dunlop and Roon (1978, 1980), in which was reported two forms of ASNases: ScASNase I, an internal constitutive enzyme, and ScASNase II, a mannan glycoprotein located in the cell wall an external enzyme which is regulated by the amount and source of nitrogen available in the growth medium. This pattern of intracellular and secreted enzymes is shared with the ASNase production in bacteria, where just the type II extracellular enzyme is effective against ALL. Even though, the phylogenetic analyses suggested that these enzymes evolved in an independent way in prokaryotes and eukaryotes. And, the amino-acid sequences analysis of the two types of ASNases (the cytosolic and the cell wall glycoprotein) present in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, classifies both enzymes as bacterial type II enzymes (BONTHORN AND JASKÓLSKI, 1997; BOREK AND JASKOLSKI, 2001; MICHALSKA AND JASKOLSKI, 2006). Recently, Costa et al., (2016) reported the cytotoxicity activity in the MOLT-4 leukemic cells of a recombinant ScASNase I (cytosolic) demonstrating the promising antineoplastic properties of these kinds of enzymes.

In this context, the present study aims the screening and characterization of ASNase produced in yeast isolated from samples collected in the Antarctic Peninsula, during the expeditions realized by the PROANTAR program. The search and studied of new ASNases enzymes with improved kinetic properties that could generate a new biopharmaceutical used for the treatment of ALL.

1.7 MATERIALS AND METHODS

1.7.1 Microorganisms

The 40 yeast strains used in this study were isolated from diverse samples obtained during an expedition to Antarctica peninsula in the austral summer of 2010 by the Brazilian Antarctic Program team (Table 5) and the microorganisms were identified using the 26S rDNA sequencing (DUARTE et al, 2013).

1.7.2 Preparation of inoculum culture and enzyme production

The yeast strains were reactivated in potato dextrose agar (PDA; Difco^{TM}) and incubated at 15°C for 72 h. The activated strains were incubated in submerged culture in potato dextrose broth (Difco^{TM}) at 15°C and 150 rpm in an orbital shaker during 48 hours. The cells were harvested by centrifugation at 3400 x*g* for 15 minutes at 5°C, and washed with sterile water. For ASNase production the yeast cells were inoculated at 5 g L^{-1} of initial inoculum in 100 mL flasks containing 50 mL of modified Czapeck Dox's medium (GULATI et al, 1997). After 24, 48, 72 and 96 hours, samples of the culture were collected and the cells and medium culture were separated by centrifugation. At each point, the ASNase and GLNase activity was determined in the culture medium and in the cells.

1.7.3 Assay of periplasmic activity of ASNase and GLNase by hydroxylaminolysis reaction

The ASNase and GLNase activity were determined by the hydroxylaminolysis reaction and using the whole cell for the enzymatic reaction (GROSSOWICZ et al, 1950; FERRARA et al., 2004). In a eppendorf tube was added, 1.6 mL of cells suspension at 1.0 of DO in 50 mM Tris-HCl buffer pH 7.0, 0.2 mL of 0.1 M L-asparagine and 0.2 mL of 1.0 M solution of hydroxylamine hydrochloride at pH 7.0, the mixture was agitated on a thermomixer at 850 rpm at 37ºC, after 30 minutes the enzymatic reaction was stopped by adding 0.25 mL of TCA/$FeCl_3$ reagent (100 g L^{-1} $FeCl_3$, 50 g L^{-1} TCA in 0.66 M HCl). The tube was centrifuged at 3400 x*g* for 5 minutes and 1.0 mL of the supernatant was measured at 500 nm. Controls were prepared in the same way as samples, but the hydroxylamine and asparagine solutions were added after the ferric chloride reagent. The GLNase activity was realized using the same process but 0.1M of asparagine was substituted for 0.1 M glutamine solution. One unit of ASNase and GLNase was defined as the amount of enzyme that produces 1μmol of β-aspartohydroxamic acid or 1μmol of γ-glutamohydroxamic acid correspondently, formed by minute by gram of dried cell weight (U g^{-1}cdw).

1.7.4 Thin Layer Chromatography (TLC) of reaction products from *Leucosporidium scottii* cells

The reaction of ASNase was performed by the addition of 0.2 ml of 0.1M L-asparagine, 0.2 mL of 1.0 M hydroxylamine pH 7.0 and 1.6 mL of cells suspension at 1.0 of OD in 50 mM Tris-HCl buffer pH 7.0. The mixture was agitated on a thermomixer at 850 rpm at 37ºC.After 30 minutes the enzymatic reaction was stopped by centrifuged the reaction at 3400 x *g* for 5 minutes, and 1.0 mL of supernatant was sample and stored at 5°C.The blank reaction was prepared in the same way but yeast cells were inactivated by heating them at 80 °C for 15 minutes. The reaction and controls were run on a silica gel plate according to the method reported by Ramakrishnan et al., (1996). It was used phenol/water (4:1) (w/v) as the mobile phase. The plate was revealed spraying 0.5% (w/v) of ninhydrin dissolved in acetone and heated using a hair dryer until the appearance of the points.

1.7.5 ASNase assay by hydrolysis reaction

The enzyme activity was assayed by the quantification of the ammonia released using the Nessler's reagent (IMADA et al., 1973). In a eppendorf tube, 50 µL of enzyme were incubated in 0.5 mL of 50 mMTris-HCl buffer pH 8.0, with 50 µL of 189 mM L-asparagine and 0.45 mL of ultrapure water, the mixture was agitated on a thermomixer at 850 rpm at 37ºC, after 30 minutes the reaction was interrupted by the addition of 50 µL of 1.5 M trichloroacetic acid (TCA). The solutions were mixed by inversion and centrifuged at 3400 *g* for 5 minutes. The release of ammonia was determined by adding 0.25 mL of Nessler's reagent to 0.1 mL of the sample diluted in the final volume of 2.5 mL of ultrapure water. The color developed was quantified at 436 nm. One unit of ASNase activity is the amount of enzyme, which produced 1 µmol of ammonia per minute.

1.7.6 Enzyme extraction

Cultures of *L. scottii* were centrifuged at 3400 xg, the cells were harvest and washed twice with sterile water, resuspended at 50% (v/v) in lysis buffer containing 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM cysteine, and 1 mM PMSF. 25 mL of cells suspension was passed through a French press homogenizer at 1500 psi during 10 cycles, maintaining the suspension in an ice bath between each cycle to avoid the rise of temperature. The homogenized suspension was centrifuged at 18514 x*g* for 20 minutes and the supernatant was collected.

1.7.7 **Enzyme purification**

1.7.7.1 **Polyethylene glycol 4000 for extract clarification**

A Polyethylene glycol 4000 solution (PEG 4000) (66 % w/v) was added to the supernatant the cell homogenate obtained to reach the 6.6% of final concentration of PEG 4000. The mixture was left in an ice bath for 30 minutes and latter centrifuged at 18514 x *g* for 15 minutes. The supernatant was filtered tough 0.45 μm membrane and stored at 4º C for subsequent analysis.

1.7.7.2 **Ion exchange chromatography**

The enzyme extract was passed through to a DEAE-HiTrap® GE 5 mL column (DEAE-sepharose) using the AKTA purifier system. The column was previously equilibrated with 20 mM Tris-HCl (pH 8.0) buffer and the protein was eluted with step gradients of NaCl (50, 100, 150, 200, 250 and 500 mM) in 20 mM Tris-HCl pH 8.0 buffer, fractions of 1 mL were collected. Protein quantification was measured using Bradford reagent and the ASNase activity was assayed by the quantification of the aspartic hydroxamic acid formed, using the method described before. The fractions with greater activity were pooled and concentrated within an ultrafiltration membrane cartridge of 10 kDa of molecular weight cut-off.

1.7.7.3 **Size exclusion chromatography (SEC)**

The concentrated protein fraction from the previous step was passed through Superdex® 200 Increase 10/300 GL column (cross-linked agarose-dextran resin) using the AKTA purifier system. The column was equilibrated with 20 mM Tris-HCl (pH 8.0) buffer added with 100 mM glycerol and eluted with the same buffer at the flow of 0.50 mL min^{-1}. Fractions of 0.25 mL were collected and the protein fractions were assayed for ASNase activity. SDS-PAGE was realized to check the purity of protein in each fraction collected.

1.7.8 **Molecular weight determination by size exclusion chromatography**

The molecular weight of the enzyme was determined using the elution volume (V_e) presented in the Superdex® 200 Increase 10/300 GL for the fraction that presents the enzyme activity. The column was calibrated by molecular weight size-marker of proteins: thyroglobulin 669 kDa, ferritin 440 kDa, aldolase 232 kDa, ribonuclease 137 kDa, conalbumin 75 kDa, ovalbumin 45 kDa, and the void volume (V_0) was determined using blue dextran. The standard proteins were run at the same conditions assayed for the enzyme fraction, buffer 20mM Tris-HCl pH 8.0 added with 100 mM glycerol, at 0.75 mL min^{-1} of flow rate. The molecular mass of the enzyme was determined by the linear relationship

obtained by plotting the K_{av} value of the proteins calculated by the equation 1, and the logarithms of their molecular weights (MW) (TAYYAB et al., 1991) See Figure 8.

$$K_{av} = \frac{V_e - V_0}{V_C - V_0} \qquad \text{equation (1)}$$

1.7.9 Determination of molecular weight in reduced conditions and purity of the fractions obtained

The different fractions obtained from the subsequent steps of purification were analyzed by the SDS-PAGE technique in a 12% polyacrylamide gel under reducing conditions in accordance with the method reported by Laemmli (1970). Molecular weight protein marker Precision Plus Protein was used (BioRad). Gels were stained with Coomassie Blue R-250 and subsequently with silver staining. The approximate enzyme molecular weight was estimated by determining the relative migration distance (R_f) and interpolating the value from the linear relation of Log_{Mw} vs R_f. The purity of the enzyme was confirmed by the presence of a single band in the fraction analyzed.

1.7.10 Enzyme glycosylation analysis by SDS-PAGE

The purified enzyme was denatured by adding 1µL of SDS 5%, 1µL of DDT 1M, and heating at 95°C for 5 minutes and cooled for 5 minutes at room temperature. The pH of the reaction was adjusted by adding 2µL of sodium phosphate buffer 0.5 M, pH 7.5 and added with 2µL of Triton X-100 at 10%. Finally, 2µL of peptide N-glycosidase F (PNGase F; Promega™) was added to the reaction and incubated at 37°C for 3 hours. The samples were treated and visualized by SDS-PAGE.

1.7.11 Determination of specific activity of ASNase

The protein concentration of enzyme fractions was assayed using the QuantiPro™ BCA assay kit (Sigma Aldrich®) To determine the ASNase specific activity quantities of the enzyme purified (0.2-1.5 µg) were incubated at 37ºC for 30 minutes in the presence of L-asparagine 20 mM and the ammonium released was quantified through Nessler's reaction as described above. The glutaminase activity was determined the same conditions at the same concentrations of the enzyme using L-glutaminase at 20 mM instead L-asparagine.

1.7.12 **Effect of pH and Temperature**

To determined the optimum pH for LsASNase I were used the following buffers at 50 mM concentration: acetate (pH 4.0- 5.5) phosphate (6.0-7.0) Tris-HCl buffer (7.5-9.0), Glycine-Sodium hydroxide (pH 9.5-10.5), Sodium bicarbonate (11.0). The enzyme was incubated in the corresponding buffer for 24 hours at 4 ºC and after the activity was determine by the hydrolysis reaction described above. To determine the optimum temperature, the enzymatic reaction was performed at different temperatures from 5°C to 65° C and after 30 minutes the enzyme activity was determined by the hydroxylaminolysis reaction, method reported by Grossowicz et al. (1950) described above.

1.7.13 **Effect of metal ions on enzyme activity**

The effect of different metal ions including K^{+} (50mM), Mg^{+2} (50 mM), Na^{+} (50mM, 1M), Ca^{+2} (10mM), Ni^{+2} (10mM), Cu^{+} (10 mM), and EDTA (1 mM) on the partial purified ASNase was studied. The enzyme was incubated during 30 minutes at 37°C in the phosphate buffer added with the corresponding metal ion and L-asparagine 20 mM, the residual activity was assayed and compared with the control, which was considered as 100% activity.

1.7.14 **Circular Dichroism for secondary structure comparison of LsASNase and *E.coli* ASNase type II (ELSPAR®)**

Circular Dichroism (CD) spectra of the samples were obtained in a Jasco J-815 Spectropolarimeter (Jasco, Tokyo, Japan). The final spectra were the average of 6 scans, following subtraction of the spectrum of the buffer 20 mM Tris-HCl pH 8.0 obtained under the same conditions. CD spectra were obtained in the far-UV range (190-260 nm). Samples were placed in 5.00 mm optical length quartz cells with a concentration of 0.28 µM (LsASNase) and 1.92 µM (Control – EcASNase II). Spectra intensities (θ, mdeg) were converted to residual molar ellipticity ([θ], deg.cm^{2}.dmol^{-1}) using the equation 2. Where "C" is the protein concentration in mol L^{-1}, "l" is the optical length in cm and "n" is the estimated number of residues in the protein.

$$[\theta] = \frac{\theta}{10 \times C \times l \times n} \qquad \text{equation (2)}$$

1.7.15 **Circular Dichroism for thermal stability of LsASNase**

Thermal stability studies of the enzyme samples were performed in a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan).The temperature was scanned from 30°C to 95°C, at a rate of 1°C/minute, and back from 95°C to 30°C to study unfolding and refolding processes, respectively. Samples with a concentration of 0.28 μM (LsASNase) or 1.92 μM (Control – EcASNase II) were placed in a 5.00 mm optical length quartz cells, and the intensities of ellipticity at 222 nm (θ_{222}, mdeg) were registered throughout the experiment. Intensities of ellipticity at 222 nm were converted to residual molar ellipticity ($[\theta]_{222}$, deg.cm^2.dmol^{-1}) using the equation 3. Where "C" is the protein concentration in mol L^{-1}, "l" is the optical length in cm and "n" is the estimated number of residues in the protein.

$$[\theta]_{222} = \frac{\theta_{222}}{10 \times C \times l \times n} \qquad \text{equation (3)}$$

1.7.16 **Determination of kinetic enzymatic parameters**

The enzyme kinetic behavior was followed by oxidation of β-NADH in present of glutamate dehydrogenase and ammonia, a product of L-asparagine hydrolysis by the ASNase action. The β-NADH depletion was monitored spectrophotometrically at 340 nm and the change in absorbance over time is proportional to the ASNase rate reaction (BALCÃO et al., 2001). The reaction quantities were modified for its use in microtiter plate. The L- asparagine concentration was varied from 0 to 2 mM to determine the kinetic parameters. In each well **t**he enzyme reaction mixture was prepared by combining 87.5 μL of 200 mM Tris-HCl buffer pH 7.5, 53.75 μL destilled water, 3.5 μL of 110 μMα-ketoglutarate (solved in 100 mM Tris-HCl buffer pH 8.0), 8.75 μL of 128 μMβ-NADH, and 17.5 μL of 0.148 μM of GDH (solved in phosphate buffer pH 7.5 with glycerol at 50% (v/v)) and 159 μL of the corresponding L-asparagine solution (2.0, 1.0, 0.75, 0.5, 0.25 0.1, 0.075, 0.05, 0.025, 0.0125, and 0 mM). For started the reaction were added 20 μL of the purified LsASNase I (≈ 2.7 x 10^{-8} M). The final volume per well was 350 μL. The molar extinction coefficient of β-NADH was experimentally determined as 6100 mol^{-1} cm^{-1}. The substrate affinity and turnover number were estimated using non-linear regression fitting in the GraphPhad Prism software version 5.0.

1.8 RESULTS AND DISCUSSION

1.8.1 Screening of enzyme activity

The activity for ASNase and GLNase by the yeast strains was determinate by the corresponding formation of β-aspartohydroxamic acid (AHA) and γ-glutamohydroxamic acid (GHA) by the action of the enzyme in the presence of hydroxylamine and the corresponding amide. Also, extracellular activity in the cell-free cultures was tested, however, no extracellular activity was detected in any yeast strain cultures. From the 40 strains analyzed 23 corresponding to Ascomycota and 17 to Basidiomycota phylum. In this screening, 16 strains were able to form the corresponding hydroxamic acid from one or both substrates, and 15 of these 16 strains correspond to the genus Basidiomycota.

Table 5. Yeast strain isolated from samples collected in Antarctic Peninsula tested for the production of ASNase and GLNase

Strain code	Specie	Phylum
L3, L6	*Debaryomyces hansenii*	Ascomycota
L4, L5, L9, L10, L11, L13, L17,L18,L19, L20, L21	*Myerozyma guillermondii*	Ascomycota
L7, L26, L38	*Rhodotorula mucilaginosa*	Basydiomycota
L32, L42, L43, L92, L124, L97, L122, L123	*Cryptococcus victoriae*	Basidiomycota
L35	*Cryptococcus laurentii*	Basidiomycota
L36, L37a, L 55	*Wickerhamomyces anomalus*	Ascomycota
L58	*Candida sake*	Ascomycota
L79, L83, L84	*Candida glaebosa*	Ascomycota
L81, L82,	*Debaryomyces macquariensis*	Ascomycota
L88	*Guhomyces pullulans*	Basidiomycota
L94	*Cryptococcus albidiosimilis*	Basidiomycota
L114	*Candidada viasina*	Ascomycota
L115, L117, L118	*Leucosporidium scottii*	Basidiomycota

The Figure 4 shows the summary of activities obtained ASNase and GLNase, the activity is expressed as units for grams of cell dry weight (U g^{-1} cdw). As was expected, some strains present both activities of ASNase and GLNase, and strains from *Rhodotorula mucilaginosa* presented the highest activity from both substrates. The L-38 strain was that shows the highest activity for ASNase 13.72 U g^{-1}cdw with a GLNase activity of 30.50 U g^{-1} cdw. The highest GLNase activity was presented by the L-26 strain of *R. mucilaginosa*, which was equal to 33.19 U g^{-1} cdw and a low ASNase activity of 2.63 U g^{-1} cdw. The genus *Cryptococcus* was that present more positive strains with activity from both substrates, 8 from the 16 positives strains. The Sixteen strains of different species were identified as: *Cryptococcus victoriae* (n= 7), *Rhodotorula mucilaginosa* (n=3), *Leucosporidium scottii*, (n=2), *Cryptococcus albidosimilis* (n=1), *Candida glaebosa* (n=1), *Guehomyces pullulans* (n=1), *Meyerozyma guillermondii* (n= 1) present enzymatic activity.

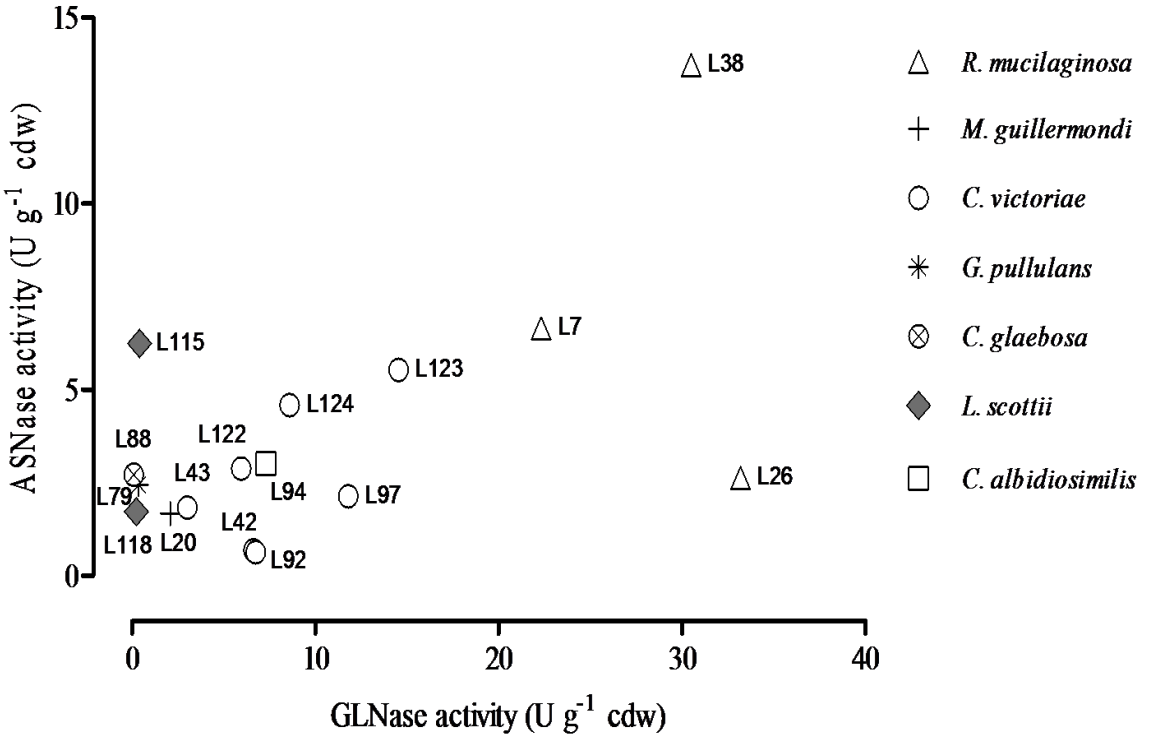


Figure 4. Enzyme activity of ASNase and GLNase in yeast strains isolated from samples collected in Antarctic Peninsula.

The results obtained from the screening coincide with that reported by Imada et al. (1973), wherein in a qualitative study of 1326 yeast strains tested, the 12% presented ASNase activity, GLNase activity, or both activities, most of the strains belonged to the genus *Hansenula, Cryptococcus*, and *Rhodotorula*. And, was reported the GLNase activity without ASNase by one *Leucosporidium scottii* strain, formerly known as *Candida scottii*. In other study realized by Arima et al. (1972) strains of *Meyerozyma guillermondii* formerly known as *Candida guillermondii* were reported with ASNase activity and the *Rhodotorula rosa* strains were those that presented more ASNase activity.

By this characterization we can imply that the amido-hydrolases activities were more common in the basidiomycetous yeasts isolated from samples collected in the Antarctic Peninsula, and the genus with more positive strains were *Cryptococcus* and *Rhodotorula*. These ASNase yeasts present affinity for both substrates in which case are denominated as glutaminase-asparaginases (EC. 3.5.1.38) (SANCHES et al., 2007). The use of the GLNase-ASNase enzymes for clinic applications has been poorly studied. But, earlier studies were realized *in-vitro* (ROBERTS et al., 1972) and *in-vivo* with humans (SPIERS and WADE, 1976) having anti-leukemia effects and recently, new interesting proposals were addressed for this kind of enzymes (RAMYA et al., 2012; SAMUDIO et al., 2013).

As result of the enzyme screening analysis, the *Leuscosporidium scottii* L115 strain was selected for further purification and characterization of the ASNase yeast, since it presented an interesting combination of enzyme activities: 6.24 U g^{-1}cdw of ASNase with a low GLNase activity of 0.41 U g^{-1}cdw. But, some yeasts can produces iron chelators under certain metabolic conditions, *Cryptococcus, Leucospordium, Rhodosporidium, Rhodotorula, Sporidiobolus,* and *Sporobolomyces* strains were reported as rhodoturolic acid an hydroxamic acids producers and the production of hydroxamic siderophrores by marine microorganisms is well known (ATKIN et al 1970; BAAKZA, et al 2003).

To confirm the production of the enzyme by *L. scottii* L115 strain was evaluated qualitatively by TLC the formation of β-AHA and the depletion of L-asparagine during the enzyme reaction. As can be seen in the Figure 5, the first track (ASN) and the second track (β-AHA) correspond to the L-asparagine (20 mM) and the β-aspartohydroxamic acid (20 mM) standards respectivetly. In the reaction blank track (B) just the presence of L-asparagine is detected, but in the reaction track (R) the depletion of L-asparagine is detected after 30 minutes reaction and can be observed the presence of β-AHA formed by the reaction of L-asparagine with hydroxylamine in presence of the enzyme, proving the presences of ASNase enzyme in the yeast cells.

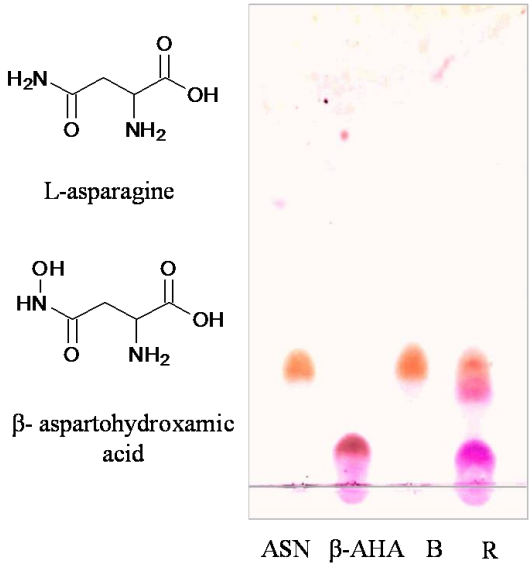
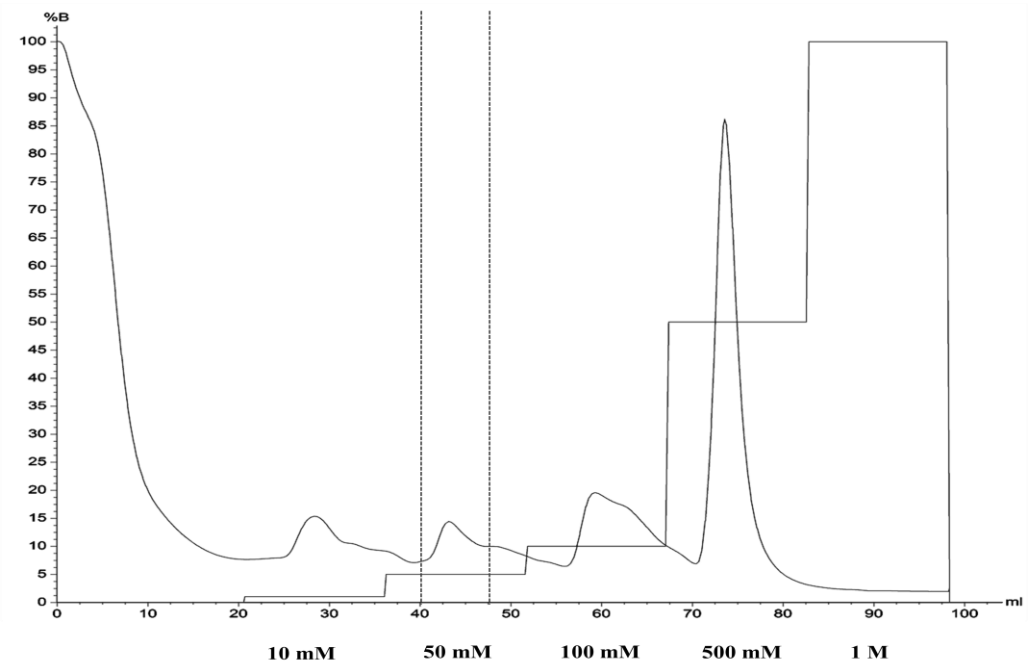


Figure 5. Thin layer chromatography of the reaction with cell suspension of *L. scottii* L115.

1.8.2 **Enzyme purification**

The enzyme was extracted by mechanical disruption by high-pressure homogenization of the yeast cells and the purification was carried out the following separation steps: homogenized extract PEG treatment, anionic exchange, and size exclusion chromatography. The polyethylene glycol 4000 (PEG 4000) at 6.6 % (w/v) of saturation was added to remove the unwanted macromolecules and lipids, clarifying the cell homogenize prior to further processing by chromatography methods. The PEG can be easily removed during the subsequent ion-exchange chromatography step since it does not absorb to the column resin (HONING and KULA, 1976; INGHMAN, 1990).

A



B

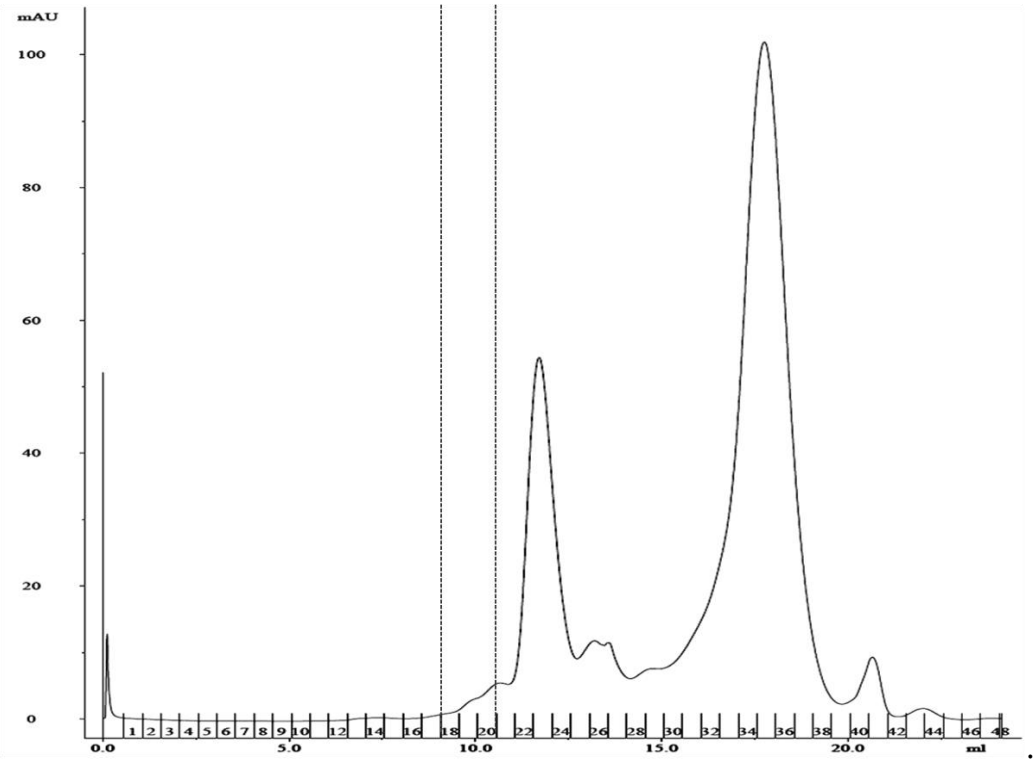


Figure 6. DEAE chromatogram, enzyme eluted at 50 mM of NaCl gradient step, (A) SEC chromatogram, the enzyme elute at 9.8 mL of the column volume (B).

The extract clarified was subjected to anionic exchange chromatography passed through a DEAE column and eluted using step gradient at 10, 50, 100, 500 mM of NaCl. The enzyme activity was found only in the 50 mM eluted fractions with a 25 % fold of recovery and a specific activity of 14.65 U mg^{-1} was obtained (Figure 6 A). The fractions with activity obtained by DEAE chromatography were pooled and concentrated within an ultrafiltration cartridge of 10 kDa of molecular weight of cut-off, and passed through SEC column Superdex® 200 Increase 10/300 GL, and the enzyme was eluted at 9.9 mL of the column volume (Figure 6B), obtaining a 228 fold of purification and a specific activity of 137.0 U mg^{-1}. The results of the different steps of the purification of LsASNase are shown in Table 6.

Table 6. Purification scheme of ASNase from *Leucosporidium scottii* .

Step	Activity (U mL^{-1})	Protein (mg/mL)	Total proteins (mg)	Total Units (U)	Specific activity (U mg^{-1})	Purification fold	Recovery (%)
Culture extract*	7.23	12.01	1082.79	650.80	0.60	1	100
anion exchange chromatography DEAE	11.02	0.75	11.28	165.33	14.65	24.38	25.40
Size Exclusion chromatography (SEC)	6.85	0.05	0.25	34.25	137.01	227.95	5.26

*From an initial 90 mL of culture extrac

1.8.3 **Determination of molecular weight by SDS-PAGE and SEC**

The purity of the purified enzyme fractions was analyzed by SDS-PAGE and the equation the of molecular weight was determined by plotting the relative protein mobility (Rf) to the dye front versus the natural logarithm of molecular weight (MW) of the protein standards. The MW of the single band corresponding to the LsASNase under reduced conditions was 54.5 kDa of MW and the native MW by SEC was 462 kDa. This provides the envidence that the enzyme purified from LsASNase is a high-mass multimeric enzyme that could be an octamer (Figure 7, 8). This result is in agreement with the intracellular ASNase reported from *S. cerevisiae* which is also reported as a decameric enzyme with a molecular weight of 400 kDa determined by SEC and 41.4 kDa in SDS-PAGE (DUNLOP and ROON, 1978; SINCLAIR et al., 1994), demonstrating that even among different groups (basidiomycetous and ascomycetous), yeast species could share the same ASNase enzyme.

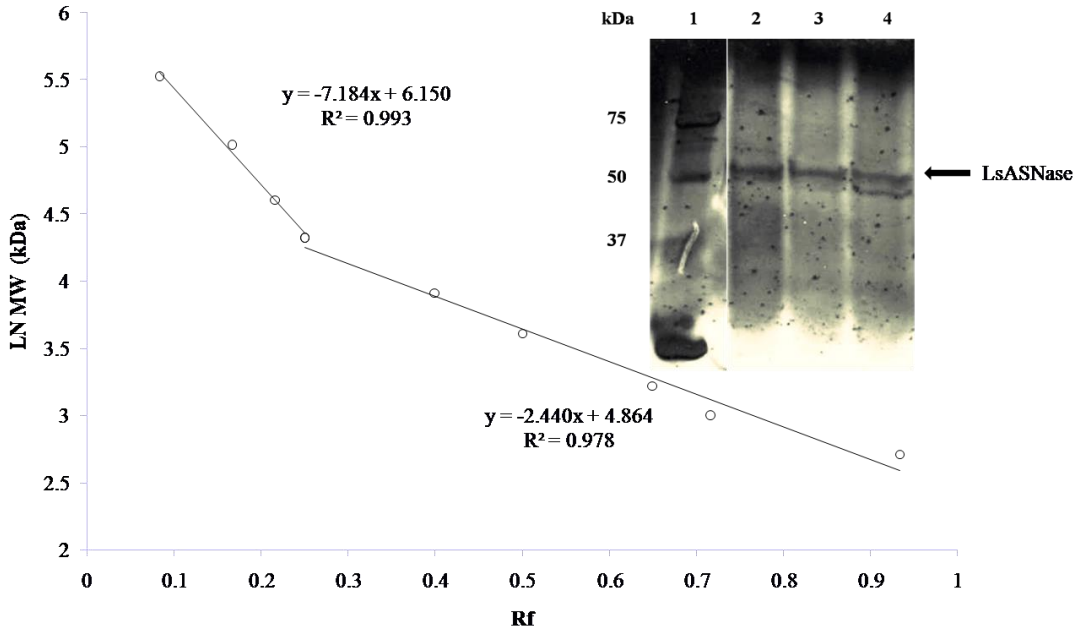


Figure 7. Molecular weight determination of the purified enzyme LsASNase **(A)** SDS-PAGE in reduced conditions of the corresponding fractions purified by SEC fraction 18 (2), fraction 19 (3), fraction 20 (4)

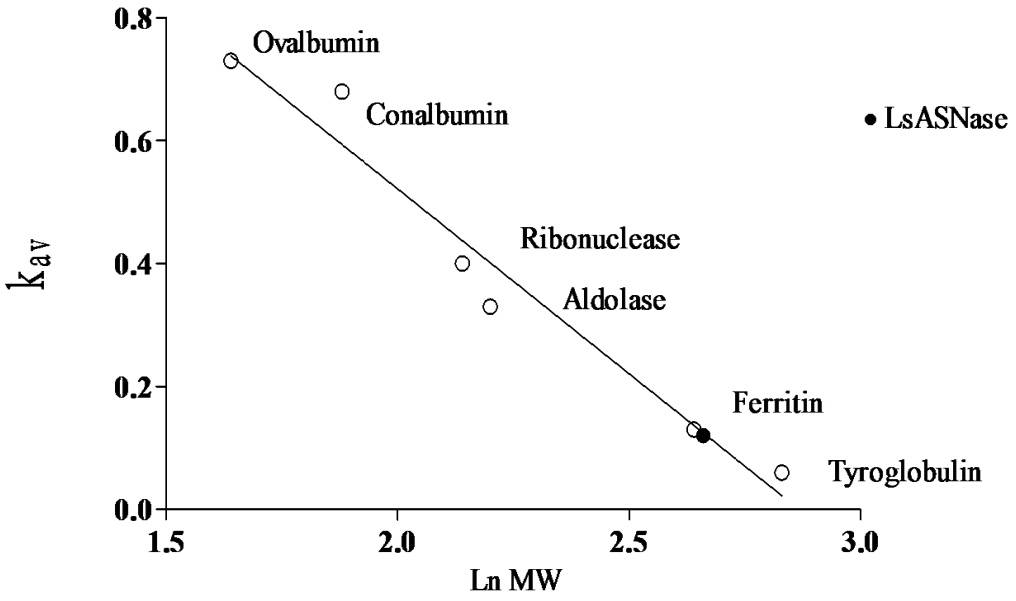


Figure 8. Molecular weight determination of the purified enzyme LsASNase in native conditions by SEC The K_{av} obtained for LsASNase I was of 0.12.

1.8.4 **Glycosylation analysis of the enzyme by SDS-PAGE**

The best characterized ASNase from yeast is the ScASNase type II is an external enzyme mannan glycoprotein located in the cell wall. The glycosylation is a posttranslational modification that is found on about 50% of all proteins, in secreted and transmenbrane proteins. To check if the LsASNase is a glycosylated enzyme form was treated with the enzyme PNGase F (Promega), which removes the N-glycosylation moieties reducing their apparent molecular weight of the enzyme. The results indicate that the treatment with the PNGase F has an effect on the mobility of the proteins treated, presenting new bands with a molecular weight lower than 25 kDa (track 5). As can see in the Figure 9 the enzyme was not completely pure, and two contaminating protein bands of lower weight appears (track 4). Even so, it can be observed that the treatment with the PNGase F modified the molecular weight of the three proteins and a fourth band appears, corresponding to the glycan sugar. This result showed that the LsASNase is a highly glycosylated enzyme and the half of the molecule weight corresponds to the sugar attached covalently to the enzyme. And, this suggests that the LsASNase is an external ASNase located in the cell wall.

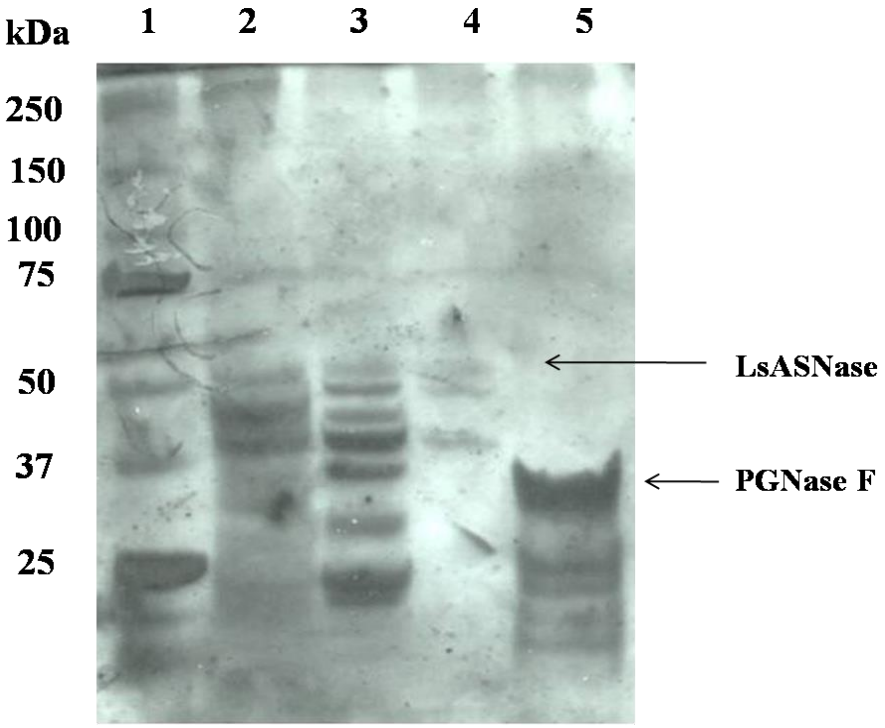


Figure 9. SDS-PAGE in reduced conditions for each step of the enzyme purification.(1) Standard proteins, (2) Homogenized extract treated with PEG 4000, (3) Anionic exchange chromatography DEAE-column,(4) LsASNase purified by SEC (5)) LsASNase purified by SEC treated with PGNase F enzyme.

1.8.5 **Determination of specific activity**

In order to determine the specific activity was used the purified enzyme by SEC. The activity was measured by the hydrolysis reaction with the Nessler's reagent and the protein concentration was determined by the micro BCA method. The specific activity of LsASNase obtained was 139.8 ± 5.094 $U \cdot mg^{-1}$ of protein and GLNase activity was 0.93 $U \cdot mg^{-1}$, reasonable activity compared with the bacterial ASNase (270- 700 $U \cdot mg^{-1}$) (NARTA et al 2001) and the yeast ASNase (196 $U \cdot mg^{-1}$) (COSTAS et al, 2016). Both graphics were made by plotting the initial velocity of the substrate hydrolysis vs the mg of purified enzyme. The specific activity corresponds to the slope obtained (Figure 10). But, an interesting point is that LsASNase present a very low GLNase activity, just the 0.6% of the activity in comparison with the bacterial ASNases that have 2 until 10 % of the GLNase side activity. That makes an interesting topic to continue studying the ASNase from yeasts species.

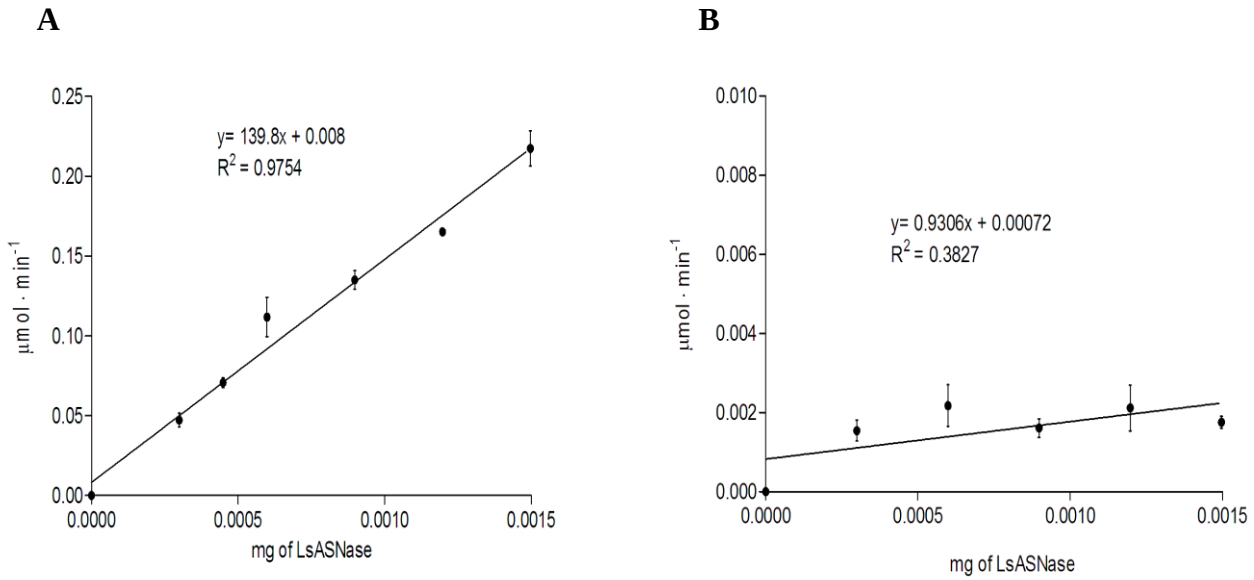
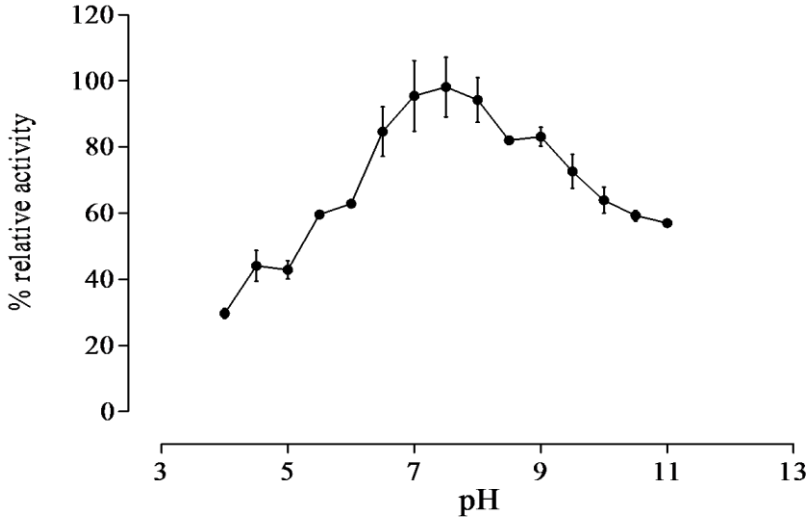


Figure 10. Determination of specific activity for LsASNase enzyme. (A) ASNase specific activity, (B) GLNase specific activity.

1.8.6 **Effect of pH and temperature on LsASNase**

The pH activity profile of the purified enzyme is presented in the Figure 11A. The LsASNase exhibited a pH optimum at 7.5 (buffer 50 mM Tris-HCl), with more than 80% of maximum activity in a pH ranges of 6.5 to 9. This pH profile suggests a potential use of the enzyme in the treatment of ALL, since it presented the maximum activity at the neutral pH. For optimum temperature, contrary to what was expected, that the maximum enzyme activity was at the optimal physiological temperature of microorganism growth (15 ºC), the optimum temperature for LsASNase was 55ºC (See Figure 11B). But, it has been reported that cold-adapted enzymes generally have their temperature optimum above of their physiological temperature, suggesting that the evolutionary pressure acted to allow the enzyme to be active at low temperatures but not to optimize its structure to present its maximum activity at low temperatures. So, different studies indicate that these changes in the enzyme structure are not related to the catalytic site, but to other regions of the protein that increases its structure flexibility (BJELIC ET AL., 2008; ISAKSEN ET AL., 2016).

A



B

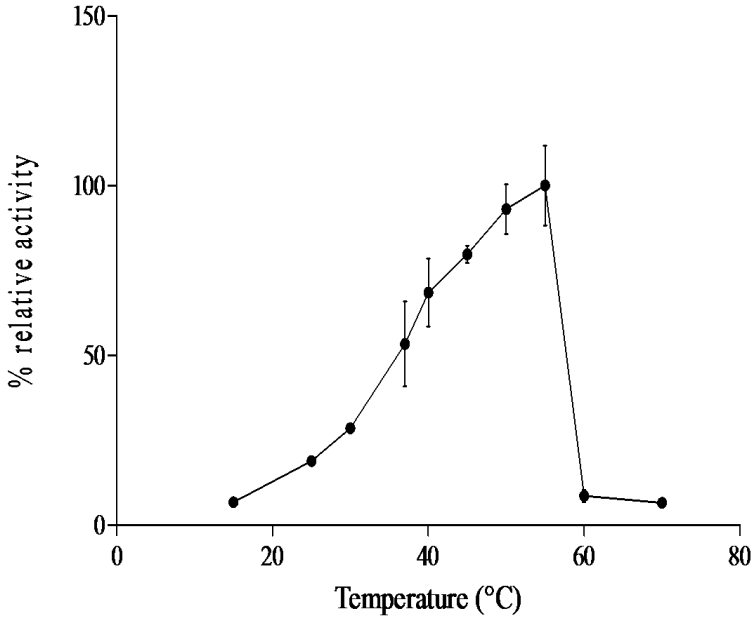


Figure 11. Effect of physical parameters on purified LsASNase. **(A)** Influence of temperature on the assay reaction. **(B)** Influence of pH on enzymatic activity determined in different buffers: acetate (pH 4.0- 5.5) phosphate (6.0-7.0) Tris-HCl buffer (7.5-9.0), Glycine-Sodium hydroxide (pH 9.5-10.5), Sodium bicarbonate (11.0).

1.8.7 **Effect of the metal ions in the LsASNase activity**

The effect of K^{+}, Na^{+}, Mg^{+2}, Ni^{+2}, Ca^{+2}, Cu^{+2}, Zn^{+2} on the partial purified ASNase (DEAE ion-exchange chromatoghaphy step) was studied. Compared to the control, Ni^{+2}, Ca^{+2}, Cu^{+2}, Zn^{+2}, the divalent ions affect the enzyme activity being the Cu^{+2} ions the highest inhibitor producing 40% of the inhibition of activity. Unlike, the of K^{+}, Na^{+}, Mg^{+2}, do not affect the enzyme activity, and interestingly the activity in present of Na^{+} 1 M just was slightly reduced by a 10 %. And, in the presence of EDTA 1 mM the activity is not affected. This results indicated that the enzyme not dependent on metallic ions.

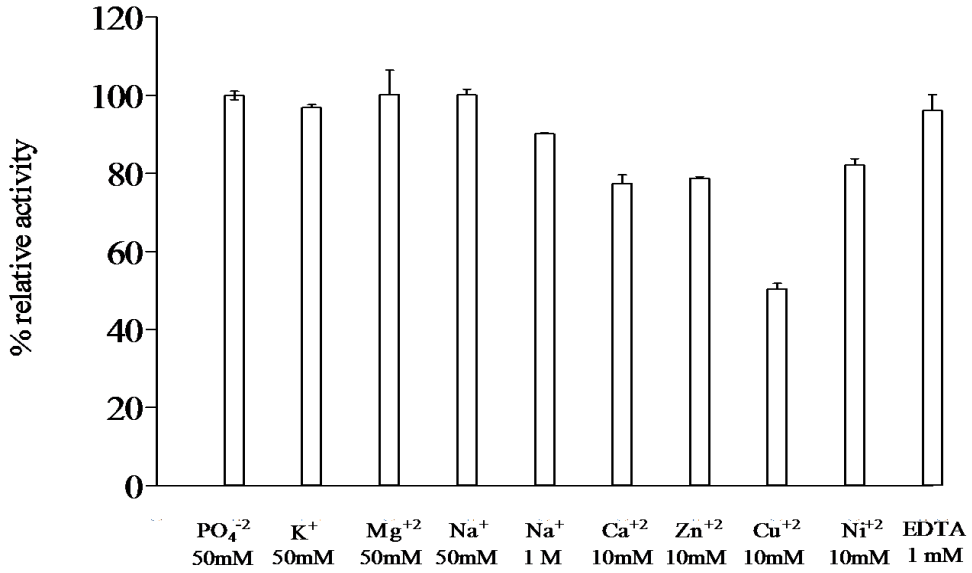


Figure 12. Effect of metal ions and EDTA on the enzyme activity of the LsASNase.

1.8.8 **Secondary structure and thermal stability assays**

Since LsASNase has never been reported in the literature, it is important to understand the secondary structure of this novel enzyme. As it can be seen in Figure 13, the spectra indicated that the conformation of LsASNase is similar to EcASNase II (ELSPAR® commercial biopharmaceutical produced by Escherichia coli). Thus, confirms that the integrity of ASNase was not affected by increasing in monomers, being the secondary structure preserved when compared to other ASNase. Moreover, thermal stability assays were performed in order to identify the denaturation temperature of the protein and understand the refolding process. The Fig. 14A and 15B depicted respectively the CD spectra of both EcASNase II and LsASNase. From the gather results, the EcASNase II has a slightly higher melting temperature (around 60°C), when compared to this novel enzyme (around 50°C).

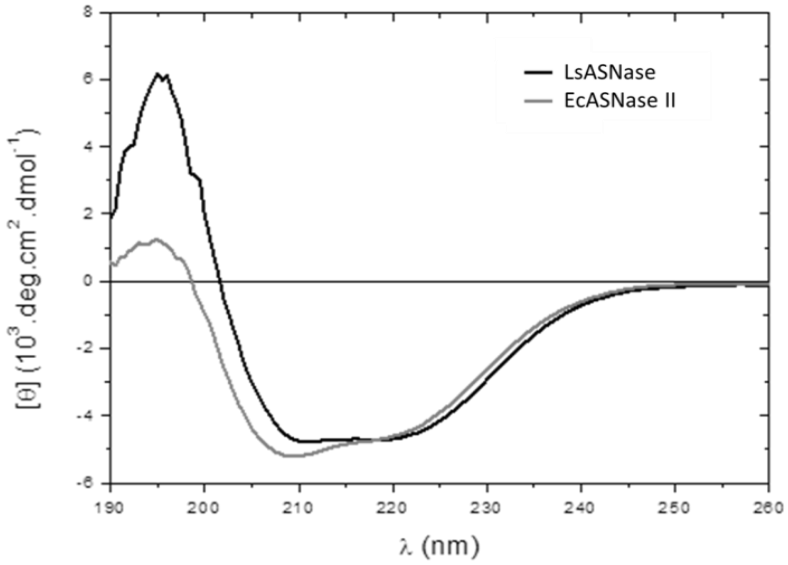
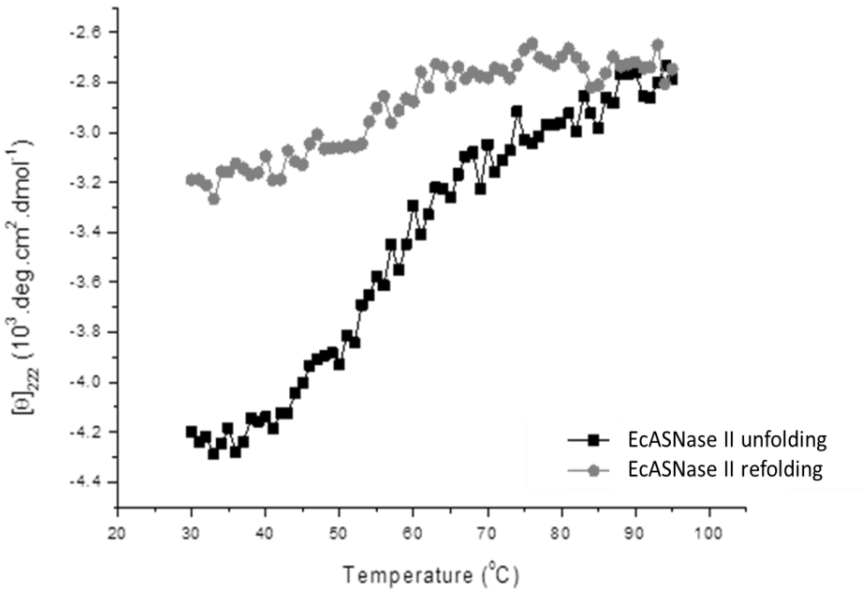


Figure 13. Far-UVCD spectrum (190 -260 nm) of LsASNase and EcASNase II (control).

Moreover, it is also possible to conclude that the refolding process of the EcASNase II is much more efficient when compared to LsASNase, this can easily justify the higher amount of monomers that the novel enzyme possess, making it difficult to refolding, due to the higher structural complexity of this latter enzyme. Also, is reported that the cold-adapted enzymes unfold according to an all-or-none process, because these enzyme structures are stabilized with fewer weak interactions and the disruption of some interactions strongly influence the whole structure, triggering the unfolding of all molecule (FELLER, 2013) for this reason can be observed a sharply loss of the activity after the reaching the melting point (Fig. 5B).

A



B

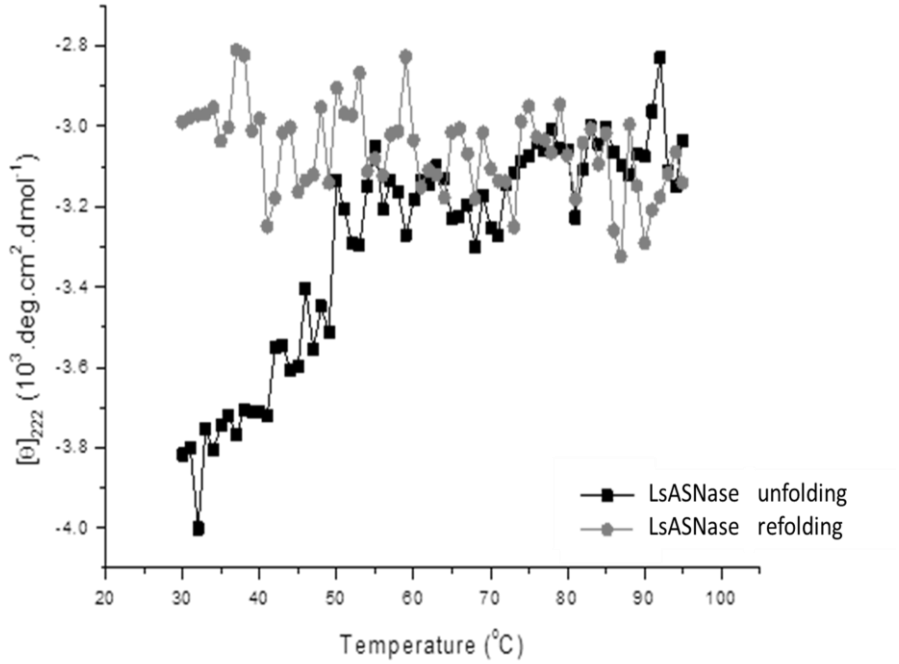


Figure 14. Thermal stability assay of: A) EcASNase II (control) B) LsASNase. CD spectrum at 222 nm and temperatures ranging from 20-95ºC.

1.8.9 **Kinetic parameters determination**

The kinetic behavior of the enzyme was better fitted to the allosteric sigmoidal model (R^2 = 0.9856; p< 0.0001) compared to the Michaelian model (R^2= 0.9653) using the F-test. The kinetic parameters obtained were $K_{0.5}$ = 233 μM a k_{cat}= 53 s^{-1} and a Hill coefficient (n_H) of 1.52. The n_H indicate the kind of cooperativity between the enzyme subunits and also, the number of substrate molecules that bind to the enzyme complex, a value of n_H> 1 pointed that the L-asparagine not only acts as substrate also acts as positive effectors of the enzyme (homotropic regulation). In general, the cytoplasmatic ASNases (ASNases type I) reported in yeast and bacteria species has a higher K_M or $K_{0.5}$ compared with the extracellular ASNases (ASNases type II) and presents an allosteric regulation, without L-glutamine affinity (See Table 7).

This behavior is shared by metabolic enzymes that act as control devices for flux alterations in metabolic pathways (HELMSTAEDT et al 2001). So, this regulation is necessary to maintain an intracellular concentration of L-asparagine for protein synthesis, and is effective at concentrations under 1 mM levels of L-asparagine (DUNLOP and ROON, 1978; YUN et al., 2007). Costa et al. (2016) report this regulation in ScASNase I at substrate levels below at 0.3 mM and for the human L-asparaginase (hASNase I) this allosteric behavior occurs at levels of 5 mM, for the LsASNase the allosteric regulation was not observed markedly but could be present at level below 50 μM (Figure 15). In Table 7 can be observed the comparison of LsASNase with the properties reported for others intracellular ASNases from yeast (ScASNase I) and bacterial (EcASNase I). For extracellular ASNases recently has been reported the heterotropic regulation by the L-glutamine, this kind regulation was important for the depletion of L-asparagine at μM levels (ANISHKIN et al., 2015; COSTA et al., 2016). In our case the affinity of the purified enzyme by L-glutamine was not observed at levels below at 10.0 mM.

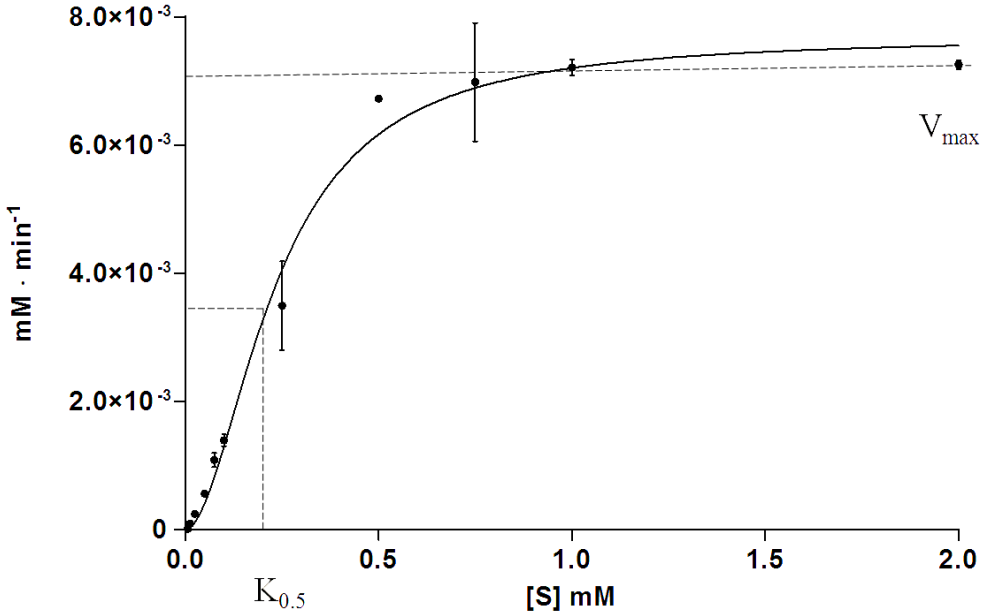


Figure 15. The LsASNase kinetic behavior with L-asparagine as substrate at pH 7.0. Points on the graphic represent the mean (n=3). The kinetic parameters were: $K_{0.5}$ = 233 μM, k_{cat} = 53 s^{-1} and n_H = 1.52.

The analysis of the crystal structures obtained for type I and type II ASNases exhibit a difference in the flexibility of the loop structures found in the N-terminal region between the oligomers; this might produce a change in the accessibility of the substrate to the active site. This structural features could produce a large difference in the affinity constant value (K_M or $K_{0.5}$) between the two enzyme types (YUN et al., 2007). The enzyme-substrate interactions are disrupted by both increases and drecreases in temperature (SOMERO, 1977). It was observed that the enzyme reaches the highest activity close to the melting point, we speculated that as a cold-adapted enzyme the flexibility of the structure will be reduced as the temperature increases, in this rigid state the allosteric regulation will be modified, because the allosteric interactions frequently appear to be correlated with alterations of the quaternary structure of the proteins. These conformational changes switch the allosteric regulation, closing the n_H to 1, and the enzyme could present a non-cooperative behavior following a Michaelis-Menten kinetics.

The glycans can alter the substrate recognition, the specificity and binding affinity as well as the turnover rates of the enzymes. In recent years, increasing evidence was found that glycans have distinct effects on the activity of many enzymes, in particular as regulatory modules for substrate binding and turnover can have both stimulating and inhibiting effects on activity (GOETTING, 2016). The interactions of glycosylation with the substrate may be a possible explanation for the behavior of the LsASNase enzyme. Because, being an extracellular enzyme present an allosteric behavior, when a Michaelian regulation should be expected.

The Table 7 shows the efficient catalytic (k_{cat} /$K_{0.5}$) of diverse ASNase isolated from different species. It is generally believed that to achieve a therapeutic effect the catalytic efficiency has to reach or be close to a 10^6 ratio, but there are other factors that must be taken into account such as the half-life, antigenicity, optimum pH, that determine the usefulness of the enzyme for therapeutic purposes. The LsASNase has an efficient catalytic of 2.3 x 10^5, only an order of magnitude lower than that of the bacterial ASNase, and one greater than the ASNase of *Saccharomyces cerevisiae*, that one that demonstrated anti ALL activity in vitro studies. This results proves the possibility that the enzyme has anti-ALL activity.

Table 7. Kinetic parameters of different ASNase enzymes reported that presents allosteric regulation and the *E. coli* enzyme.

ASNase type	K_M , $K_{0.5}$* (µM)	k_{cat} (s^{-1})	V_{max} U min^{-1}	n_H	Specific activity (U mg^{-1})	k_{cat} /$K_{0.5}$ (M^{-1} s^{-1})	Reference
EcASNase II	18*	60	------	1.5	------	3.3 x 10^6	Anishkin et al., 2015
EcASNase I	1200*	-----	0.016	2.6	173	-------	Yun et al., 2007
ScASNase I	75*	217	0.042	2.2	196.2	1.6 x 10^4	Costa et al., 2016
ScASNase I	740	-----	0.06	----	5.4	-------	Dunlop and Roon, 1978
ScASNase II	240	-----	57.8	----	30.5	-------	Dunlop et al, 1980`
hASNase I	11500*	6.7	------	3.9	------	5.8 x 10^2	Karamitros et al., 2014
LsASNase I	233*	54.7	0.007	1.52	150	2.3 x 10^5	

1.9 **CONCLUSION.**

Few ASNases in yeasts have been described, so far the results show that the enzyme isolated from the psychrotolerant yeast strain Leucosporidium scottii L115 presents different characteristics from those reported in bacteria and other yeast species. As a cold-adapted enzyme the LsASNase present a characteristic behavior affecting the enzyme kinetic parameters. This particular behavior can still be studied at the structural level by characterizing the structures that increase the flexibility of the protein and the catalytic site that has low affinity for the L-glutamine.

A further structural characterization will allow elucidate the characteristics and similarities with other ASNases and analyze the use of this enzyme as a possible anti-ALL drug. Being a biopharmaceutical born before the era of genetic engineering, the re-study of the ASNase can improvement all drawbacks intrinsic to the enzyme and can be re-planned to generate new ASNases engineered from the accumulated knowledge of the study and characterization of the different ASNase enzymes. The knowledge of the structure and function is necessary in order to increase activity, selectivity, and stability of the enzyme, and this will improve the treatment of the ALL.

.

2 CHAPTER III. MEDIUM CULTURE COMPONENTS OPTIMIZATION FOR PRODUCTION OF L-ASPARAGINASE ENZYME BY A PSYCHROTOLERANT YEAST *Leucosporidium scottii* L115

2.1 INTRODUCTION

Leucosporidium scottii is a psychrotolerant yeast, has been isolated mainly from cold environment around the world, including terrestrial and marine Antarctic ecosystems (SUMMERBELL, 1982; DUARTE et al, 2013). Marine and psychrophilic yeasts provide a unique potential for the synthesis of new biomolecules. These yeasts have been investigated for the production of pharmaceutical and enzymatic products but still remain poorly explored, besides, the marine and cold-adapted microbial enzymes can offer novel biocatalyst with extraordinary properties (ZHANG and KIM, 2010; ABDELRAHMAN et al, 2014). Few yeast strains have been studied as producers of ASNase and still many potential microorganisms in diverse ecosystems remain uninvestigated. To date, *L. scotti* has not been reported as L-asparaginase producer, for this reason, the yeasts from Antarctic continent could be an interesting eukaryotic source of ASNase.

The interest for studied new fonts of asparaginases is promoted basically by the applications anti-cancerogenic properties of these enzymes. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi are commonly reported in scientific literature for produce asparaginase with possible less adverse effects (PINHEIRO, et al 2002). The demand for ASNase could increase in the coming years due to its potential applications if the adverse effects are reduced. Also, the use of ASNase for acrylamide mitigation in food industry, makes the application of ASNase more attractive for industrial commerciallization in other fields aside from biopharmaceutical industry (XU et al., 2016). To develop a low-cost production is an important step in the development of this biopharmaceutical (GURUNATHAN AND SAHADEVAN 2011).

2.1.1 **Development of culture medium**

In the development of industrial biotechnological processes, the design of fermentation medium is the critical importance. The composition of the culture medium is the most important factor in the production of enzymes, growth, and physiology of the microorganism and allows the formation of bioproducts. The carbon and nitrogen sources can dramatically influence the metabolite formation, changes in nutrients and their concentrations have different effects on the accumulation of different metabolites, which are controlled by intracellular effectors (WANG and XU, 2005; DINARVAND et al 2013). The screening of different sources and selection of the optimum concentration of medium components are very important to determine the production and economic feasibility of the production process. Nonetheless, each organism has their particular nutritional and environmental condition for maximum enzyme production (SHARMA and HUSAIN, 2015). Table 8 shows the average composition of the microorganisms, these quantities must be taken into account to develop a medium with enough nutrients so as not limit the growth of the microorganisms.

Table 8. Average composition of microorganisms. (% dry mass)

Component	**Bacteria**	**Yeast**	**Fungi**
Carbon	48 (46-52)	48 (46-52)	48 (45-55)
Nitrogen	12.5 (10-14)	7.5 (6- 8.5)	6 (4-7)
Proteins	55 (50-60)	40 (35-45)	32 (25-40)
Carbohidrates	9 (6-15)	38 (30-45)	49 (40- 55)
Lipids	7 (5-10)	8 (5-10)	8 (5-10)
Nucleic acids	23 (15-25)	8 (5-10)	5 (2-8)
Minerals (same composition for the three microorganisms)			
Phosphorous		1.0- 2.5	
Sulfur, Magnesium		0.3-1.0	
Potassium, Sodium		0.1- 0.5	
Ferrum		0.01-0.1	
Zinc, Copper, Manganesium		0.001- 0.01	

As previously mentioned, the medium composition plays an important role in the improvement of productivity of the enzyme and it is also important to reduce the cost of the medium as much as possible. A diverse medium composition has been reported for the production of ASNase in eukaryotic microorganisms like yeasts and fungi species (Table 10).

2.1.2 **Carbon sources**

Carbohydrates are an excellent carbon and energy sources for microbial fermentations. However, although the patways of sugar utilization follow the same theme in all yeasts, important biochemical and genetic variations on it exists The diffent sugars produce signals which modify the conformation of centain proteins that in turn, directly or through regulatory cascade affect the expression of the genes subject to catabolite repression (GANCEDO 1998). Most yeasts use sugar hexoses, where only a few use lactose. The use of molasses, beet molasses, cereal grains, starch, glucose, sucrose and lactose as carbon sources and salts of ammonia, urea, nitrates, corn solids, bean meal, slaugther house waste and fermentation residues as source tend to be the production criteria because they are cheap substrates (OKAFOR , 2007).

2.1.3 **Nitrogen sources**

The nitrogen source in the culture medium regulates several genes in bacteria, yeast, and filamentous fungi. This phenomenon is known as regulation of nitrogen and occurs when the micro-organisms are able to select more easily metabolizable source, for example, ammonia, glutamine, and asparagine, and inhibit the expression of transporters and enzyme systems that allow the assimilation of sources known as poor such as nitrate, urea, and proline (ARIMA et al, 1972; GODARD et al, 2007) . The nitrogen source regulation enzymes typically under such control are proteases, amidases, ureases and those that degrade amino acids. The nitrogen font selected results in the expression of the permeases and the activation of the enzymes responsible for the uptake, synthesis, and interconversion of the compounds, and additionally the transcription factors and membrane trafficking proteins that regulate the activity of these enzymes and permeases (MAGASANIK and KAISER, 2002). For this, the correct selection of a nitrogen font for culture medium plays an important role in the production of bio-industrial products such as intra and extracellular enzymes (SANCHEZ and DEMAIN, 2002). This is the case of L-ASNase and different enzymes regulation that his production is closely related to the nitrogen fonts present in the culture medium.

Table 9. Medium composition for ASNase production by eukaryotic microorganisms

Microorganisms	Medium composition (g/L)	Reference
Different species of yeasts	20 g glucose, 5.0 g asparagine, 1.0 g K_2HPO_4, 0.5 g KCl, 0.01 g $MgSO_4$. $7H_2O$, 1.0 g yeast extract , pH 6.0.	Arima & Tamura 1972
Different species of yeasts	30 g Sucrose, 5 g polypepton, 5 g beef extract, 2 g yeast extract, 2 g malt extract, 5 g KH_2PO_4, 1.5 g K_2HPO_4, 0.5g $MgSO_4.7H_2O$.	Imada 1973
S. cerevisiae X-2180-A2	Growth medium: 20 g of D-glucose, 2 g of yeast nitrogen base (without amino acids and ammonium sulfate), and 1.32 g of ammonium sulfate. Nitrogen-free medium (production medium): 20 mM potassium phosphate buffer, pH 7.0 and 3% glucose and allowed to incubate 3h at 25º C.	Dunlop and Roon 1975
Candida utilis	20 g of D-glucose, 2g of yeast nitrogen base (without amino acids and ammonium sulfate), and 2 g of peptone	Kil J. O 1995
Rhodosporidium toruloides CBS14, ATCC10788, Rhodotorula glutinis NCYC59, ATCC90950, Rhodotorula rubra MTCC248	20 g D-mannitol, 5g L-asparagine, 1,2 g K_2HPO_4, 6 g KH_2PO_4, 0.5 g KCl, 0.01 g $MgSO_4$•$7H_2O$, 1 g yeast extract, pH 6.0.	Rmamakrishnan e Joseph 1996.
Aspergillus terreus MTCC1782	Soy bean meal fluor (not reported) glucose 6 g, L-asparagine 1.0 g, NaNO3 4.0 g, KCl 1.0g, 0.052 g $MgSO_4.7H_2O$ and 0.02 g FeSO4.7H2O pH 6.	Gurunathan and Sahadevan 2011.
Aspergillus tamarii, Aspergillus terreus.	Czapek- Dox modified with different nitrogen sources: L-asparagine 0.1g, L- proline 0.02 g or 0.2g, urea 0.02g, L- glutamine 0.1 g.	Sarquis et al 2004.
Fusarium ssp.	First grown in M agar : sucrose 30g, Polypeptone 5g, beef extract 2g, yeast extract 5g, malt extract 2g. Production medium: dextrin 30g, Pharmamedia 40g, KH_2PO_4 5g, K_2HPO_4 1.5 g, $MgSO_4$•$7H_2O$ 0.5 g, NaCl 5 g.	Nakahama et al 1973.

2.1.1 **Experimental design in the optimization of medium composition**

The use of mathematical modeling by using design of experiments (DOE) followed by response surface methodology (RSM) is commonly used by many companies in order to achieve the best combination of factors that will render the best characteristic of a product and or process response. In the initial formulation of the culture medium, a study has to be made to select the best source of carbon and nitrogen to the production of the enzyme. The screening of the culture medium compounds is time-consuming and costly, this is due to a large number of substrates available and the number of possible component combinations is immense. The Plackett-Burman (PB) design may be an excellent option, once it has been widely used to develop process conditions and to allow the understanding of the effects of various physicochemical, biochemical and sensory variables using a minimum number of experiments. The Placket Burman design allows the screening of main factors from a lage number of variables that can be retained in the further optimization process (HYMAVATHIM et al, 2013; GRANATO and CALADO, 2014).

Since the optimization of nutritional requirements and operating conditions is a very important step for bioprocess development. So far, most applications of DoE have concerned optimization of the composition of growth and production culture media. The intention is to find the most favorable mix of nutrient factors to maximize the cellular productivity by supplying a well balanced composition of nutrients that enhances the maximum yield of the product molecule (MANDENIUS and BRUNDIN, 2008).

The objective of the present work was to apply statistical DOE methods to optimize the medium composition for the production of ASNase by *L. scottii* by submerged fermentation. Several nutritional factors for enzyme production were screened by factorial fractional design and the optimum medium composition was obtained by response surface methodology.

2.2 MATERIALS AND METHODS

2.2.1 Microorganism and inoculum preparation for ASNase production for PB design

The yeast L115 strain used in this study was isolated from Antarctic marine sediments obtained during expeditions to Antarctica in the austral summer (2009 and 2010) by the Brazilian Antarctic Program team and identified as *Leucosporidium scottii* on the basis of 26S rDNA gene (Duarte et al, 2013). The L-115 *L. scottii* stock culture was maintained on Potato-dextrose broth (PDB, Difco^{TM}) added with glycerol (20% final volume) and stored at -80ºC. The Inoculum was prepared by incubating the microorganism in 200 mL of PDB in 500 mL baffled Erlenmeyer flask for 72 hours (log phase) incubated at 15ºC in a rotatory shaker at 200 rpm of agitation. The different production medium (Table 10) were inoculated at 1 % v/v of final concentration with the cells harvested. After 24, 48, 72, 96, and 120 hours of culture the cells were harvest by centrifugation at 3400 X *g* for 15 minutes and washed with distilled water for further ASNase activity determination. All medium were supplemented with the same salt base composed by KCl, 0.52 g L^{-1}; $MgSO_4.7H_2O$, 0.52 g L^{-1}; $CuNO_3.3H_2O$, 0.001 g L^{-1}; $ZnSO_4.7H_2O$, 0.001 g L^{-1}; $FeSO_4.7H_2O$, 0.001 g L^{-1}.

2.2.2 Assay of ASNase Activity

The ASNase activity was determined by the hydroxylaminolysis reaction and using the whole cell for the enzymatic reaction (GROSSOWICZ et al, 1950; DRAINAS et al, 1977; FERRARA et al., 2004). In a eppendorf tube was added, 1.6 mL of cells suspension at 1.0 of OD in 50 mM Tris-HCl buffer pH 7.0, 0.2 mL of 0.1 M L-asparagine and 0.2 mL of 1.0 M solution of hydroxylamine hydrochloride at pH 7.0, the mixture was agitated on a thermomixer at 850 rpm at 37ºC, after 30 minutes the enzymatic reaction was stopped by adding 0.25 mL of TCA/$FeCl_3$ reagent (100 g L^{-1} $FeCl_3$, 50 g L^{-1} TCA in 0.66 M HCl). The tube was centrifuged at 3400 x*g* for 5 minutes and 1.0 mL of the supernatant was measured at 500 nm. Controls were prepared in the same way as samples, but the hydroxylamine and asparagine solutions were added after the ferric chloride reagent. One unit of ASNase was defined as the amount of enzyme that produces 1μmol of β-aspartohydroxamic acid formed by minute by gram of dried cell weight (U g^{-1}cdw).

2.2.3 **Plackett-Burman design**

To evaluate the effect of 16 factors of medium components on the ASNase production a PB design of 20 experimental runs was carried out. The factors were tested at two levels (-1, +1). Table 10 shows the variables studied and the concentration tested. Three unassigned variables (commonly referred as dummy variables) were evaluated in the design and used to estimate the experimental error by pooling into the error the effect values obtained for each variable. The initial concentrations of each nutrient were selected empirically based on the literature survey.

Table 10. Nitrogen, carbon and complex fonts tested and levels used in PB design

Code	Variables	+1 (g L^{-1})	-1 (g L^{-1})
Nutrients considered like nitrogen fonts			
A	Proline	5	0
B	Urea	5	0
C	Aspartate	5	0
D	Chloride ammonium	5	0
E	Arginine	5	0
F	Glutamine	5	0
G	Asparagine	5	0
H	Glutamate	5	0
Nutrients considered like carbon fonts			
I	Maltose	10	0
J	Glycerol	10	0
K	Sucrose	10	0
L	Citric acid	10	0
Complex fonts			
M	Yeast extract	2.5	0
N	Soybean peptone	2.5	0
O	Step corn solids	2.5	0
P	Potato dextrose broth	2.5	0
Dummy variables			
Q	D1	0	0
R	D2	0	0
S	D3	0	0

The effect of each variable on the enzyme production and biomass production was evaluated using the next equation:

$$Effect_{(x_i)} = \frac{\sum X_{i+} - \sum X_{i-}}{N} \qquad \text{equation (4)}$$

Where the effect of each variable (X_i) is the response effect for determination of ASNase ; the X_{i+} is the yield of ASNase and biomass from the experiments when variables tested were at high levels; X_{i-} the yield of ASNase and biomass when variables tested were at low concentrations; and *N* the number of experiments. The significance was determined by applying the Student's t-test, P-value, and the significance levels. The variables which were significant at 5% level ($P < 0.05$) from the regression analysis were considered to have a greater impact on ASNase production. The statistical analysis were generated using Statistica 10(StatSoft, UK).

2.2.4 Inoculum preparation and production evaluation of L-Asparaginase for CCD design study

The pre-inoculum was prepared by incubating the microorganism in 200 mL of PDB in 500 mL baffled Erlenmeyer flask for 72 hours (log phase) incubated at 15ºC in a rotatory shaker at 200 rpm of agitation. The Inoculum was prepared by adding 5 g L^{-1} the microorganism in 200 mL of medium containing proline 5 g L^{-1}, sucrose 10 g L^{-1}, glycerol 10 g L^{-1}in 500 mL baffled Erlenmeyer flask for 72 hours (log phase) incubated at 15ºC in a rotatory shaker at 200 rpm of agitation. The different production medium (Table 13) were inoculated with the cells harvested at 1 % v/v of final concentration. After 24, 48, 72, 96, and 120 hours of culture the cells were harvest by centrifugation at 3400 X *g* for 15 minutes and washed with distilled water for the further ASNase activity determination.

2.2.5 Response surface methodology

The most important factors found from the PB screening were examined in more detail using response surface methodology. The concentration of variables selected was optimized by using a central composite design (CCD). A set of 16 experiments was performed with three variables, and each variable at five levels (-α,-1, 0, 1, α) (α = 1.6817). The error was calculated incorporating two replicates of the medial point (000). In Total 17 assays were performed (Table 13). The quadratic equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \qquad \text{equation (5)}$$

Where: Y is the predicted response; β_0 the intercept; β_i the linear coefficients for linear effects; β_{ii} the squared coefficient for quadratic effects; β_{ij} the cross-product coefficient for the effects; X the independent variables; and ε the experimental error. The response surface plots and statistical analysis were generated using Statistica 10. ANOVA analysis was employed to determine the model fitness.

2.2.6 **Validation of statistical model**

To validate the composition of the medium, an additional experiment in triplicate was conducted under conditions predicted for enzyme production. ASNase activity and cell mass production were used as a dependent variable in the comparative study.

2.3 RESULTS AND DISCUSSIONS.

2.3.1 Evaluation of influence of different founts of nitrogen and carbon for L-asparaginase production using the Placket-Burman design

The PB design was very useful for finding the main substrates for the culture medium by studying the main effect of each factor in the enzyme production, which is time-saving and can be evaluated more than one factor in a single experiment. The orthogonality of the design implying that the effect of each variable worked out is pure in nature and not confounded with interaction among variables. The results founded from the screening are shown in Table 11. According to the results of the experimental design, the nitrogen sources that have a positive effect in the production of ASNase were proline and ammonium chloride at 5 g L^{-1} and the corn steep liquor at 2.5 g L^{-1}. The carbon sources that have a positive effect were sucrose, glycerol, citric acid at 10 g L^{-1} and those that affected the production were urea, arginine at 5 g L^{-1} and yeast extract at 2.5 g L^{-1}, the other sources were not statistically significant for the production of ASNase. The nutrients that contribute effectively to the production of biomass were sucrose and glycerol at 10 g L^{-1} and proline at 5 g L^{-1}. The urea at 5 g $L^{-1}$was the only nutrient that has materially affected the production of biomass, the other sources were not significant.

In the conducted screening is clearly observed that the enzyme production is affected by the different sources of nitrogen and carbon selected and shows that the ASNase produced by *Leucosporidium scottii* is a nitrogen-regulated enzyme as the enzyme produced by *Saccharomyces cerevisiae.* Commonly the L-proline is repoted as the non-preferred sources of nitrogen used in most nitrogen catabolic repression studies realized in yeasts (MAGASANIK and KAISER, 2002). Also, in *S. cerevisiae* is reported that glycerol is a solute compatible with the amino acid proline in terms of efficiency of energy metabolism and redox balance of the cells (TAKAGI, 2008). The PB design allowed the reduction of sixteen possible nitrogen and carbon sources preselected from literature to only three. As was expected, an augment in the enzyme production was not observed in the presence of good sources of nitrogen such as asparagine, glutamine, aspartate, and glutamate. The urea considered a not preferred nitrogen source did not produce an increase in the enzyme production, on the contrary, it affected the production of the enzyme significantly.

Table 11. Plackett-Burman design and responses of ASNase and Biomass production

Run	Variables																		ASNase	cdw
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	(U L^{-1})	(g L^{-1})
1	-	+	-	-	-	+	+	-	+	+	+	-	+	+	-	-	+	+	-	166.6	5.96
2	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+	-	-	+	+	141.6	6.18
3	-	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+	-	-	+	206.0	7.04
4	+	-	+	-	+	+	-	+	-	+	+	-	+	+	-	+	-	-	-	181.8	10.36
5	+	+	-	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	212.0	4.23
6	+	+	+	-	+	-	+	-	+	-	+	-	-	-	+	+	+	-	-	152.7	5.97
7	+	+	+	+	-	+	-	-	+	+	-	+	-	-	-	+	-	+	-	161.9	3.64
8	-	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	-	+	73.1	1.29
9	-	-	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-	+	-	119.5	5.37
10	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	+	296.8	11.33
11	+	+	-	+	+	-	-	+	-	+	-	-	+	-	+	-	+	+	-	117.8	3.24
12	-	+	+	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	165.7	3.73
13	+	-	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+	+	180.6	6.98
14	+	+	-	-	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+	40.05	2.50
15	-	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	+	90.01	1.89
16	-	-	+	+	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	173.1	6.24
17	-	-	-	-	+	+	-	+	+	-	-	+	-	+	+	+	+	+	-	101.0	2.94
18	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	234.4	11.23
19	+	-	-	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	+	70.25	2.98
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39.77	1.07

The possibilities that give us the PB design and the technical and scientific criteria facilitate the choice of the variables evaluated to continue with a further step of optimization. The proline was the only nitrogen source that favored the ASNase and biomass productions, the sucrose and glycerol besides presented the highest positive effects in the enzyme production also are relatively inexpensive sources of carbon. The proline, sucrose, and glycerol were selected over complex sources since it is an advantage to use a defined medium in industrial production, avoiding the variation of production between each batch. In addition, the defined medium can be used for further studies of the process modeling.

Table 12. Effects of the variables and statistical analysis of the Plackett-Burman design

	ASNase production (U L^{-1})				cdw production (g L^{-1})			
	Effect	S.E	t student	p-Value	Effect	S.E	t student	p-Value
Intercept	146.27	5.82	50.21	0.00001	5.20	0.31	33.77	0.00005
YE	-33.39	5.82	-5.73	0.010*	-0.07	0.31	-0.21	0.841
SP	11.20	5.82	1.92	0.150	0.49	0.31	1.59	0.209
CSS	19.33	5.82	3.31	0.045*	0.60	0.31	1.96	0.144
PDB	4.91	5.82	0.84	0.461	0.90	0.31	2.94	0.060
Glutamine	-8.17	5.82	-1.40	0.255	-0.28	0.31	-0.91	0.429
Asparagine	-15.79	5.82	-2.71	0.073	-0.02	0.31	-0.08	0.939
Maltose	-1.24	5.82	-0.21	0.845	0.08	0.31	0.26	0.808
Glycerol	58.18	5.82	9.99	0.002*	2.89	0.31	9.36	0.002*
Glutamate	-14.71	5.82	-2.52	0.085	-0.94	0.31	-3.06	0.054
Sucrose	90.78	5.82	15.58	0.001*	4.02	0.31	13.04	0.000*
Proline	18.63	5.82	3.20	0.049*	1.06	0.31	3.46	0.040*
Urea	-25.31	5.82	-4.34	0.022*	-2.68	0.31	-8.70	0.003*
Aspartate	14.51	5.82	-2.49	0.088	-0.25	0.31	-0.80	0.478
Citrate	20.33	5.82	3.49	0.039*	-0.10	0.31	-0.34	0.755
Arginine	-21.08	5.82	-3.62	0.036*	0.64	0.31	2.08	0.128
Ammonium Chloride	25.76	5.82	4.42	0.021*	0.19	0.31	0.63	0.570

*significance at 5 %

R2= 0.9920, R adjusted 0.9499

PDB- Potato Dextrose broth, CSS- Corn step solids, SP- soybean peptone, YE- yeast extract.

2.3.2 **Optimization of nutritional factors on L-asparaginase production using Central Composite Design**

The 3 nutrients selected from the 16 evaluated were significant for the both responses evaluated, this means that proline, glycerol and sucrose contributive significant for the enzyme and cell mass production. The CCD was employed to evaluate the interaction between these three variables and to determine their optimal concentrations for ASNase production. The CCD is formed from two-level factorials by the addition of just enough points to estimate curvature and interaction effects. The designs can be viewed as partial factorials with factors at five levels and is extensively used to build second-order response surface models (KENNEDY and KROUSE, 1999).

The three nutrients were evaluated at the five levels: proline (2, 4, 7, 10 and 12 g L^{-1}) glycerol (0, 10, 25, 40, and 50 g L^{-1}) and sucrose (0, 10, 25, 40, and 50 g L^{-1}). The design matrix, results, and predicted values are presented in Table 13. The maximum ASNase production was obtained in the middle level for the factors, further increase in the factor above the middle level showed a decrease in the ASNase production.

Table 13. Experimental design and responses from ASNase production and biomass production

Run	Proline	Sucrose	Glycerol	cdw (g L^{-1})		ASNase (U L^{-1})	
	(g/L)	(g/L)	(g/L)	Experimental	Predicted	Experimental	Predicted
1	4	10	10	7.77	6.42	2489.46	2283.89
2	4	10	40	9.68	10.66	2563.13	2254.16
3	4	40	10	11.65	11.76	3012.48	2955.93
4	4	40	40	12.57	12.27	2328.63	2367.27
5	10	10	10	7.17	8.22	2110.09	1879.50
6	10	10	40	10.53	11.23	1939.43	1812.33
7	10	40	10	11.25	11.04	2048.70	2169.23
8	10	40	40	8.27	10.33	1535.50	1543.12
9	2	25	25	8.76	9.42	2081.85	2290.97
10	12	25	25	11.13	9.30	1259.26	1267.19
11	7	0	25	10.12	9.60	1456.93	1856.54
12	7	50	25	13.96	13.30	2374.05	2192.24
13	7	25	0	8.37	8.91	3108.25	3212.95
14	7	25	50	13.56	11.85	2556.99	2666.41
15	7	25	25	11.87	12.37	3202.20	3366.92
16	7	25	25	11.84	12.37	3382.46	3366.92
17	7	25	25	12.50	12.37	3251.87	3366.92

A regression analysis based on the least squares method was performed using STATISTICA software Version 10.0. The regression equation coefficients were calculated and the data was fitted to a second-order polynomial (equation 5). The non-significative effects were ignored. The relative magnitude of the coefficients indicate that the influence of the parameters individualy influence more that the interactions between them. The response ASNase production (Y) can be expressed in terms of the following regression equation:

$$\text{ASNase (U L}^{-1}) = -799.51 + 732.54 \cdot PROLINE - 59.64 \cdot PROLINE^2 + 121.99\ SUCROSE - 1.99\ SUCROSE^2 + 31.034\ GLYCEROL - 0.53\ GLYCEROL^2 - 0.62\ \text{SUCROSE} \cdot \text{GLYCEROL}$$

The statistical analysis of the model was performed by the analysis of variance (ANOVA) performance at the 90% confidence limit. The ANOVA results of the CCD experiment and can be observed in Table 15. The results indicate that the concentration of the proline, glycerol and sucrose has a direct relation to the enzyme production. The Linear terms of proline, sucrose and glycerol ,the quadratic terms of proline, sucrose and glycerol and just the sucrose-glycerol interaction was significative for the ASNase production.for the ASNase production were significative ($p < 0.1$). The ANOVA also suggested that the obtained experimental was a good fit whit the model presenting a correlation coefficient $R^2 = 0.93$ and $R^2_{adj} = 0.840$. Removing the not significant terms, was obtained a lack of fit *p*-value of 0.1087 ($p>0.1$), implying that the lack of fit of model was not significant relative to the pure error, it means that the proposed model fit with the experimental data. The contour graphs (Figure 16) show in the dark regions the optimal ASNase production. As can be observed the best ASNase production is localized near to the central region, contrary for the cell biomass production were the optimal zones are present at higher levels of carbon sources, these higher concentrations of sucrose and glycerol have a negative impact in the enzyme production.

Table 14. ANOVA analysis of the production of L-asparaginase by *L. scottii* according to CCD design

	SS	*DF*	*,MS*	*F*	*p- value*
Proline (L)	1251591	1	1251591	144.37	0.0068*
Proline (Q)	3141439	1	3141439	362.37	0.0027*
Sucrose (L)	137444	1	137444	15.85	0.0576*
Sucrose (Q)	2195471	1	2195471	253.25	0.0039*
Glycerol (L)	360911	1	360911	41.63	0.0231*
Glycerol (Q)	154271	1	154271	17.79	0.0518*
Proline x Sucrose	70724	1	70724	8.15	0.1038
Proline x Glycerol	679	1	679	0.07	0.8059
Sucrose x Glycerol	151267	1	15267	17.44	0.0528*
Lack off it	452698	5	90540	10.44	0.1087
Pure error	17338	2	8669		
Total SS	6734472	16			

*significance at 10 % ; $R^2 = 0.932$

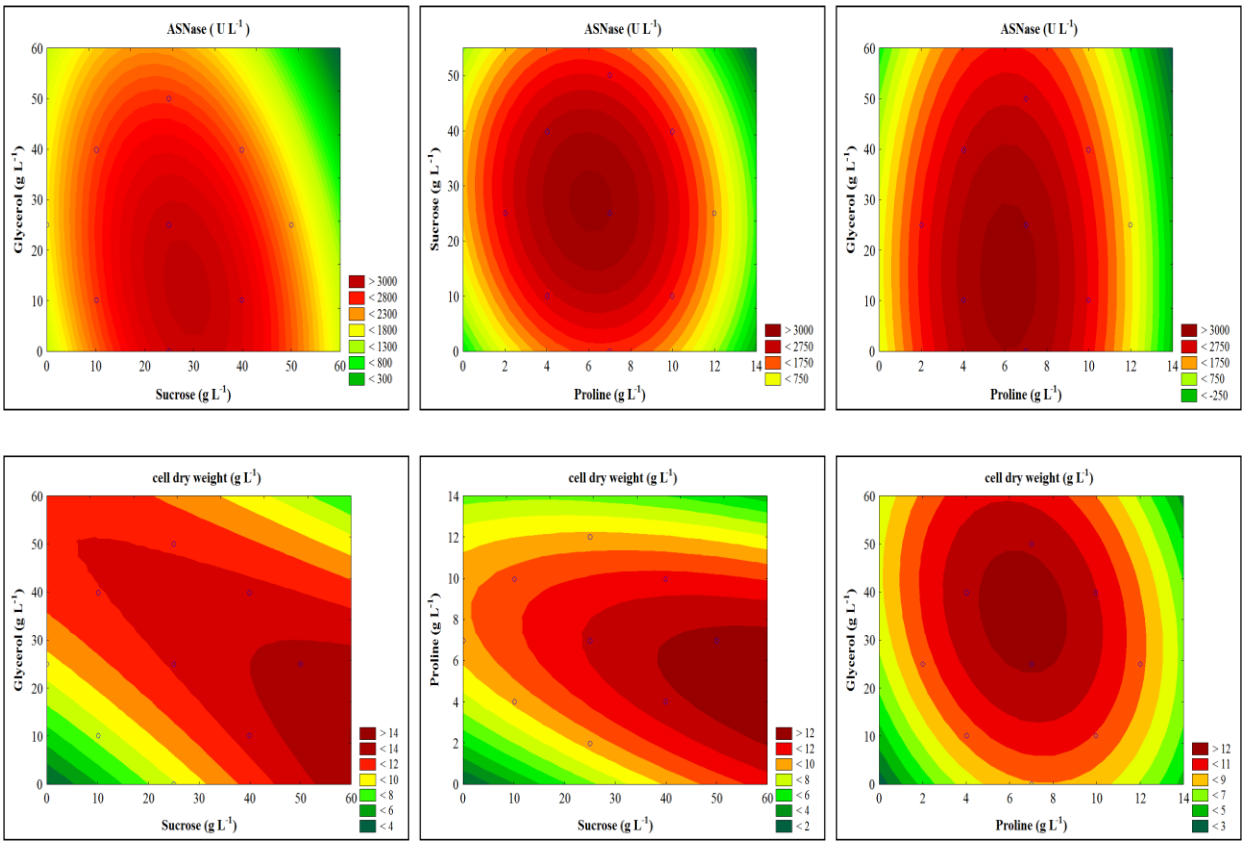


Figure 16. Contour plots for ASNase (U L^{-1}) and cell dry weight (g L^{-1}) production.

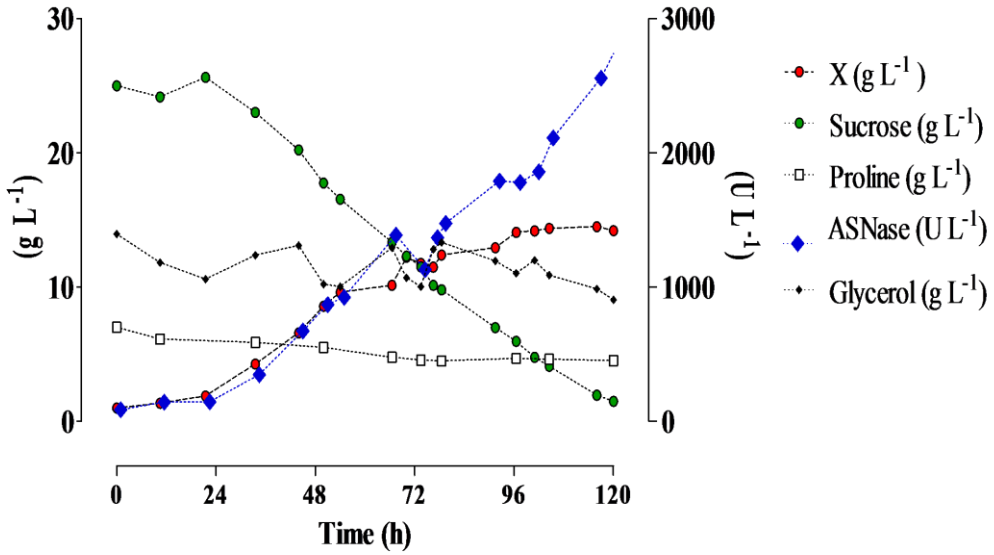


Figure 17 Substrate comsumption and ASNase production in the optimized medium.

2.3.3 **Validation of the model**

The CCD design used to optimize the ASNase production provided the predicted values of 6.15 g L^{-1} of proline, 28.34 g L^{-1} sucrose, and 15.61 g L^{-1} of glycerol for a maximal production. The culture was performed using 200 mL of the synthetic medium containing the optimized quantities added with the salts: KCl, 0.52 g L^{-1}; $MgSO_4.7H_2O$, 0.52 g L^{-1}; $CuNO_3.3H_2O$, 0.001 g L^{-1}; $ZnSO_4.7H_2O$, 0.001 g L^{-1}; $FeSO_4.7H_2O$, 0.001 g L^{-1}, the pH was adjusted at 5.0. Under these conditions the predicte response for ASNase production was 2850 U L^{-1} the predicted response was 3379.41 ± 337.9 U L^{-1}, even the model fail in predict the response in around to 200 U L to be inside the optimized confidence interval (90%) the model can improved the production of the enzyme in a almost in 50 fold, compared with the started medium used by Gulati et al (1997). The figure 17 shows the comsuption of substrates during the culture process, is observed that the sucrose finish at the 120 hours of culture, unlike the glycerol was not completely comsumed. Due to this it is necessary to continue the study in bioreactor to work in the required oxygenation conditions and to observe the consumption of the different carbon sources present in the culture medium.

CONCLUSION

The results show the potential of the Antarctic yeast to produces new molecules, in particular, the ASNase, that has potential uses in the pharmaceutical and food industries It is to highlight that the psychrotolerant yeasts are few demanding in terms of nutritional requirements and can grow well in a simple culture medium. The production of ASNase by *Leucosporidium scottii* was improved in flask cultures after using different DoE methodologies. The application of two well-planed set of experiments increased the enzyme production till in 50-fold compared whit the initial culture medium used. The rational use of DoE in bioprocess optimization and understanding how the nutrients and its concentration influence in the microbial metabolite production during fermentation is a powerful tool to develop a culture medium. This study allowed to develop a defined culture medium with 3 main components to increase the ASNase production with substrates such as glycerol and sucrose a relative cheap components easily available at industrial levels, and that it can be used for further metabolic studies.

4 CHAPTER IV EVALUATION OF L-ASPARAGINASE PRODUCTION AND LIPID ACCUMULATION BY THE PSYCHROTOLERANT YEAST *Leucosporidium scottii* L115

4.1 INTRODUCTION

Leucosporidium scottii is a non-fermentative, aerobic basiodiomycetous yeast considered as a psychrotolerant microorganism, isolated from low –temperature environments, generally in polar regions or temperate regions during the cold weather seasons, and in different habitats in the Antarctic such as seawater, soils and mosses and saline lakes. Able to thrive in relatively extreme environmental conditions, like high salinity, low temperature or a high concentration of aromatic compounds (SUMMERBELL, 1983; SAMPAIO, 2011). Psychrotolerant and psychrophilic yeasts have attracted the attention of scientists for their potential application in various industries because the capacity to produces valuable metabolites such as extracellular hydrolytic enzymes, pigments, polyunsaturated fatty acids PUFAs, polyols, antifreeze proteins, and single cell oils (SCOs) (BRIZZIO, et al 2007; de GARCIA, et al 2007, SPENCER, et al 2002, ALCAINO et al 2015). For this reason, the study of yeasts isolated from cold environments could provide new strains with biotechnological potential (MARGESIN and SCHINNER, 1994).

The *Leucosporidium scottii* L115 strain was isolated from marine sediment collected in the Antarctic Peninsula and our research group found it as capable of produces L-asparaginase (ASNase) enzyme. This enzyme has gained attention for its anti-leukemia potential and for its uses in the mitigation of acrylamide production during the food processing. Also, *L. scotti* is reported as producer of valuable metabolites like lipases and coenzymes Q9 and Q10 (WOO-HONG, 1991; DUARTE, et al 2013) is nutritionally versatile and capable of utilizes alternative carbon sources as xylose and glycerol (SAMPAIO, 2011). And, recently was characterized as an oleaginous yeast species able to accumulated lipids with a suitable fatty acid profile for the production of biodiesel (PEREYRA et al 2014). The oleaginous yeasts are advantageous hosts for the development of platforms to produce lipid-based chemicals and, new molecular techniques are studied to exploit the potential of microorganisms as a lipid producer for biodiesel production (ADRIO 2017; TSAI et al 2017). The yeasts have the benefits of single-cell fermentation and there are a large number of genetic tools to study and

manipulate them and, exist a continuously increasing interest for the discovery of new yeast strains capable of processing low-cost feedstocks like glycerol, molasses, and lignocellulosic hydrolysates, to transform and storing high levels of SCOs amenable to being converted into 2nd generation biodiesel (TCHAKOUTEU, et al 2015; HUANG, et al 2017).

Microbial oils are an attractive alternative source of PUFAs and according to experimental evidence, psychrophilic microorganisms like yeast may be are a promising source (RATLEDGE 2004). And, as cold-adapted yeasts are able to synthesize polyunsaturated fatty acids (PUFAs) of medical and dietetical interest, over-expressed at low temperatures The production of yeast lipids with composition similarities with high added-value has been considered as a process potentially economically viable, specifically if various low or negative cost raw materials are utilized as substrates (ROSSI, et al 2009; PAPANIKOLAU and AGGELIS, 2011; AGEITOS, et al 2011).

The aims of this work were to evaluate the ASNase production by *L. scottii* L115 in a bioreactor under different conditions of initial cell concentration (X_0) and carbon source, and optimization of process parameters for maximum enzyme production. This work contributed to add knowledge about psychrotolerant yeasts as potential producer of enzymes and fatty acids.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism and inoculum

Psychrotolerant yeast *Leucosporidium scottii L115* was isolated from marine sediment of Antarctic Peninsula by the expedition team of the Brazilian Antarctic Program (PROANTAR) and was identified using the 26S rDNA sequencing (DUARTE et al , 2013). The yeast strain was incubated in 250 mL Erlenmeyer flask for 72 h at 15°C and 200 rpm in an orbital shaker (New Brunswick™ Innova® 42) in YPD medium: dextrose 20 gL-1, peptone 20 g L-1, yeast extract 10 g L-1. The cells were harvested by centrifugation (Thermo Scientific, Multifuge X3 FR) at 3400 xgfor 15 minutesat 5°C,and washed with milli-Q water. These cells were growth in Erlenmeyer flasks to produce the mass required to inoculate in the bioreactors. The propagation media was composed by: sucrose, 28.34 g L^{-1}; glycerol, 15.6 g L^{-1}; L-proline, 6.12 g L^{-1}; KCl, 0.52 g L^{-1}; MgSO4.7H2O, 0.52 g L^{-1}; CuNO3.3H2O, 0.001 g L^{-1}; ZnSO4.7H2O, 0.001 g L^{-1}; FeSO4.7H2O, 0.001 g L^{-1}, in phosphate buffer 50 mM pH 7.0. The propagation was carried at the same condition described above. After 48 h, the cells were harvested by centrifugation and suspended in milli-Q water to inoculate the bioreactors.

4.2.2 Bioreactor assays

All batch cultures were performed in a multi-bioreactor system (BIOSTAT®Qplus, Sartorius Stedim, Germany) of 1.0 L equipped with automatic monitoring and control units for temperature, pH, aeration, and agitation. The bioreactor vessel was equipped with two six/bladed Rushton turbines and, the working volume used was 0.5 L.

4.2.3 Effect of initial cell concentration in enzyme production

All experiments to assess the initial cell concentration on enzyme production were performed at 15°C under cascade control at 30% of oxygen saturation using compressed air where the range of agitation was set up at 50 – 500 rpm, and the airflow was set up at 0.1 – 1 Lmin-1.The initial cell concentration was evaluated at 1, 3, and 5 g L-1 (dry basis). The media used was the same for the propagation. Antifoam was used in the case of need. Samples were collected regularly and frozen at -20°C immediately for further analysis.

4.2.4 **Effect of the carbon source in the enzyme production**

The effect of the carbon source in the L-asparaginase production was assessed in glycerol and sucrose separately as sole carbon source and the mixture of them in the same concentration of 43.94 g L^{-1}. The concentration of each carbon source in the mixture was 15.6 g L^{-1} of glycerol and 28.34 g L^{-1} of sucrose, this proportional was previously optimized in flasks (unpublished results). L-proline (nitrogen source), minimum mineral medium, an initial cell concentration of 5 g L^{-1} was applied for all experiments. The bioreactor parameters were set up in the same condition cited previously.

4.2.5 **CCD design for optimum k_La determination**

The airflow and the agitation speed were evaluated using a central composite design. A set of 11 experiments were performed, and each variable was assayed at five level (-α,-1, 0, +1, +α) (α = 1.414). The error was calculated incorporating replicates of the central point (00). The statistical analyses were generated using Statistica 10 (StatSoft, Tulsa, OK). The significance of the estimated effects was tested by analysis of variance (ANOVA). The significance of each coefficient was determined by a p-value test ($p< 0.1$) considering 90% of confidence.

Table 15. Factor Levels for a CCD Design

Variable	**Code level**				
	-1.41	-1.0	0	+1.0	+1.41
Agitation(rpm)	77.5	150	325	500	572.5
Air flow(L min^{-1})	0.02	0.10	0.30	0.50	0.58

Table 16. Experimental design for the optimization of enzyme production

Assay	**Agitation (rpm)**	**Air flow (L min^{-1})**	**k_La (h^{-1})**
1	150	0.10	1.42
2	150	0.50	74.11
3	500	0.10	41.75
4	500	0.50	123.0
5	78	0.30	4.19
6	572	0.30	88.92
7	325	0.02	23.25
8	325	0.58	91.72
9 (C)	325	0.30	53.21
10 (C)	325	0.30	53.21
11 (C)	325	0.30	53.21

4.2.6 **Analysis**

4.2.6.1 *ASNase assay by the hydroxylaminolysis reaction*

The ASNase activity was determined by the hydroxylaminolysis reaction and using the whole cell for the enzymatic reaction (Ferrara et al., 2004). In a eppendorf tube was added, 0.8 mL of cells suspension at 1.0 of DO in 25 mMTris-HCl buffer pH 8.0, 0.1 mL of 0.1 M L-asparagine and 0.1 mL of 1.0 M solution of hydroxylamine hydrochloride at pH 7.0, the mixture was agitated on a Thermomixer at 850 rpm at 37ºC, after 30 minutes the enzymatic reaction was stopped by adding 0.25 mL of TCA/$FeCl_3$ (100 g L^{-1} $FeCl_3$, 50 g L^{-1} TCA in 0.66 M HCl).The tube was centrifuged at 3400 x *g* for 5 min and 0.3 mL of the supernatant was measured at 500 nm in a plate reader (Synergy MX, Biotek). Controls were prepared in the same way as samples, but the hydroxylamine and asparagine solutions were added after the ferric chloride reagent. One unit of ASNase was defined as the amount of enzyme that produces 1μmol of β-aspartohydroxamic acid, formed for a minute by gram of dried cell weight (U g^{-1}cdw).

4.2.6.2 *Quantification of biomass production*

The cells growth was accompanied by measuring the optical cell density (OD) at 600 nm using spectrometer detector (Synergy MX, Biotek) using water as a blank. The dry weight was measure through gravimetrical analysis, 1 mL of each sample was centrifuged at 3400 *g*, re-suspended twice in milli-Q water, and placed to dry at 60°C in the oven until constant weight in the analytical balance.

4.2.6.3 *Substrate quantification*

The samples were centrifuged at 5000 *g* and the supernatant was collected. The glycerol and sucrose concentrations were determined by HPLC (Ultimate 3000, Dionex) with IR detector 50 °C, Aminex column HPX-87H 300 mm x 7.8 mm at 60º C, and 0.5 $mL.min^{-1}$ of 5mM H_2SO_4 as eluent phase. The D-glucose and D-fructose were determined using the enzymatic assay kit K-SUFRG (Megazyme) according to the manufacturer's protocol (K-SUFRG 06/14).

4.2.7 **Staining of yeast cell with Nile Red Dye for microscopy analysis**

In order to detect the accumulation of lipids, the method of staining neutral lipids in yeast cells by Nile red was used according to Rostronand Lawrence (2017). A stock solution of Nile Red (2 $mgmL^{-1}$) in acetone was prepared and stored at -20ºC. Yeast cells were re-

suspended and washed twice in the in PBS 1X buffer pH 7.4 and the final concentration was adjusted at 1.0 of DO. At 250 µL of cell suspension was added 25 µL of DMSO:PBS 1:1 and 25 µLof Nile Red in acetone at 60 µg mL^{-1} mixed and incubated at room temperature in the dark for 5 min. The cells were centrifuged at 5000 x *g*, the supernatant discarded and the cell washed twice with PBS 1X. The cells were re-suspended in 100 µL of 10% of formaldehyde (37%) and fix for 15 min at room temperature in the dark. The cells were centrifuged and washed twice with PBS 1X buffer and re-suspended in 100 µL of the same buffer. The stained cells were analyzed under a fluorescence microscope (Leica, Germany) using the filter EGFP.

4.2.8 **Lipid extraction**

The yeast cultures at different conditions were harvest and washed twice with distilled water and the final concentration of cells suspension was adjusted at the 10- 30 mg· mL^{-1} of dcw, 2 mL of the suspension were transferred to an eppendorf tube, centrifuged at 4.7 x *g* for 5 minutes the supernatant was discarded, 500 µL of glass beads (0.22mm) and 500 uL of distilled water were added. The yeast cell were homogenized using an homogenizer Precellys 24 (BertinTechnologies) during 10 cycles of 6000 rpm for 20 sec each, with a period of 10 sec in bath ice avoiding the over heat of the samples. The homogenized was recovered washing the eppendorf tube with 10 mL of methanol, and stored at -20ºC.

4.2.9 **Total lipids quantification and determination of fatty acid composition**

Lipids were extracted from all samples using a modified form of the method described by Bligh and Dyer (1959), with a reduced amount of solvent (30.0 mL methanol and chloroform, 1:1). Lipid content was determined by gravimetry after evaporation of chloroform and is expressed as the percentage. Lipid extracts were used for the preparation of fatty acid methyl esters using AOCS Official Method CE 2-66 (1998). C 23:0 methyl ester was used as an internal standard. Fatty acid methyl esters (FAME) were analyzed using the Agilent 7890A GC system equipped with a column X from Supelco, using AOCS official method Ce 1b-89 (1998).

4.2.10 **Fermentation parameters**

Considering a determined time of culture in the bioreactor, the corresponding values of cells (X), substrate (S) and product (P) concentrations, can be related though the conversion factors. For comparison of the trials, the production parameters were determined using the equation described below.

- *Conversion factor of limitant substrate to cells ($Y_{x/s}$)*

$$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f} \qquad \text{equation (6)}$$

Where: X_f = cell concentration at the time evaluated (g L^{-1}); X_0 = initial cell concentration (g L^{-1}); S_f = substrate in the medium at the time evaluated (g L^{-1}); S_0= initial substrate concentration (g L^{-1});

- *Conversion factor of limitant substrate to product ($Y_{P/s}$)*

$$Y_{P/S} = -\frac{P_f - P_0}{S_0 - S_f} \qquad \text{equation (7)}$$

Where: P_f = concentration of product formed at the time evaluated (g L^{-1}); P_0 = initial concentration of the product (g L^{-1}); S_f = substrate in the medium at the time evaluated (g L^{-1}); S_0= initial substrate concentration (g L^{-1});

- *Volumetric productivity (Q)*

$$Q = -\frac{P_f - P_0}{V \times t}\ , \left(\frac{g}{L \cdot h}\right) \qquad \text{equation (8)}$$

Where P, is the maximum of enzyme produced and for lipid was measured at the end of the culture, S_0 is the initial concentration of glycerol + sucrose, X_f is cell concentration at time t of maximum production of the enzyme, and X_0 is the initial cell concentration, V the volume of liquid culture and t is the culture time for maximum enzyme production.

4.2.11 **Determination of k_La**

The volumetric oxygen transfer coefficient (k_La) was determined experimentally using the method of "gas out- gas in" (TRIBE et al, 1994), in a 1L bioreactor(BIOSTAT®Qplus, Sartorius Stedim, Germany) at 15ºC containing the 0.5 L of medium culture. The oxygen probe was set to zero in each determination by bubbling nitrongen into the medium culture.

The aeration an agitation parameter were seted accoerding to the experiment design conditions (Table 17) and the values of oxygen saturation were register at different times until the probe reads 100% of saturation.

The variation of the dissolved oxygen (DO) concentration with the time can be expressed as

$$\frac{dC}{dt^{o}} = -k_L a \cdot (C_s - C) \qquad \text{equation (9)}$$

Integrating the equation 9 is obtained the equation 1

$$Ln\left(1-\frac{C}{C_s}\right)=-k_L a\cdot t \qquad \text{equation (10)}$$

Were C_s is the oxygen saturation concentration (mg L^{-1}) at the set temperature, and C is the dissolved oxygen concentration during the gas-out period. Thus, the k_La can be determinted from the slope of $Ln(1- C/C_s)$ versus time.

4.3 RESULTS AND DISCUSION

4.3.1 Effect of initial cell concentration in the L-asparaginase production

Figure 18 shows the substrate consumption, biomass and enzyme production, as can be observed the consumption of substrates is sequential and not in parallel. The glucose was preferred consumed, followed by the fructose and glycerol. The uptake of glucose was clearly observed during the first hours of the culture the sucrose was broken down and the glucose was consumed simultaneously, in contrast, the fructose was consumed until the glucose was almost depleted. The *lag* phase was absent in all experiments, and the ASNase was concurrently produced with cellular growth. All sugars were depleted in 114 h under initial cell concentration of 1 g L^{-1} and 3 g L^{-1}, whereas starting with 5 g L^{-1} of inoculum, the carbon sources were depleted in approximately 70 h. The sequential metabolism of the carbon founts suggests that the *L. scottii* yeast produces an extracellular invertase or a fructofuranosidase located in the cell wall, breaking first the sucrose present in the medium and latter occurs the sequential consumption of substrates. We observed that the uptake of glycerol occurs during the stationary phase, this means that glycerol is not used for the growth of the cell, instead is used for a stage of accumulation, it is reflected in the increase of intracellular lipids during the stationary phase. In addition, it was not observed a marked diauxic growth phenomenon during the consumption of the substrates.

At 5% of initial inoculum the substrates are consumed in 70 hours with an enzyme productivity of 17.4 $U \cdot L^{-1} \cdot h^{-1}$, with an initial inoculum of 3%, the carbon sources ends around 94 hours with a productivity of 10.45 $U \cdot L^{-1} \cdot h^{-1}$ and with 1% of inoculums, the substrates are consumed in 114 hours with a productivity of 8.93 $U \cdot L^{-1} \cdot h^{-1}$. As can be observed the inoculum size at 5% increases the productivity of the process. Also, can be observed biomass production was not significantly different, however, the production of ASNase was affected by the concentration of the initial inoculum, the experiments showed that the production of enzyme increases with the cell growth reaching the pick with the stationary phase. The maximum production of the ASNase and yeast growth were achieved with an inoculums size of 5% (v/v) producing 2595 $U \cdot L^{-1}$ at the end of the culture.

As shown in Table 17, the production of the enzyme per gram of cell produced increases relative to the concentration of the inoculum increasing by almost 50 Ug^{-1} from 1 to 5% of the initial inoculums increasing. In addition, the initial cell concentration influenced the ASNase volumetric and activity yields. Therefore, the yield increases linearly with cell concentration and it suggests that the higher value could attain under high cell density.

Table 17. Enzyme and lipid production at different concentration of inoculum

X_0	Time	μ_{max}	Q_X	P_{ASNase}	Q_{ASNase}	$Y_{X/S}$	$Y_{ASNase/S}$	$Y_{E/X}$	$Y_{L/S}$	$Y_{L/X}$
g L^{-1}	**h**	**h^{-1}**	**g L^{-1} h^{-1}**	**U L^{-1}**	**U L^{-1} h^{-1}**	**g g^{-1}**	**U g^{-1}**	**U g^{-1}**	**g g^{-1}**	**g g^{-1}**
1	114	0.05	0.081± 0.002	1699 ± 85	13.99 ± 0.74	0.31± 0.01	39.03± 8.25	109.3± 9.07	0.21± 0.04	0.60± 0.04
3	94	0.036	0.0089± 0.008	2199 ±180	16.79± 1.91	0.29± 0.04	39.74± 4.09	116.7± 18.09	0.18± 0.03	0.56± 0.05
5	70	0.030	0.133 ± 0.007	2595 ±350	27.24± 5.12	0.31± 0.02	40.93± 6.53	122.2± 21.82	0.14± 0.01	0.52± 0.02

$Y_{ASNase/S}$,enzyme yield, U g^{-1} substrate assimilated, $Y_{X/S}$, cell yield, g g^{-1} substrate assimilated, $Y_{L/S}$, lipids yield, g g^{-1} substrate assimilated, Q_{ASNase} , volumetric enzyme productivity , U $L^{-1}h^{-1}$, Q_X , volumetric cell productivity , U $L^{-1}h^{-1}$

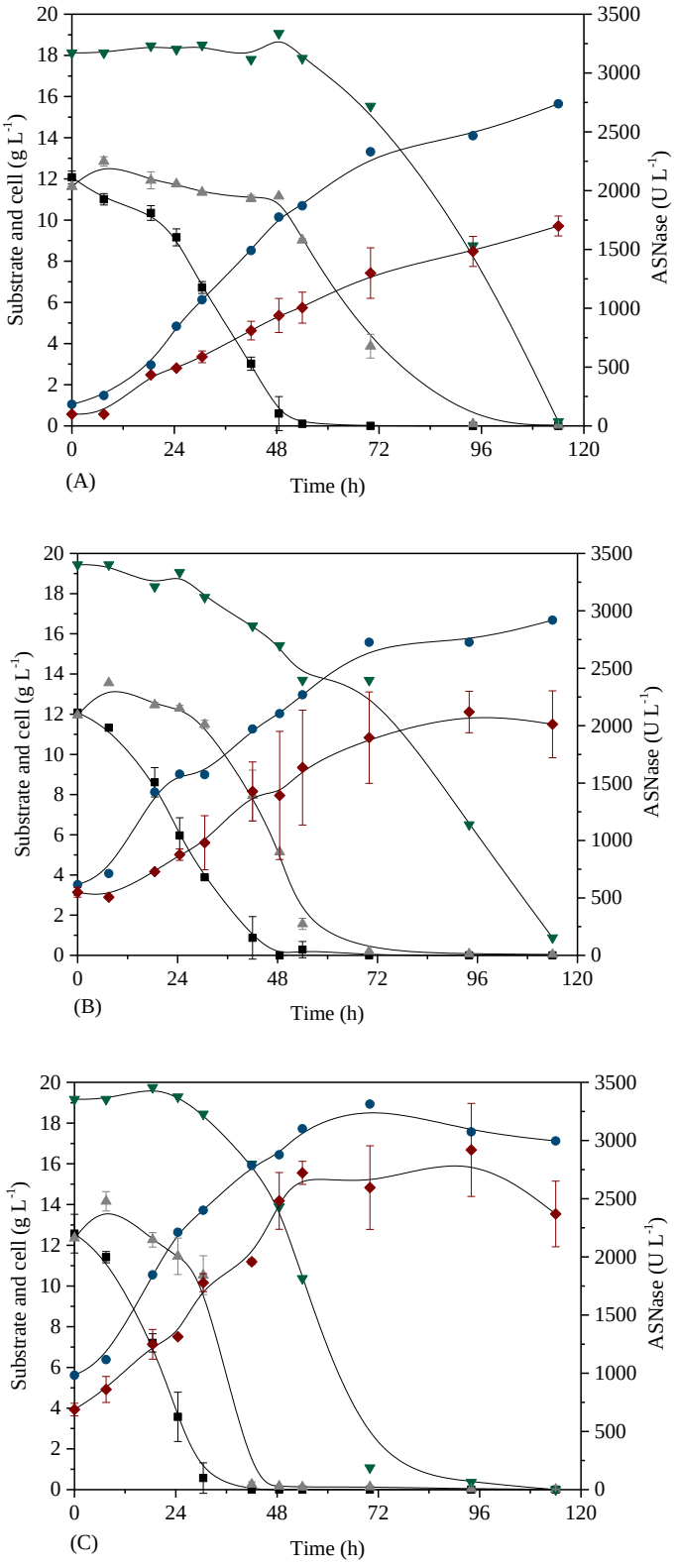
4.3.2 **Effect of carbon source in enzyme production**

The composition of culture medium was previously optimized in shaker resulting in the ratio sucrose: glycerol (28.34:15.6 g L^{-1}) for the best production of the enzyme. Even so, the effect of glycerol and sucrose were tested individually at the initial concentration of 43.94 g L^{-1}. This study was conducted to evaluate in bioreactor the influence of both carbon sources in the ASNase and lipid production. According to Figure 18, the cell growth was similar independently of carbon source, and the enzyme production initiated since the inoculation. For the conditions tested, the production of the enzyme was gradually increased during the exponential phase and the maximum was reached in the stationary phase it means that the yeast grew firstly due to the availability of nitrogen and the carbon source, then when were exhausted the enzyme production was increased by the activation of the genes for survival mechanism. The ASNase production using glycerol (1085 ± 55 U L^{-1}) or sucrose (1534 ± 358 U L^{-1}) separately was less than their mixture (2595 ± 359 U L^{-1}) and the final cell concentration ranged from 16.1 to 17.45 g L^{-1} the biomass production was not significantly different from that obtained using sucrose or the mixture of both substrates.

The glycerol and sucrose together caused a positive synergistic effect on enzyme production, this means that combination of both substrates improves the production of the enzyme modifying the internal metabolism of the yeast. This interaction could be explained because in high sugar concentration medium with a high expression of invertase the hydrolysis of sucrose to glucose and fructose augment the osmotic pressure in the medium, this stressing osmotic pressure can inhibit the yeast growth triggering a complex response that enhanced the glycerol production and retention and the ASNase production. Osmotolerant yeasts are able to retain and synthesize glycerol to act as an osmoregulator, and some yeasta have active glycerol uptake pumps (FLORES et al, 2000). This behavior was observed in the *L. scottii* culture were was produced a small quantity of glycerol in the presences of sucrose at 49 g L^{-1}(figure 2A). Synthesis and retention of glycerol by yeast strains has been shown previously to correlate strongly with fermentation in media with high concentrations of either glucose and fructose, or equivalent levels of sucrose (MYERS, et al 1997). In *S. cerevisiae* is reported that the ability to adapt to high osmotic pressures is critical to the efficiency of fermentation of high sugar concentration media producing high levels of glycerol 3-phosphate dehydrogenase (GD3P) (ATTFIELD and KLETSAS, 2000). This is in agreement the capacity of the L. scottii to survive in marine environments where intracellular osmotic regulation is very important due to the hypertonic characteristics of the sea water.

4.3.3 **Effect of aeration and agitation**

The oxygen is a factor that influences the growth and biosynthesis of the yeast enzymes, as well as the production of different metabolites such neutral lipids. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities, however, each microorganism varies in its oxygen requirements. Measurements of k_La provide important information about a bioprocess on bioreactor and the determination of the coefficient ensure that processing conditions are such that an adequate supply of oxygen is available for cell growth (GARCIA-OCHOA et al, 2010). Being non-fermentative and strictly aerobic yeast (WATSON et al, 1978) is interesting the study of the behavior of *Leucosporidium scottii* L115 under different k_La conditions and at 15°C of culture temperature which favors the dissolution of oxygen in the medium.



.

Figure 18. Experimental data from the batch cultures of *L. scottii* L115 (◆ASNase; ●cell; ▲fructose; ■glucose; and ▼glycerol) to assess the initial cell concentration A) X_0: 1 g L^{-1}, B) X_0: 3 g L^{-1}, C) X_0: 5 g L^{-1}.

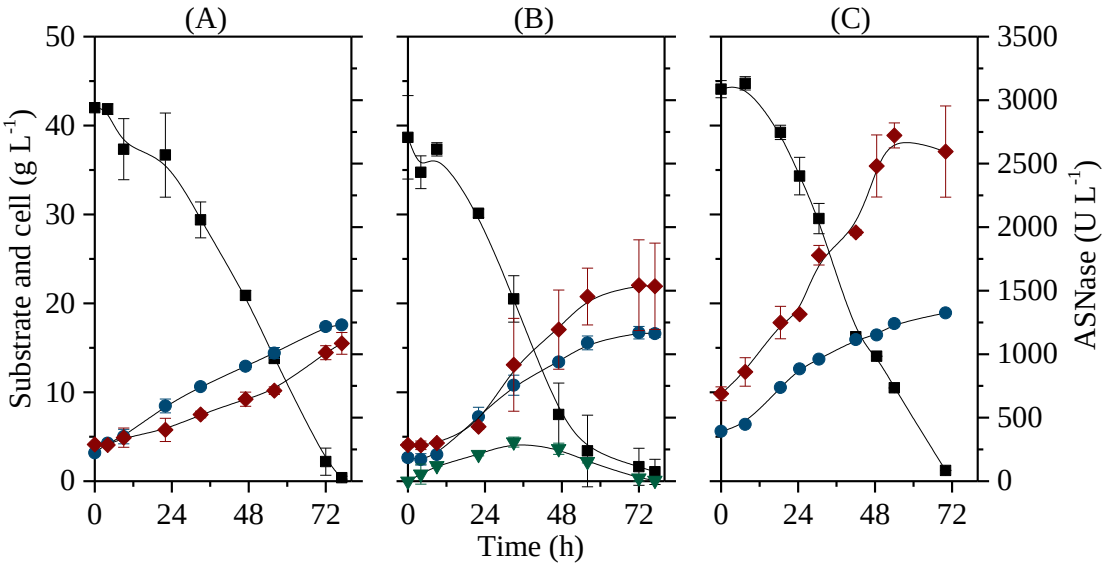
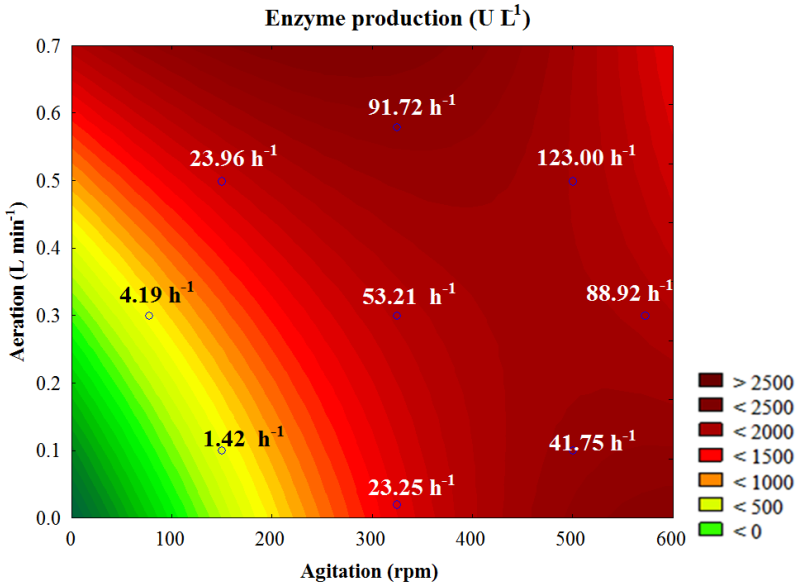


Figure 19. Experimental data from the batch cultures of *L. scottii* L115 (◆ASNase; ● cell; and ■ substrate) to assess the carbon source A) Glycerol, B) Sucrose, C) Glycerol + sucrose.

From the bioprocess point of view, the effect of oxygen transfer condition on *L. scottii* L115 culture was evaluated in the bioreactor. The effect of the aeration and agitation on the production of the enzyme and the lipids accumulation was studied through RSM using a Central Composition Design (CCD). According to Table 18, higher values of k_La benefited ASNase and lipids production. The maximum ASNase production, approximately 2613 U L^{-1}, corresponded to assay 6 (k_La: 89 h^{-1}) and assay 8 (k_La: 92 h^{-1}), and the maximum productivity of the enzyme (36.30U·L^{-1}·h^{-1}) was obtained with a k_La coefficient of 89 h^{-1} obtained at 572.5 rpm of agitation speed and 0.3 L min^{-1} of airflow. These conditions were more efficient to produce cell as well, 0.31 and 0.27 g g^{-1}, respectively, also, more efficient in terms of specific enzyme production (U g^{-1} cell) that corresponded to 169 and 200 U g^{-1}, respectively. The conditions of the assay 8 (325 rpm, 0.58 L min^{-1}) are more suitable parameters due to less foam was produced and in consequence, less anti-foam will be required, instead of assay 6 that corresponds to higher agitation. At lower agitation rate, the oxygen transfer is limited resulting in decreased cell mass growth, while at higher agitation rate, although growth is more, there is a reduction in enzyme activity. It may be because of the variation in the agitation speed influences in the nutrient uptake. Thus, analyzing the data obtained we can establish that the working conditions for the optimization of work for the ASNase production were 88.92 – 91.72 h^{-1} of k_La.

This design allowed to explore a wide range of oxygen transfer conditions, find the effect caused by this factor on kinetic parameters in ASNase production and in the lipid accumulation, with this information set a specific condition that can serve as a starting point to the culture of *L. scottii* L115 within a bioreactor using conditions that guarantee sufficient oxygen for the cultures.

A



B

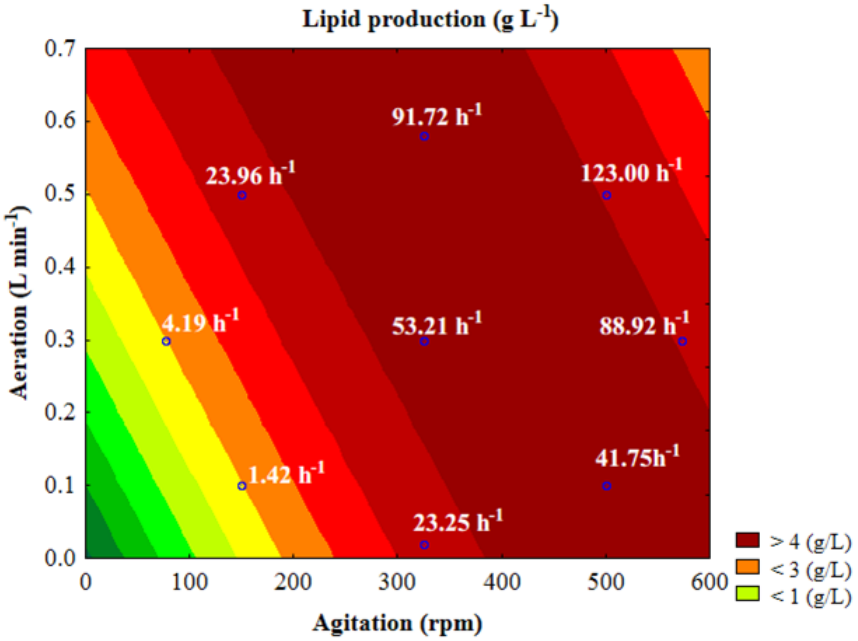


Figure 20. Countor surface of k_La determination at different conditions of aeration and agitantion in Bioractor 1L. Biostat. Sartourius

Table 18. CCD design to optimize the ASNase production and results of productivity and conversion

Assay	Agitation	Airflow	k_La	μ_{max}	ASNase	Lipids	Q_{Cell}	Q_{ASNase}	P_L	$Y_{X/S}$	$Y_{ASNase/S}$	$Y_{L/S}$	$Y_{ASNase/X}$	$Y_{L/X}$
	rpm	L min^{-1}	h^{-1}	h^{-1}	U L^{-1}	g L^{-1}	g L^{-1} h^{-1}	g L^{-1} h^{-1}	g L^{-1} h^{-1}	g g^{-1}	U g^{-1}	g g^{-1}	U g^{-1}	g g^{-1}
1	150	0.1	1.42	0.028	523	1.67	0.065	7.30	0.03	0.13	13.78	0.05	105	0.41
2	150	0.5	74.11	0.046	1893	5.08	0.151	26.30	0.07	0.25	38.04	0.10	155	0.42
3	500	0.1	41.75	0.035	1880	5.98	0.160	26.10	0.08	0.24	37.82	0.12	155	0.51
4	500	0.5	123.0	0.053	1880	5.56	0.175	26.10	0.08	0.26	38.66	0.12	147	0.46
5	78	0.3	4.19	0.025	772	1.91	0.078	10.70	0.03	0.20	27.09	0.07	133	0.35
6	572	0.3	88.92	0.057	2613	5.04	0.183	36.30	0.06	0.31	52.93	0.09	169	0.33
7	325	0.02	23.25	0.041	2062	5.11	0.183	28.60	0.06	0.26	40.47	0.09	154	0.34
8	325	0.58	91.72	0.046	2583	5.27	0.176	35.90	0.07	0.27	53.13	0.10	200	0.39
9	325	0.3	53.21	0.044	2179	5.66	0.156	22.70	0.08	0.24	34.11	0.12	143	0.49

$Y_{ASNase/S}$,enzyme yield, U g^{-1} substrate assimilated, $Y_{X/S}$, cell yield, g g^{-1} substrate assimilated, $Y_{L/S}$, lipids yield, g g^{-1} substrate assimilated, Q_X , volumetric cell productivity , g $L^{-1}h^{-1}$, Q_{ASNase} , volumetric enzyme productivity , U $L^{-1}h^{-1}$.

4.3.4 **Lipids accumulation**

The accumulation of lipids in the different psychrotolerant yeast has been reported with a fatty acid profile for the production of biodiesel and uses in food industry. As is well know the accumulation of lipids in yeast is produced in certain conditions like low nitrogen concentration in the medium and the adequate oxygen transfer rate (BUZZINI et al 2012; PEREYRA et al 2014). The maximum productivity of lipids was 0.08 $g \cdot L^{-1} \cdot h^{-1}$ obtained at 41.75, 53.21 and 123.0 h^{-1} of K_La, but comparing the lipid accumulated by gram of cell produced the best condition was $41.75h^{-1}$.As can be observed in the figure 22 the accumulation of lipids could be accompanied by the staining of the cells using nile red.

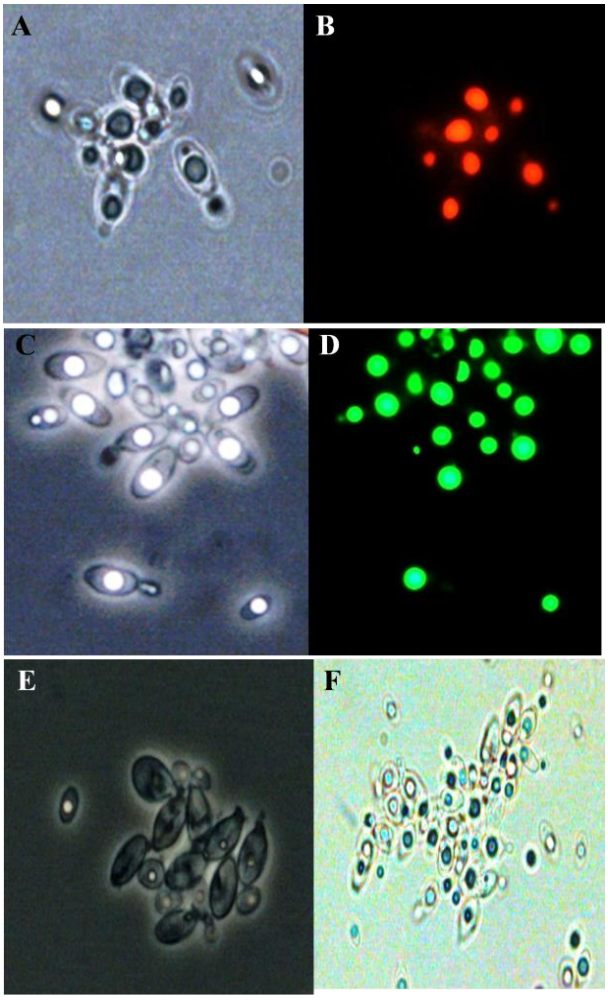


Figure 21. Lipid accumulation of Nile red stained cells of *Leucosporidium scottii* L115 observed trough phase-contrast microscopy and fluorescence microscopy (63x) **A**) Bright-field microscopy (cells cultivated in high k_La condition), **B**)Fluorescence microscopy YFP filter (Ex54/Em572 nm), **C**) Phase-contrast microscopy (cells cultivated in high k_La condition), **D**)Fluorescence microscopy EGFP filter (Ex488/Em509 nm), (**E**) Phase-contrast (low k_La.) (**F**) Bright-field microscopy (cells cultivated in high k_La condition).

The lipids production was similar for all assays, except in low k_La (Assay 1 and 5) between 0.06 – 0.08 g L^{-1} h^{-1}. Moreover, the maximum lipids production not corresponded to maximum enzyme production. The better lipid production corresponded to assay 3 (k_La: 42 h^{-1}) and assay 9 (k_La: 53 h^{-1}) that produced 0.51 and 0.49 g of lipids per g of cell, respectively. Therefore, *L. scotti* L115 is well producer of ASNase or accumulate lipids depending on k_La value for maximum lipids accumulation was 42 – 53 h^{-1}. Analyzing the generated response surfaces, at lower values of 23 h^{-1} of k_La, the lipids production will decrases considerably, this means that a good oxygenation of the medium is necessary for the production of lipids.

4.3.5 **Fatty acid profile produced by *Leucosporidium scottii* L115**

The *Leucosporidium scottii* recently has been reported as oleaginous yeast (PEREYRA 2014). These kinds of species are capable of forming lipid bodies for storage molecules such as triglycerides (TAGs) and steryl esters (SE). Those are not suitable for integration into pholipid bilayers (BEOPOULOS , et al 2008). Among the total fatty acid (FA) quantified in *L. scottii* the oleic acid (18:1) was the principal acid in the composition (50.15%) followed by the palmitic acid (16:0) (16.72%) and linoleic acid (18:2) (14.88%). The FA profile (Table 19) produced by *L. scottii* L115 containe monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA).

Table 19. Fatty acids composition produced by *Leucosporidium scottii* L115 (Average composition)

Fattyacids	**Average value (%)**
Myrisitic acid (C14:0)	0.61 ± 0.08
Palmitic acid (C16:0)	16.72 ± 1.68
Palmitoleic acid (C16:1)	0.53 ± 0.21
Stearic acid (C18:0)	8.56 ± 2.51
Oleic acid (C18:1)	50.15 ± 4.40
Linoleic acid (C18:2)	14.88 ± 1.59
Octadecatrienoic acid (C18:3 n-4)	4.46 ± 1.09
Arachidic acid (C20:0)	0.54 ± 0.11
Gadoleic acid (C20:1) (n-11)+(n+9)	0.23 ± 0.06
Arachidonic acid (C20:4) (n-6)	0.10 ± 0.06
cis-11-docosenoic acid (cetoleic) (C22:1) (n-11)	0.96 ± 0.42
Docohexanoic acid (DHA) (C22:6) (n-3)	1.61 ± 0.95

The total lipid quantified reaches the 50 % of the total weight and the results of the composition obtained are similar with that reported by Pereyra et al (2004), but unlike, we found that *L. scotti* L115 produces small amounts of DHA at a level of 1%, whereas in others studies the DHA was not detected in psychrotolerant yeasts species. This could be explained because the previous studies the temperature of culture was at 25°C, instead our experiments were carried at 15°C and it is know that the lipid profile can be modified by adjusting the culture temperature because the degree of saturation generally decreases with the temperature. The psychrophiles are generally endowed with a higher proportion of unsaturated fatty acids, especially hexadecenoic (16:1) and octadecenoic acid (18:1), that mesophilic and increase in the DHA (22:6) and/or eicosapentanoic acid (20:5) is observed in psychrophiles and barophiles when grown at temperatures closed to 0°C (RUSSELL 1992). From the samples of cultures obtained at different oxygen transfer rates analyzed the composition of the fatty acids not changes, but the production was affected significantly.

4.4 CONCLUSION

In conclusion, the study confirms that the *Leucosporidium scottii* isolated from Peninsula Antarctica show the capacity to produce ASNase and accumulate lipids using substrates like glycerol and sucrose, substrates that could be used for a large-scale production. These findings emphasize that the cold-adapted yeasts are a potential source of enzymes suitable for biotechnological processes at low and mild temperatures. The development of the bioprocess for ASNase production by the psychrotolerant yeast *Leucosporidium scottii* using the DOE strategies for increasing the volumetric yield of the enzyme, the optimization of the dissolved oxygen level, results in an increment of the enzyme production. Under optimized conditions, the highest enzyme production was obtained (growth temperature 15º C, X_0: 5 g L^{-1}, pH 7.0, 48 h, k_La 89-92 h^{-1}).There are few psychrotolerant yeasts studies in bioreactor cultures at temperatures of 15ºC, for this very reason, this study can be taken into account for the next studies of different psychrotolerant yeasts and to better understand the culture process of these microorganisms.

The lipid accumulation critically depends on the carbon used and the dissolved oxygen levels. The cultivation of *L. scottii* L115 at 15ºC in cheap and convenient carbon sources generating an interesting lipid profile, where it presents monounsaturated and polyunsaturated lipids that can be studied in greater depth for their possible use in the production of biodiesel or with uses in the food industry. Also can be explored the use of molasses from biomass production and recycling glycerol from the biodiesel industry to produces biodisel in a sustainable way.

5 GENERAL CONCLUSION

The L-Asparaginase from *Leucosporidium scottii* L115 has potential to uses at therapeuctic level and in the food industry, in further years the need for new ASNase molecules with improved properties will need to get a better ALL treatment. Further studies of the LsASNase molecule can help to understand the mechanisms whereby presents a low glutamine affinity and the structure to favore its kinetics parameters. Also this non-conventional yeast have the capacity to use alternative sources of carbon, to generate value-added molecules, such as enzymes and lipids.

The microbiological potential of Antarctica can be exploited through the Brazilian Antartic Program (PROANTAR) which can provide microorganisms that have been little studied and that can generate new molecules of great interest for the pharmaceutical, chemical and food industries. The psychrophiles and psychrotolerant microorganism, in special the yeasts have shown that they are capable of producing new enzymes used at industrial levels, these cold adapted enzymes presents different characteristics that deservs be studied in deep for the improved structures and kinetic parameters that can result in the generation of new molecules with more specificity and greated catalitc efficience.

For this reason, the study of the cold-adapted microorganisms opens a wide range of options to generate new molecules, through the study of the mechanisms and molecular structures developed to compensate the cold-environments.

6 FUTURE PERSPECTIVES

The production of recombinant LsASNase is necessary to study the effect of glycosylation on the structure and its change in kinetic behavior since the allosteric behavior can be produced by the presence of glycosylations. These could also affect the affinity of the substrate and the Kcat can increase by double considering the reduction of molecular weight by almost half.

The structural analysis of the enzyme, will identificate the the sequences that increase the flexibility of the structure and improve the kinetic parameters, not only of this but also of other enzymes as well.

In relation to the lipid profile obtained from *L. scottii*, studies can be made of the possible applications of these as SCOs or for the production of biodiesel from the uses of cheap carbon sources such as glycerol, molasses or xylose.

7 REFERENCES

1. Abd El Baky H.H., El Baroty G. S., Optimization of Growth Conditions for purification and production of L-asparaginase by *Spirulina maxima*, Evidence-Based complementary and Alternative Medicine, Article ID 1785930, Vol. 2016.
2. Adrio, J.L., Oleaginous Yeasts: Promising platforms for the production of Oleochemicals and Biofuels, Biotechnology and Bioengineering, Vol. 114, No. 9, pp. 1915-1920, 2017.
3. Aghaiypour K., Wlodawer A., Lubkowski J., Structural basis for the activity and substrate Specificity of *Erwinia chrysanthemi* L-Asparaginase, Biochemistry, 15 40 (19) pp. 5655-5664, 2001.
4. Alcaino J., Cifuentes V., Baeza M., Physiological adaptations of yeast living in cold environments and their potential applications, World J Microbiol Biotechnol, 31, pp 1467-1473, 2015.
5. Aldelrahman S. Z. Gregory A. Chenyu D., Marine yeast isolation and industrial application, FEMS Yeast Research 14 pp 813-825, 2014.
6. Andreote F.D., Pereira S. M., Melo M. V., Roesch L., The Brazilian Soil Microbiome, in Pylro V., Roesch L., (eds) Soil Biodiversity in Amazonian and Other Brazilian Ecosystems, Current status and Perspectives, Springer International Publishing AG, 2017.
7. Anishkin,A., Sukharev, S., Rempe, S.B., Catalytic role of the substrate defines specificity of therapeutic L-asparaginase, J Mol Biol, 427, 2867-2885, 2015.
8. Arima, K., Sakamoto, T., Araki, C., Tamura, G., Production of extracellular asparaginases by microorganisms. Agric Biol Chem 36, 356-361, 1972.
9. Atkin C.L., Neilands J.B., Phaff H.J., Rhodotorulic Acid from Species of *Leucospordium, Rhodosporidum, Rhodotorula, Sporidiobolus,* and *Sporobolomyces,* and a new alanine-containing ferrichrome from *Crytococcus melibiosum,* Journal of Bacteriology, Vol. 103, No.3., pp. 722-733, 1970.
10. Attfield, P.V., Kletsas, S., Hyperosmotic stress response by strains of baker's yeasts in high sugar concentration medium, Letters in Applied Microbiology, 31, 323-327, 2000.

11. Avramis V.I., Tiwari, P. N., Asparaginase (native ASNase or pegylated ASNase) in the treatment of acute lymphoblastic leukemia, International Journal of Nanomedicine, I (3) 241-254, 2006.
12. Baakza A., Vala A. K., Dave B. P., Dube H.C., A comparative study of siderophore production by fungi from marine and terrestrial habitats, Journal of Experimental Marine Biology and Ecology 311, pp 1-9, 2004.
13. Balcão,V.M., Mateo, C., Fernandez-Lafuente, R., Malcata, X., Guisán, J.M., Structural and functional stabilization of asparaginase via multisubunit immobilization onto highly activated supports. Biotechnol Prog 17(3), 537- 542, 2001.
14. Beopoulos A., Mrozova, Z., Thevenieau, F., Le Dall M.T., Hapala, I., Papanikolau, S., Chardot, T., Nicaud, J.M., Control of lipid accumulation in yeast *Yarrowia lipolitica*, Applied and Enviromental Microbiology, pp. 7779-7789, 2001.
15. Birgisson H., Delgado, O., García A., L., Hatti-Kaul, R., Mattiasson, B., Cold-adapted yeasts as producers of cold-active polygalacturonases, Extremophiles (7), pp. 185-193, 2003.
16. Bjelic, S., Brandsdal, B.O., Aqvist, J., Cold adaptation of enzyme reaction rates. Biochem 47, 10049-10057, 2008.
17. Bonthron, D.T., Jaskólski, M., Why a benign mutation kills enzyme activity. Structure-based analysis of the a176v mutant of *Saccharomyces cerevisiae* asparaginase I.Acta Biochim Pol 44(3),491-504, 1997.
18. Borek, D. Michalska K., Brzezinski K., Kisiel A., Podkowinski J., Bonthron D.T., Krowarsch D., Otlewski J., Jaskólski, M., Expression, purification and catalytic activity of *Lupinus luteus* asparagine β-amidohydrolase and its *Escherichia coli* homolog, European Journal of Biochemistry, 271, 15, pp 3215-3226, 2004.
19. Borek, D., Jaskólski, M., Sequence analysis of enzymes with asparaginase activity. Acta Biochim Pol 48(4), 893-902, 2001.
20. Borek, D., Kozak, M., Pei J., Jaskolski M., Crystal structure of active site mutant of antileukemic L-asparaginase reveals conserved zinc-binding site, FEBS Jounal, 281, pp 4097-4111, 2014.
21. Brizzio, S., Turchetti, B., de García, V., Libkind, D., Buzzini, P., van Broock, M., Extracellular enzymatic activities of basidiomycetous yeasts isolated from glacial

and subglacial waters of northwest Patagonia (Argentina), Can. J. Microbiol. Vol. 53, pp. 519-525, 2007.

22. Buzzini, P., Branda, E., Goretti, M., Turchetti, B., Psychrophilic yeast from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol 82 (2), 217-241, 2012.
23. Canal Saude/ Fiocruz, Ministerio de Saúde garante medicamento contra leucemia infantil, consulta: 15 de maio 2014. http://www.canal.fiocruz.br/destaque/index.php?id=1430#head-brasil
24. Carrasco, M., Rozas, J.M., Barahona, S., Alcaíno, J., Cifuentes, V., Baeza, M., Diversity and extracellular enzymatic activities of yeasts isolated from King George island, the sub-antartic region. BMC Microbiol 12, 251, 2012.
25. Cavello, I., Albanesi, A., Fratebianchi, D., Garmedia, G., Vero S., Cavalitto, S., Pectinolytic yeasts from cold enviroments: novel findings of *Guehomyces pullulans, Cystofilobasidium infirmominiatum* and *Cryptococcus adeliensis* producing pectinases, Extremophiles (21) pp. 319-329, 2017.
26. Cavicchioli, R., Charlton, T., Ertan, H., Mohdomar, S., Siddiqui, S., Williams, T.J., Biotechnological use of enzymes from psycrhrophiles. Microbiol Biotechnol 4(4), 449-460, 2011.
27. Chan, W. K. et al, The Glutaminase Activity of L-Asparaginase Is Not Required for Anticarcer Activity against ASNS-Negative Cells, Blod , v. 123, n. 23, p. 3596- 3606., 2014.
28. Cooney D.A., Handschumacher R.E., L-asparaginase and L-aspagine Metabolism, Annu. Rev. Pharmacol., v. 10, 421-440, 1970.
29. Costa, I.M., Schultz, L., De Araujo, B., Leite, M.S., Farsky, S.H. De Oliveira, M.A., Pessoa, A., Monteiro, G., Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity. Sci Rep 6: 36239, doi:10.1038/srep36239, 2016.
30. Covini,D., Tardito, S., Bussolati, O., Chiarelli, L.R., Pasquetto, M.V. Diglio, R., Valentini, G., Scotti,C., Expanding targets for a metabolic therapy of cancer: L-asparaginase. Recent Pat Anticancer Drug Discover 7,4-13, 2012.
31. D´amico, S., Gerday, C., Molecular basis of cold adaptation. Phil. Trans. R. Soc Lond B 357, 917- 925, 2002.

32. De García, V., Brizzio, S., Libkind, D., Buzzini, P., Van Broock, M., Biodiversity of cold–adapted yeast from glacial melt water rivers in Patagonia, Argentina. FEMS Microbiol Ecol 59(2), 331-341, 2007.
33. De Mot, R., Verachtert, H., Purification and characterization of extracellular α-amylase and glucoamylase from yeast *Candida Antarctica* CBS 6678, Eur. J. Biochem, 164, pp. 643-654, 1987.
34. Dinarvand M. Ariff A. B., Effects of C/N Ratio and Media Optimization through Response Surface Methodology on Simultaneous Productions of Intra- and Extracellular Inulinase and Invertase from *Aspergillus Níger* ATCC 20611, BioMedical Research International Journal. Article ID 508968. p.13, 2013
35. Drainas C., Kinghorn J.R., Pateman J.A., Aspartic Hydroxamate Resistance and Asparaginase Regulation in the Fungus *Aspergillus nidulans,* Journal of General Microbiology , Vol. 98, 493-501, 1977.
36. Drainas, C., Pateman, J.A., L-Asparaginase Activity in the Fungus *Aspergillus nidulans,* Biochemical Society Transactions,v. 5, p. 259- 261, 1977.
37. Duarte, A.W.F., Dayo-Owoyemi,I., Nobre, F.S., Pagnocca, F.C., Chaud, L.C.S, Pessoa, A., Felipe, M.G.A., Sette, L.D., Taxonomic assessment and enzymes production by yeast isolated from marine and terrestrial Antarctic samples. Extremophiles 17, 1023-1035, 2013.
38. Dunlop, P.C., Roon, R.J., Characterization of two forms of asparaginase in *Saccharomyces cerevisiae.*J Biol Chem 253(4), 1297- 1304, 1978.
39. Dunlop,P.C., Meyer, G.M., Roon, R.J., Reactions of asparaginase II of *Saccharomyces cerevisiae*. A mechanistic analyisis of hydrolysis and hydroxylaminolysis. J Biol Chem 255(4), 1542-1546, 1980.
40. Egler, R.A., Ahuja, S.P., Matloub, Y., Asparaginase in the treatment of patients with acute lymphoblastic leukemia. J Pharmacol Pharmacother 7,62-71, 2016.
41. El-Ahmady N., El-Nagga R, Microbial L-asparaginase as a Potential Therapeutic Agent for the Treatment of Acute Lymphoblastic Leukemia: The Pros and Cons, Internal Journal of Pharmacology v. 10, n.4,p.182-199, 2014.
42. El-Ghonemy D., Microbial Amidases and their Industrial Applications: A Review, Journal of Medical Microbiology & Diagnosis, vol. 4:1, 2014.
43. Feller, G., Psychrophilic enzymes: from folding to function and biotechnology. Scientifica (Cairo) 2013, 512840, 2013.

44. Feller, G., Gerday, C., Pychrophilic enzymes: molecular basis of adaptation. Cell. Mol Life Sci53, 830-841, 1997.
45. Ferrara, M.A., Pereira, N.J., Kinetics of Asparaginase II fermentation in *Saccharomyces cerevisiae ure2dal80 mutant.* Appl Biochem Biotechnol 113-116, 299-305, 2004.
46. Flores C-L., Rodríguez C., Petit T., Gancedo C., Carbohydrate and energy-yielding metabolism in non-conventional yeasts, FEMS, Microbiology Reviews, 24,pp. 507-529, 2000.
47. Foda, M.S., Zedan, H.H., Hashem, S.A., Formation and properties of L-glutaminase and L-asparaginase activities in Pichia polymorpha, Acta Microbiologica Polonica 29 (4): 343-352, 1980.
48. Gancedo J. M., Yeast carbon catabolite repression, Mricrobial Molecular Biology Reviews, v. 62 (2), pp.334-361, 1998.
49. Garcia-Ochoa F., Santos M. V.E., Merchuk, J.C., Oxygen uptake rate in microbial processes: An overview, Biochemical Engineering Journal, 49, pp. 289-307, 2010.
50. Gerday, C., Aittaleb, M., Arpigny, J.L., Baise, E., Chessa, J.P., Garsoux, G., Petrescu, I., Feller, G., Psychrophilic enzymes: a thermodynamic challenge. Biochim Biophys Acta1342(2), 119-131, 1997.
51. Gerday,C., Aittaleb, M., Bentahir, M., Chessa, J.P., Claverie, P., Collins, T., D´Amico, S., Dumont, J., Garsoux, G., Georlette, D., Hoyoux, A., Lonhienne, T., Meuwis, M.A., Feller, G., Cold adapted enzymes: from fundamentals to Biotechnology.Trends Biotechnol 18(3), 103-107, 2000.
52. Gervais D., Allison N., Jennings A., Jones S., Marks T., Validation of a 30-years old process for the manufacture of L-asparaginase from *Erwinia chrysanthemi* , Bioprocess Biosyst Eng, 36, 453-460, 2013.
53. Goetting, P., Effects of Glycosylation on the Enzymatic Activity and Mechanisms of Proteases, Int. J.Mol. Sci. 17,1969, 2016
54. Gomes, J., Gomes, I., Steiner, W., Thermolabile xylanase of the Antarctic yeast *Crytococcus adeliae*: production and properties, Extremophiles, 4: 227-235, 2000.
55. Godard P., André B., Effect of 21 Different Nitrogen Sources on Global Gene Expression in the Yeast *Saccharomyces cerevisiae,* Molecular and Cellular Biology, Vol. 27, No. 8, p. 3065-3086, 2007.
56. Granato D., Araújo Calado V.M., The use and importance of design of expermients (DOE) in process modelling in food science and technology, in

Granato D., Ares G., (eds) Mathematical and Statistical Methods Food Science and Technology, First Edition, Wiley & Sons, 2014.

57. Grossowicz, N., Wainfan, E., Borek, E., Waelsch, H., The enzymatic formation of hydroxamic acids from glutamine and asparagine. J Biol Chem187, 111-125, 1950.
58. Gulati,R., Saxena, R.K., Gupta, R., A rapid plate assay for screening asparaginase producing micro-organisms. Lett Appl Microbiol 24, 23-26, 1997.
59. Gurunathan B., Sahadevan R., Optimization of Media Components and Operating Conditions for Exogenous Production of Fungal L-asparaginase, Chiang Mai Journal Science, v. 38, n. 2, p. 270-279, 2011.
60. Helmstaedt, K., Krappmann, S., Braus, G.H., Allosteric regulation of catalytic activity: *Escherichia coli* asparate transcarbamoylase versus yeast chorismate mutase. Microbiol Mol Biol Rev 65(3), 404-421, 2001.
61. Huang, C., Chen X., Xiong L., Chen X., Ma L., Chen Y., Single cell oil production from low-cost substrates: The possibility and potential of its industrialization, Biotechnology Advances, 31, pp. 129-139, 2017.
62. Honing, W., Kula, M.R., Selectivity of protein precipitation with polyethylene glycol fractions of various molecular weights. Anal Biochem 72, 502-512, 1976.
63. Hoyoux, A., Gerday, C., Review Extreme Catalysts from Low-Temperature Enviroments., J Biosci Bioeng 98 (5), 317-330, 2004.
64. Hymavathi M. Et Al, Impact Of Carbon and Nitrogen Sources on L-Asparaginase Production by Isolated *Bacillus circulans* (MTCC 8574): Application of Saturated Plackett-Burman Design., Chem. Biochem. Eng. Q. v.24, n. 4, p. 473-480, 2010. .
65. Isaksen,G.V., Aqvist, J., Brandsdal, B.O., Enzyme surface rigidity tunes the temperature dependence of catalytic rates. Proc Nat l Acad Sci USA 113(28), 7822-7827, 2016.
66. Inghman, K.C., Precipitation of proteins with polyethylene glycol. Methods Enzymol 182, 301-306, 1990.
67. Imada, A., Igarasi, S., Nakahama, K., Isono, M., Asparaginase and glutaminase activities of micro-organisms. J Gen Microbiol 76, 85-99, 1973.
68. Johnson, E.A., Biotechnology of non-saccharomyces yeast- the basidiomycetes. Appl Microbiol Biotechnol 97, 7563- 7577, 2013.
69. Joseph B., Ramteke, P.W., Thomas G., Cold active microbial lipases: Some hot issues and recent developments, Biotechnology advances, 26, pp. 457-470, 2008.

70. Karamitros, C.S., Konrad, M., Human 60 kDa lysophospholipase contains an N-terminal asparaginase domain that is allosterically regulated by L-asparagine. J Biol Chem 289(19), 12922-12975, 2014.

71. Kennedy, M.J., Krouse, D., Strategies for improving fermentation medium performance: A review, Journal of Industrial Microbiology and Biotechnology, 23, pp. 456-475, 1999.

72. Kil, J. O., Kim, G.N., Park, I., Extraction of Extracellular L-asparaginase from *Candida utilis*, Biosci, Biosc. Biotech. Biochem., 59 (4), 749-750, 1995.

73. Kin Chan, W., Lorenzi, L.P., Anishkin, A., Purwaha, P., Rogers, D.M, Sukharev, S., Rempe, S.B., Weinstein, J.N., 2014. The glutaminase activity of asparaginase is not required for anticancer activity against ASNs-negative cells. Blood 123(23), 3596-3606, 2014.

74. Kotzia, G.A., Lappa K., Labrou N.E., Tailoring structure-function properties of L-asparaginase: engineering resistance to trypsin cleavage, Biochem. J., 404, pp. 337-343, 2007.

75. Krysiak, J., Bialkowska, A., Turkiewicz, M., Aspartic protease- Unique enzyme from psychrotolerant yeast *Sporobolomyces roseus*, New Biotechnology, v. 33, p.109.

76. Laemmli, U., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 670–685, 1970.

77. Lario, L.D., Chaud, L., Almeida, G. M., Converti, A., Sette, D. L, Pessoa Jr., A., Production, purification, and characterization of an extracellular acid protease from the marine Antarctic yeast *Rhodotorula mucilaginosa* L7, Fungal Biology, v. 119, (11), pp. 1129-1136, 2015.

78. Livingston B., Krakoff I., L-Asparaginase: A new type of anticancer Drug, The American Journal of Nursing, v. 70, n. 9, p. 1910-1915, 1970.

79. Liu, C., Yang, W., Devidas, M., Cheng, C., Pei, D., Smith, C., Carroll, W.L., Raetz, E.A., Bowman, M.P., Larsen, E.C., Maloney, K.W., Martin, P.L., Mattano, L.A., Winik, N.J. Mardis, E.R. Relling, M.V., Clinical and genetic risk factors for acute pancreatitis in patients with acute lymphoblastic leukemia. J Clin Onco l34(18), 2133-2140, 2016.

80. Magasanik B., Kaiser C. Nitrogen regulation in *Saccharomyces cerevisiae* , Gene 290, pp.1-18, 2002

81. Mandenius C.F, Brundin A., Bioprocess Optimization Using Design of Experiments Methodology, Biotechnology Progress, 24, pp.1191-1203, 2008.
82. Margesin, R., Fauster, V., Fonteyne, P.A., Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida*, Letters in Applied Microbiology , 40, pp. 453-459, 2005.
83. Margesin, R., Miteva, V., Diversity and ecology of psychrophiic microorganisms. Res Microbiol162(3), 346-361, 2011.
84. Michalska, K., Jaskolski, M., Structural aspects of L-asparaginases, their friends and relations. Acta Biochim Pol 53 (4), 627-640, 2006.
85. Michiyo, M., WO, Patent 8802775, 1986.
86. Myers, D.K., Lawlor, D.T.M., Attfield P.V., Influence of Invertase activity and Glycerol Synthesis and retention on fermentacion of media with a high sugar concentration by *Saccharomyces cerevisiae*, Applied and Enviromental Microbiology, v.63, No.1, pp. 145-150, 1997.
87. Nakahama K., Imada A., Igasari S., Formation of L-Asparaginase by Fusarium Species, Journal of General Microbiology, Vol 75, p 269-273, 1973.
88. Narta U. K., Kanwar S.S., Azmi W., Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia, Critical Reviews in Oncology/Hematology 61, pp. 208-221, 2007.
89. Nakagawa, T., Ikehata, R., Uchino, M., Miyaji, T., Takano, K., Tomizuka, N., Cold-active acid β-glactosidase activity of isolated pyschrophilic-baidiomycetous yeast *Guehomyces pullulans*, Microbiological Research, v. 161 (1), pp. 75-79, 2006.
90. Nestl, B.M., Hauer B., Engineering of Flexible loops in Enzymes , ACS Catal, 4, pp. 3201-3211, 2014.
91. Nguyen H.A, Durden D.L., Lavie A., The differential ability of asparagine and glutamine in promoting the closed/active enzyme conformation rationales the Wolinella succinogenes L-asparaginase substrate specificity. Scientific reports 7:41643, 2016.
92. Okafor N., Modern Industrial Microbiology and Biotechnology, Science Publishers, USA, pp. 54-55, 2007.
93. Ohnuma, T., Holland, J.F., Freeman, A., Sinks, L.F., 1970. Biochemical and pharmacological studies with asparaginase in man. Cancer Res 30(9), 2297-2305.

94. Ollenschlager, G., Roth, E., Linkesch, W., Jansen, S., Simmel, A., Modder, B., Asparaginase-induced derangements of glutamine metabolism: the pathogenetic basis for some drug-related side-effects. Eur J Clin Invest 18, 512-516, 1988.

95. Panosyan EH, Avramis VI., Asparaginase antibody and asparaginase activity in children with higher-risk acute lymphoblastic leukemia: Children's Cancer Group study CCG-1961. J Pediatr Hematol Oncol,, 26 (4) pp. 217-226, 2004.

96. Pazgier, M., Turkiewicz, M., Kalinowska, H., Bielecki, S., The unique cold-adapted extracellular subtilase from psychrophilic yeast *Leucosporidium antarcticum*, Journal of Molecular catalysis B: Enzymatic, v. 21 (1-2), pp. 39-42, 2003.

97. Pereyra, V., Martinez, A., Rufo, C., Vero, S., Oleaginous yeasts form Uruguay and Antarctica as renewable raw material for biodiesel production, American Journal of BioScience, v.2, n 6 , pp. 251-257, 2014.

98. Petrescu, I., Lamotte-Brasseur J., Chessa, J.P., Ntarima, P., Claeyssens, M., Devreese, B., Marino, G., Gerday, C., Xylanase from the psychrophilic yeast *Cryptococcus adeliae* , Extremophiles, 4: 137-144, 2000.

99. Paul J.H., Isolation and characterization of a *Chlamydomonas* L-asparaginase, Biochem. J. 203, 109-115, 1982.

100. Pieters, R., Hunger, S.P., Boss, J., Rizzari, C., Silverman, L., Baruchel, A., Goekbuget, N., Schrappe M., Pui, CH., L-asparaginase treatment in acute lymphoblastic leukemia. A focus on Erwinia asparaginase. Cancer, 117 (2), 238-249, 2011.

101. Ramakrishnan, M., Joseph, R., Characterization of an extracellular asparaginase of *Rhodosporidium toruloides* CBS14 exhibiting unique physicochemical properties. Can J Microbiol 42, 316-225, 1996.

102. Ramya, L.N., Pulicherla, K.K., L-asparaginase as potent anti-leukemic agent and its signficance of having reduced glutaminase side activity for better treatment of acute lymphoblastic leukaemia. Appl Biochem Biotechnol 167, 2144-2159, 2012.

103. Roberts, J., Holcenberg, J.H., Dolowy, W.C., Isolation, Crystallization, and Properties of *Achromobacteraceae* Glutaminase-Asparaginase with antitumor activity. J Biol Chem 247 (1), 84-90, 1972.

104. Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U.M., Matteuzzi D., Growth, lipid accumulation, and fatty

acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts, FEMS MicrobiolEcol 69, pp. 363-372, 2009.

105. Russell, N. J. Physiology and molecular biology of psychrophilic microorganisms. In molecular biology and biotechnology of Extremphiles (R. A. Herbert and R. J. Sharp, Eds), 1992.
106. Sampaio, J.P., 2011, *Leucosporidium* Fell, Statzell, I.L. Hunter &Phaff (1969), In: Kurtzman, C.P., Fell, J.W, Boekhout, T. (Eds), The Yeast. A taxonomic study v1, Elsevier, 5thedn, pp. 1485-1494, 2011.
107. Samudio I., Kopopleva M., Asparaginase unveils glutamine-addicted AML, Blood, v.122, n. 20. pp. 3388-3400, 2013.
108. Sanches, M., Krauchenco, S., Polikarpov, I., Structure, substrate complexation and reaction mechanism of bacterial asparaginases.CurrChemBiol1, 75-86, 2007.
109. Sánchez S., Demain A.L., Metabolic regulation and overproduction of primary metabolites, Microbial biotechnology, 1 (4), pp . 283-319, 2008.
110. Sanson, E., Jaskolski, M., Structure, Dynamics and Electrostatics of the L-asparaginase Catalytic Centre: Implications for Reaction Mechanism, Departament of Crystallography, Bickberg College, London and Venus Internet Ltd., London, 2004.
111. Santiago,M., Ramirez-Sarmiento, C.A., Zamora, R.A., Parra, L.P. Discovery, molecular mechanisms and industrial applications of cold-active enzymes, Front Microbiol 7, 1408, doi: 10.3389/fmicb.2016.01408.
112. Sarquis M. I. , Oliveira M., E. M., Santos S., A., Costa L., G., Production of L-asparaginase by Filamentous Fungi, Mem Inst Oswaldo Cruz, v. 99 (5). Pp 489/492, 2004.
113. Savitri, Asthana N., Azmi W., Microbial L-Asparaginase: A Potent Antitumour Enzyme, Indian Journal of Biotechnology, v. 2, p. 184-194, 2003.
114. Shivaji, S., Prasad, G.S., Antarctic Yeasts: Biodiversity and Potential Applications, in: Satyanarayana, T., Kunze, G. (Eds.), Yeast biotechnology: diversity and applications, Springer., Amsterdam, pp. 3-18, 2009.
115. Siddiqui K. S., Cavicchioli R., Cold-Adapted Ezymes, Annual Review of Biochemestry, n. 75 p. 403-433, 2006.
116. Sinclair, K., Warner, J.P., Bonthron, D., The ASP1 gene of *Saccharomyces cerevisiae*, encoding the intracellular isozyme of asparaginase. Gene 144, 37-43, 1994.

117. Shrivastava, A., Singhal, P.K., Recent developments in asparaginase discovery and its potential as anticancer agent. Crit Rev Oncol Hematol100, 1-10, 2015.
118. Spencer J.F.T., Ragout de Spencer, A. L., Laluce, C., Non-conventional yeasts, ApplMicrobiolBiotechnol, 58: 147-156, 2002.
119. Spiers, A.S., Wade, H.E., Bacterial glutaminase in treatment of acute leukemia. Br Med J 1, 1317-1319, 1976.
120. Somero G. N., Temperature as a selective factor in protein evolution: the strategy of "compromise". J. Exp. Zool. 194. Pp. 175-188, 1977.
121. Song, C., Liu G.L., Xu, J.L., Chi, Z.M., Purification and characterization of extracellular β- galactosidase from the psychrotolerant yeast *Guehomyces pullulans* 17-1 isolated from sea sediment in Antarctica, Process Biochemistry, V. 45, (6) , pp. 954-960, 2010.
122. Summerbell R., The heterobasidiomycetous yeast genus *Leucosporidium* in an area of temperate climate, Canadian Journal of Botany 61: 1402-1410, 1983.
123. Takagi H., Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications, Appl Microbiol Biotechnology, v. 81, p. 211-223, 2008.
124. Tayyab, S., Qamar, S., Islam, M., Size exclusion chormatography and size exclusion HPLC of proteins. BiochemEduc 19(3), 149-152, 1991.
125. Tchakouteu S.S., Kalantzi, O., Gardeli Chr., Koutinas, A. A., Aggelis G., Papanikolau s., Lipid production by yeast growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency, Journal of Applied Microbiology , 118, 911-927. 2015.
126. Tribe L.A., Briens C.L, Margaritis A., Determination of the volumetric mass transfer coefficient (k_La) using the dynamic "gas out- gas in" method: Analysis of errors caused by dissolved oxygen probes, Biotechnology and Bioengineering, v. 46, pp. 388-392, 1994.
127. Tsai Y. Y., Ohashi, T., Kanazawa, T., Polbureee, P. Misaki R., Limtong, S., Fujiyama, K., Development of a sufficient and effective procedure for transformation of an oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16, Curr Genet v.63, pp. 359-371, 2017.
128. Turkiewicz M., Pazgier, M., Kalinowska, H., Bielecki, S., A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*, Extremophiles,7: 435-442 , 2003.

129. Turkiewicz M., Pazgier, M., Donachie S. P. , Kalinowska H., Invertase and α-glucosidase production by the endemic Antarctic marine yeast *Leucosporidum antarticticum*, Polish Polar Research, v. 26 no. 2, pp 125-136, 2005.
130. Van Den Berg H., Asparaginase revisited, Leukemia and Lymphoma, v. 52 n. 2, p. 168-178, 2011.
131. Verma N. et al, L-Asparaginase: A Promising Chemotherapeutic Agent, Critical Reviews in Biotechnology, v. 27, p. 45-62, 2007.
132. Wada H., Imamura I., Sako M., Katagiri S., Tarui S., Nishimura H., Inada Y., Antitumor Enzyme: Polyethylene Glycol-modified Asparaginase, Annals of the New York Academy of Sciences, Vol 613, Enzyme Engieering 10, pp. 95-108, 1990.
133. Walsh, G. Pharmaceutical Biotechnology- Concepts and Applications, John Wiley & Sons Ltd, p. 131-135, 2007.
134. Watson K., Arthur H., Morton H., Thermal adaptation in yeast: Obligate psychrphiles are obligate aerobes, and obligate thermophiles are facultative anaerobes, Journal of Bacteriology, v.136, n.2, pp. 815-817, 1978.
135. Woo-Hong, J., Ubiquinone compounds of the strains in *Leucosporidium scottii* and its related taxa, Kor. J. Mycol. Vol. 19, No. 4, pp 258-265,1991.
136. Xu, F., Oruna-Concha, M.J., Elmore, J.S., 2016. The use of asparaginase to reduce acrylamide levels in cooked food. Food Chem 210, 163-171.
137. Yun, M.K., Nouse, A., White, S.W., Rock, C.O., Heath, R.J., Cristal structure and allosteric regulation of the cytoplasmic *Escherichia coli* L-asparaginase I.J Mol Biol369(3), 794-811, 2007.
138. Zhang C., Kim S.K., Research and Application of Marine Microbial Enzymes: Status and Prospects, Marine drugs, 8, 1920-1934, 2010.
139. Zuo S., Zhang T., Jiang B., Mu W. Recent research progress on microbial L-asparaginases, Applied Microbiology and Biotechnology, n. 99, p. 1069-1079, 2015.

ANEXO A

DISPENSA DE ANÁLISE DO COMITÈ DE ÈTICA EM PESQUISA

UNIVERSIDADE DE SÃO PAULO

Faculdade de Ciências Farmacêuticas
Departamento de Tecnologia Bioquímico-Farmacêutica
Laboratório de Biotecnologia Farmacêutica

São Paulo, 20 de janeiro 2018

Profa.Dra. Carlota Rangel Yagui
Coordenadora do Programa de Pós-Graduação em
Tecnologia Bioquímico-Farmacêutica

Senhora Coordenadora:

Vimos declarar que o trabalho intitulado "Produção de L-asparaginase de interesse farmacêutico a partir de cultivo de leveduras isoladas do continente Antárctico" dispensa análise do Comitê de Ética em Pesquisa e/ou Comitê de Ética em Experimentação Animal.

Atenciosamente,

Prof. Dr. Adalberto Pessoa Jr.
Faculdade de Ciências Farmacêuticas
Universidade de São Paulo

Prof. Dr. **Adalberto Pessoa Junior**
Depto. Tecnlogia Bioquímica-Farmacêutica
FBT/FCF

Ignacio Sánchez Moguel

ANEXO B
FICHA DO ALUNO

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

9134 - 8823771/1 - Ignacio Sánchez Moguel

Email:	moguel_ignacio@usp.br
Data de Nascimento:	27/11/1984
Cédula de Identidade:	RNE - V949358-L - SP
Local de Nascimento:	México
Nacionalidade:	Mexicana
Graduação:	Químico Farmacéutico Biólogo - Universidad de Guanajuato - México - 2008

Curso:	Doutorado Direto
Programa:	Tecnologia Bioquímico-Farmacêutica
Área:	Tecnologia de Fermentações
Data de Matrícula:	25/09/2013
Início da Contagem de Prazo:	25/09/2013
Data Limite para o Depósito:	23/01/2018
Orientador:	Prof(a). Dr(a). Adalberto Pessoa Junior - 25/09/2013 até o presente. Email: pessoajr@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 25/09/2013 Português, Aprovado em 21/12/2017
Prorrogação(ões):	120 dias Período de 25/09/2017 até 23/01/2018
Data de Aprovação no Exame de Qualificação:	Aprovado em 27/11/2015
Estágio no Exterior:	Technical University of Denmark, Dinamarca - Período de 01/03/2017 até 31/08/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 25/09/2013 Prorrogação em 23/08/2017

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Prorrogação em 23/08/2017
Impresso em: 20/01/2018 16:33:48

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

9134 - 8823771/1 - Ignacio Sánchez Moguel

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBT5776-4/9	Tópicos Especiais de Tecnologia Bioquímico-Farmacêutica II	07/10/2013	20/10/2013	30	2	100	A	N	Concluída
FBT5733-5/6	Uso Industrial de Enzimas	03/02/2014	06/04/2014	90	6	100	A	N	Concluída
FBT5768-4/3	Princípios de Fermentação Contínua	07/02/2014	13/03/2014	75	5	100	A	N	Concluída
FBT5706-3/1	Estratégias e Gerenciamentos de Processos Farmacêuticos e Biotecnológicos	10/03/2014	11/05/2014	45	3	100	A	N	Concluída
BTC5721-4/4	Tópicos Avançados em Química de Proteínas e Peptídeos para Aplicação Biotecnológica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	11/03/2014	14/04/2014	75	5	100	A	N	Concluída
FBT5700-3/1	Preparo de Artigos Científicos na Área de Tecnologia Bioquímico-Farmacêutica	03/04/2014	04/06/2014	90	0	-	-	N	Matrícula cancelada
TNA5801-1/2	Purificação de Proteínas e Peptídeos (Instituto de Pesquisas Energéticas e Nucleares - Universidade de São Paulo)	11/08/2014	21/09/2014	120	8	87	A	N	Concluída
FBA5728-3/10	Aprimoramento Didático	19/08/2014	15/09/2014	60	4	75	A	N	Concluída
BTC5737-3/1	Cultura de Células de Mamífero Aplicada à Obtenção de Produtos Farmacêuticos e à Terapia Gênica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	23/09/2014	03/11/2014	90	6	92	A	N	Concluída
FBF5805-1/3	Delineamento de Experimentos e Ferramentas Estatísticas Aplicadas às Ciências Farmacêuticas	24/09/2014	28/10/2014	45	3	80	A	N	Concluída
PQI5810-5/2	Delineamento Experimental Aplicado ao Desenvolvimento e Melhoria de Processos (Escola Politécnica - Universidade de São Paulo)	23/02/2015	29/05/2015	120	8	100	B	N	Concluída
FBT5773-7/5	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	02/03/2015	10/05/2015	30	2	100	A	N	Concluída
FBT5713-2/1	Biologia Molecular Aplicada à Biotecnologia Farmacêutica Industrial	09/03/2015	22/03/2015	60	0	-	-	N	Matrícula cancelada
QBQ5891-1/1	Tecnologia do DNA recombinante (Instituto de Química - Universidade de São Paulo)	06/08/2015	18/11/2015	60	0	-	-	N	Matrícula cancelada
PQI5870-1/4	Proteínas Recombinantes: Aspectos Moleculares e Desenvolvimento de Processos (Escola Politécnica - Universidade de São Paulo)	14/09/2015	14/12/2015	120	8	100	A	N	Concluída
BTC5704-7/3	Engenharia Bioquímica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	29/09/2015	11/01/2016	75	5	88	B	N	Concluída
FBA5758-1/1	Fundamentos do Planejamento Experimental e Otimização Simplex	26/01/2016	01/02/2016	30	2	100	A	N	Concluída
PQI5858-3/6	Análise Estatística Multivariável Aplicada a Processos Químicos (Escola Politécnica - Universidade de São Paulo)	22/02/2016	03/06/2016	120	0	-	-	N	Matrícula cancelada
BMM5729-2/1	Análise Sistêmica e Engenharia do Metabolismo Microbiano (Instituto de Ciências Biomédicas - Universidade de São Paulo)	16/03/2016	24/05/2016	120	8	100	A	N	Concluída

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
IBI5035-1/5	Biologia Molecular Computacional (Curso Interunidades: Bioinformática - Universidade de São Paulo)	25/07/2016	14/11/2016	120	0	-	-	N	Matrícula cancelada
BTC5835-1/1	Modelagem e Simulação Matemática de Processos Biotecnológicos (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	01/08/2016	02/10/2016	90	6	100	B	N	Concluída
BTC5800-1/2	Anticorpos Monoclonais e Futuros Desafios (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	03/11/2016	07/12/2016	120	0	-	-	N	Matrícula cancelada
FBA5905-1/1	Planejamento Experimental e Análise Multivariada	13/02/2017	26/02/2017	60	0	-	-	N	Pré-matrícula indeferida

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	40	81
Estágios:			
Total:	0	40	81

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Prorrogação em 23/08/2017

Impresso em: 20/01/2018 16:33:49

ANEXO C
CURRICULUM LATTES

Ignacio Sanchez Moguel

Endereço para acessar este CV: http://lattes.cnpq.br/4996189653152017
Última atualização do currículo em 23/09/2015

Possui mestrado em Maestria en Farmacia pela Universidad Autonoma Del Estado de Morelos(2011). Tem experiência na área de Biotecnologia, com ênfase em Biotecnologia Industrial. **(Texto gerado automaticamente pela aplicação CVLattes)**

Identificação

Nome	Ignacio Sanchez Moguel
Nome em citações bibliográficas	MOGUEL, I. S.

Endereço

Endereço Profissional	Universidade de São Paulo. Avenida Professor Lineu Prestes Butantã 05508000 - São Paulo, SP - Brasil Telefone: (11) 30913862

Formação acadêmica/titulação

2013	Doutorado em andamento em Tecnologia Bioquímico-Farmacêutica (Conceito CAPES 6). Universidade de São Paulo, USP, Brasil. Orientador: . Bolsista do(a): .
2009 - 2011	Mestrado em Maestria en Farmacia. Universidad Autonoma Del Estado de Morelos, U.A.ESTADO MOREL, México. Título: Liberación controlada de Nimesulida a partir de la elaboración de polímeros asociativos,Ano de Obtenção: 2011. Orientador: Dr. Efrén Baltazar Hernández. Bolsista do(a): .

Áreas de atuação

1.	Grande área: Ciências Biológicas / Área: Biotecnologia / Subárea: Biotecnologia Industrial.

Idiomas

Espanhol	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Português	Compreende Razoavelmente, Fala Razoavelmente, Lê Razoavelmente, Escreve Pouco.
Inglês	Compreende Razoavelmente, Fala Pouco, Lê Razoavelmente, Escreve Pouco.

Página gerada pelo Sistema Currículo Lattes em 20/01/2018 às 16:43:54