UNIVERSIDADE FEDERAL DE UBERLÂNDIA
FACULDADE DE ENGENHARIA QUÍMICA
CURSO DE ENGENHARIA DE ALIMENTOS

MAYCON DOUGLAS DE OLIVEIRA

L-ASPARAGINASE: USO TERAPÊUTICO E APLICAÇÃO NA INDÚSTRIA DE ALIMENTOS

TRABALHO DE CONCLUSÃO DE CURSO

PATOS DE MINAS
2020

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Trabalho de Conclusão de Curso como requisito para à obtenção do título de Bacharel em Engenharia de Alimentos, da Faculdade de Engenharia Química, da Universidade Federal de Uberlândia.

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PATOS DE MINAS

2020

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HOMOLOGAÇÃO

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L-asparaginase: Uso terapêutico e aplicação na indústria de alimentos

Trabalho de Conclusão de Curso aprovado nesta data para obtenção do título de Bacharel em Engenharia de Alimentos da Universidade Federal de Uberlândia (UFU) - *campus* Patos de Minas (MG) pela banca examinadora constituída por:

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Patos de Minas, 2 de outubro de 2020.

Documento assinado eletronicamente por **Carla Zanella Guidini**, **Presidente**, em 02/10/2020, às 11:44, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Ubirajara Coutinho Filho**, **Membro de Comissão**, em 02/10/2020, às 11:44, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Vivian Consuelo Reolon Schmidt**, **Membro de Comissão**, em 02/10/2020, às 11:45, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Referência: Processo nº 23117.085945/2019-08 SEI nº 2280849

AGRADECIMENTOS

Agradeço primeiramente a Deus, por tudo e por todos, principalmente por possibilitar a conclusão de mais uma nova etapa vencida em minha vida e de muitas que ainda virão, a minha família, que nos momentos difíceis sempre se mostraram presentes para superá-los, em especial aos meus pais, Maria Dalva Pires de Oliveira e Antônio Donizete de Oliveira e minha avó Onofra Maria, sendo eles meus maiores exemplos e espelhos de persistência e coragem durante toda essa jornada, e também as minhas duas irmãs, Tays Fernanda de Oliveira e Thatiane Pires de Oliveira além dos demais familiares, que sempre incentivaram e não mediram esforços para a conclusão do meu ensino superior, não há palavras que demonstrem e justifiquem o quão grato sou a todos vocês.

Gostaria de deixar meus agradecimentos às Professoras Drs., e também amigas, Carla Zanella Guidini, Liliane Maciel e Vivian Schmidt que sempre estiverem ao meu lado, e que juntamente com toda comunidade de docentes Universidade Federal de Uberlândia – Campus Patos de Minas possibilitou e cedeu todo suporte necessário e conhecimento quanto ao universo da Engenharia de Alimentos, incentivando a cada dia a se torna um profissional competente e com uma excelente qualificação.

Quero agradecer também aos colegas de faculdade que sempre estiveram juntos durante momento difíceis, de desafios e alegrias, hoje se tornaram grandes amigos que levarei para toda a vida, gratidão por ter cada um de vocês presentes comigo hoje em especial Laura, Eva Larissa, Victor Bertolanni, Maykon Júnior, Miriane, Anubia, Nayara, Amanda Cristina e outros.

Enfim, a todos os que por algum motivo contribuíram para a conclusão desse novo ciclo e também pela realização desta pesquisa, deixo registrado toda a minha gratidão por cada um de vocês e meu muito obrigado.

Eu denomino meu campo de Gestão do Conhecimento, mas você não pode gerenciar conhecimento. Ninguém pode. O que você pode fazer, o que a empresa pode fazer é gerenciar o ambiente que otimize o conhecimento. (PRUSAK, Laurence, 1997)

L-ASPARAGINASE: THERAPEUTIC USE AND APPLICATION IN THE FOOD INDUSTRY

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Abstract

Enzymes are biological molecules of fundamental importance for the survival of living beings, as they play central and essential functions in metabolic processes. They have been used in several industrial sectors, such as food, pharmaceuticals and cosmetics, with advantages in terms of saving water and energy, reducing chemicals, increasing shelf life of food products, as also to your therapeutic uses. Among all enzyme types, L-asparaginase stands out. It is used in the treatment of "Acute Lymphoblastic Leukemia (ALL)", inhibiting protein biosynthesis in lymphoblasts. L-asparaginase is also effective in reducing acrylamide formation in thermoprocessed foods. High temperature processing of food samples rich in asparagine and reducing sugars leads to a characteristic brown appearance, attributed to the formation of acrylamide and other intermediates, which has been recognized as a potent carcinogen in humans in the last years. Since acrylamide is a carcinogenic compound, the scientific community and regulatory bodies have been establishing goals and strategies for its reduction in food. The presence of L-asparaginase has been reported in several organisms, including animals, plants and microorganisms. Due to its therapeutic use and demand, microorganisms have been used to produce L-asparaginase on an industrial scale for many years. In this review, studies on sources of L-asparaginase enzymes, methods of enzyme immobilization, promising improvements in biocatalyst for therapeutic use, and application in the food industry are presented and discussed.

Keywords: Enzyme; L-asparaginase; Acrylamide; Immobilization; Food, Pharmaceutic.

Introduction

"Special Enzymes" which are those used in fine chemistry or as medicine. Special Enzymes require greater purification and specificity, as they are targeted for therapeutic, diagnostic, analytical and research use. A wide range of enzymes, from different sources and for different therapeutic and diagnostic use is available on the market (Zimmer et al. 2009; Kunamneni, A. et al. 2018). In addition to their clinical use, enzymes encounter several other applications, such as in fuels, paper, food, beverage and clothing industries, in the production of cleaning products, monitoring devices as in therapeutic use, etc. (Vlieghe et al. 2010). These applications aim to use new raw materials and improve

processes, while also improving physical and chemical properties of the raw materials and products. Therefore, from an industrial point of view, a commercially usable enzyme is one that guarantees delivery of a final product with better quality than the traditional one, allows for process improvements, reduces laboratory costs, and enables fast production of products not available on the market (Borzani et al. 2001).

In industrial scale, one of the main sources of enzymes are the processes of fermentation by microorganisms. They are considered attractive sources because of the low cost involved in production of metabolites. Microorganisms can ferment large amounts of medium in a relatively short time, and it is possible to use inexpensive raw materials in the production medium. Other advantages include not being influenced by climate conditions or geographical location, and not presenting seasonal production.

The global enzyme market size was $ 9.9 billion in 2019, and is expected to grow at an annual rate of 7.1% between 2020 and 2027. The increasing demand is related to end-use industries, such as food and beverages, biofuel, animal feed and household cleaning products. These industries are projected to contribute to the growth of the enzyme market, boosting it upward (Grand View Research 2020).

Therapeutic use of enzymes dates back to the end of the 19th century, when crude preparations of porcine pancreatic enzymes were used as digestive aids (Cruz et al. 2015). Since then, industries have been developing a wide variety of enzyme-based medications for clinical use, such as antiseptics, anti-inflammatories, reconstituting of hemostatic and other metabolic enzymes, digestive aids, inhibitors of coagulation in the treatment of cystic fibrosis, as well as cancer drugs. Therapeutic enzymes are generally available as lyophilized preparations containing biocompatible buffering salts and mannitol diluent. According to treatment purpose, enzymes can be administered topically, orally, intravaginally and parenterally (Zimmer et al. 2009; Kunamneni et al. 2018).

Among the main therapeutic enzymes of microbial origin, L-asparaginase stands out. It is an important chemotherapeutic agent used in the treatment of a variety of lymphoproliferative disorders and lymphomas, in particular, acute lymphoblastic leukemia (ALL). The use of L-asparaginase has been a cornerstone of combined chemotherapy protocols used in the treatment of pediatric ALL for almost 30 years. Blast cells need asparagine to survive but they do not have the enzyme asparagine synthase. As L-asparaginase is able to deplete plasmatic asparagine, it causes death of blast cells. (Narta et al. 2007).

In addition to their therapeutic use, the field of applications of L-asparaginases has been expanding. For instance, they are used by the food industry to reduce the formation of acrylamide in starch rich foods (Sanghvi et al. 2016). Acrylamide is a low molecular weight hydrophilic compound formed in heat-treated food products. Its formation occurs during cooking or frying of carbohydrates and asparagines rich foods at temperatures above 120 °C. Acrylamide is mainly formed from the reduction of sugars and asparagine in the Maillard reaction. This compound was considered "potentially carcinogenic to humans" by the International Cancer Research Agency (IARC 1994). (Mottram et al. 2002; Stadler et al. 2002; Zyzak et al. 2003; Liu 2018). L-asparaginase is an enzyme that catalyzes hydrolysis of asparagine in aspartic acid and ammonia, and is therefore used in the supply of some food products to reduce acrylamide content (Pedreschi 2008).

The industrial scale use of enzymes such as L-asparaginase requires the development of efficient processes in order to preserve catalytic activity while maintaining final product price, which is the main obstacle. Several internal factors such as specific characteristics of enzyme and of product of interest, reaction rate and external factors as temperature, pH and other operational conditions directly influence the final characteristics of a product (Filho et al. 2019).

In most cases, biocatalysts are relatively expensive due to the several steps involved in their production, such as fermentation, extraction, purification and stabilization. Therefore, enzyme recovery and reuse are interesting strategies to reduce industry costs in the acquisition of enzymes. Enzymatic immobilization is another attractive strategy, as it provides easy recovery of enzymes, allows reactor design simplification, provides simpler reaction control, limits use of toxic or highly reactive reagents, and makes processes simpler and more robust. It is the simplest solution to the solubility problem of biocatalysts. Thus, immobilization is generally applied to enzymes used in industrial applications (Gupta et al. 2009, Mohamad et al. 2015).

L-asparaginase is an enzyme with important applications in medical and food fields. Therefore, the aim of this study was to carry out a systematic and extensive review of different methods and studies on the immobilization of L -asparaginase since there are few studies on this subject. Different studies are here presented to compare experimental conditions with a compilation of the most interesting results of environmental reaction conditions (optimum pH and temperature) and kinetic constants (Km and Vmax),

focusing on the stability of these properties when immobilized, and when the enzyme is used for the reduction of acrylamide present in food products.

L - asparaginase

L-asparaginase, also known as aminohydrolase, belongs to the group of enzymes amidase (Kumar and Verma 2012). Normally, L-asparaginase exists as a tetramer, but it is also found in hexameric, dimeric or monomeric form. Different forms are obtained when the enzyme is isolated from different sources. Most bacterial L-asparaginases exhibit quaternary or tertiary structures (Ln et al. 2011), and the tetramer consists of four identical subunits. Altogether, the molecule has two intimate dimers within its molecular structure where non-allosteric catalytic centers are created. Every active site is modeled by the transport of amino acids in two adjacent monomers. The amino acids that constitute the active site are Thr15, Tyr29, Ser62, Glu63, Thr95, Asp 96, Ala120 and Lys168, with the amino acids Thr15 and Thr95 being the residues responsible for catalytic activity of the enzyme (Jaskólski et al. 2001).

Asparaginase is the enzyme responsible for irreversible hydrolytic catalysis of L-asparagine in L-aspartic acid and ammonium; it is found in certain plants, animals and microorganisms but never in humans. The amino acid L-asparagine comes from human diet, but it is also synthesized by the enzyme L-asparagine synthetase from L-aspartic acid, L-glutamine and Adenosine Triphosphate - ATP (Verma et al. 2007).

L-asparaginase enzymes bacterial type can be classified based on amino acid sequence, biochemical characteristics of the enzyme, group of origin (bacterial, vegetable and rhizobial), or enzymatic type (type I and type II asparaginase). Enzymes are characterized by their enzymatic activity on L-,asparagine and L-glutamine, being widely distributed in microorganisms, plants, vertebrates and animal tissue (Izadpanah et al. 2018). Type I asparaginases are found in the cytosol, and are called cytosolic asparaginase. They show low affinity for L-asparagine and a greater affinity for L-glutamine. Type II asparaginases are found in the vicinity of periplasmic spaces and are produced extracellularly, showing high affinity for L-asparagine and being less specific to L-glutamine (Hendriksen et al. 2009). Hence, type II enzyme exhibits higher specific action against L-asparagine. Also, asparaginase type II shows precisely greater antitumor activity and is used as a chemotherapeutic agent. Type II asparaginase has been better

studied and applied in the food industry to remove asparagine from primary ingredients, before food processing, in order to reduce formation of acrylamide (Kotzia and Labrou 2007; Hendriksen et al. 2009).

L-asparaginase (Lasparagine amidohydrolase, EC 3.5.1.1) has been the focus of several studies in the last three decades due to its antineoplastic activity, demonstrating effectiveness in various chemotherapeutic strategies from its high specific activity with L-asparagine that is only induced in anaerobic condition.

In addition to medical application, L-asparaginase has also been widely used as an additive in food processing. According to a researche in food technology have shown that a colorless, odorless crystalline solid called acrylamide (2-propenamide) is produced as a result of the Maillard reaction when starchy foods are fried or cooked at temperatures above 120 °C (Lingnert et al. 2002). Acrylamide is a neurotoxin and has been classified as a carcinogen for humans (Gökmen and Palazoğlu 2008). During food processing, acrylamide is mostly produced from heat-induced reactions between the α-amino group of the free amino acid asparagine and carbonyl groups of reducing sugars, such as glucose, during cooking or frying (Tyl and Friedman 2003). Due to its ability to convert L-asparagine to L-aspartate, L-asparaginase seems to be a viable method to reduce the amount of precursors of the Maillard reaction in starchy foods, such as potato dough and bakery products, thereby reducing the risk of acrylamide formation (Pedreschi et al. 2008).

L - asparaginase - Enzyme for therapeutic use

A wide variety of therapeutic enzymes have been used as oncolytic, thrombolytic or anticoagulant agents, and as substitutes in metabolic deficiencies. Currently, the pharmaceutical industry represents one of the largest producers and users of enzymes, and it is also the most promising market for this segment. The advantage of using enzymes as medicine is that small amounts of this biological catalyst can produce very specific effects under physiological conditions (Zimmer et al. 2009). However, the use of enzymes for medical purposes by pharmaceutical and cosmetics industries is not free of challenges. During product development, safety of the formulation must be ensured to avoid irritating or sensitizing reactions and possible enzyme immunogenicity. In addition, enzyme based products need to offer high activity and stability at physiological pH, without causing

damage to the components of various body fluids (Santoset al. 2017 and Zimmer et al . 2009). Despite the long list of producing microorganisms, only a minority can be used as sources of enzymes in the pharmaceutical and cosmetics industries, since they must be non-pathogenic and considered safe for humans. During enzyme source screening, it is important to avoid any possibility of undesirable contamination by incompatible material, and also to allow adequate purification (Cruz, et al. 2008).Differently from the industrial use of enzymes, therapeutic enzymes are needed in relatively small amounts but generally with a high degree of purity and specificity. The kinetics of these enzymes must be high to assure an efficient enzymatic process, even at low enzyme and substrate concentrations. Commercial therapeutic enzymes are generally prepared with pure lyophilized material with biocompatible buffering salts and mannitol thinner (Gurung et al. 2013).

L-asparaginase belongs to the group of therapeutic enzymes with antineoplastic properties that has been extensively studied by researchers and scientists. It was first observed by Lang and Uber (1904). Research studies regarding the use of L-asparaginase as medicine started when Kidd (1953) carried out his studies using serum of guinea pigs (*Cavia porcellus*) and mice with subcutaneous lymphomas. He injected the serum into the intraperitoneal region of sick mice and observed that the injection was capable of causing regression in tumor growth. Soon after, Neuman and Mccoy (1956) verified the metabolic need of "Walker carcinosarcoma 256" cancer cells in the supply of the amino acids L-asparagine and L-glutamine. After several studies, as previously reported, Broome (1961) found that the antitumor activity of guinea pig serum was due to the enzyme L-asparaginase from that serum. Furthermore, studies carried out by Mashburn and Wriston (1963) verified the potential of microbial asparaginase enzyme as a chemotherapeutic agent, which inhibits tumor growth. The study this research found that tumor cells require asparagine as a source of nutrients, and that L-asparaginase could be a method capable of depleting the amino acid asparagine through hydrolysis. Thus, L-asparaginase would indirectly inhibit or slow down the growth of malignant tumors and lymphoblastic lymphomas by reducing plasma concentration of asparagine in the body (Bakshi and Patel 2019). Physiological effects of L-asparaginase have been ongoing for more than half a century to confirm whether both normal and leukemic cells require the amino acid L-asparagine for their metabolic needs (Batool et al. 2016).

Currently, L-asparaginase is used as a drug to treat certain hematopoietic tumors, being able to reduce plasma levels of L-asparagine in patients (Pieters et al. 2011).

Lymphoblasts need large amounts of the amino acid L-asparagine to maintain their rapid and malignant growth, but they are unable to synthesize it. Neoplastic cells do not have the ability to synthesize asparagine due to the absence of L-asparagine synthetase. Therefore, they depend on the exogenous supply of asparagine for survival and growth. The use of L-asparaginase as clinical treatment causes the circulating asparagine to be drastically reduced, which leads to the starvation of cancer cells. The absence of asparagine causes inhibition in protein biosynthesis, and cells undergo apoptosis. Thereby, tumor growth is significantly controlled. (Batool et al. 2016).

Healthy cells, on the other hand, have the enzyme L-asparagine synthase, which converts L-aspartic acid into L-asparagine, being slightly affected by treatment with L-asparaginase (Pieters et al. 2011) (Figure 1). Normal cells synthesize L-asparagine using the enzyme transaminase, which performs the conversion of oxaloacetate into an intermediate aspartate. After this, the transfer of an amino group from glutamate to oxaloacetate occurs producing α-ketoglutarate and aspartate molecules. Asparagine is synthesized from aspartate and the amino group comes from glutamine (Batool et al 2016).

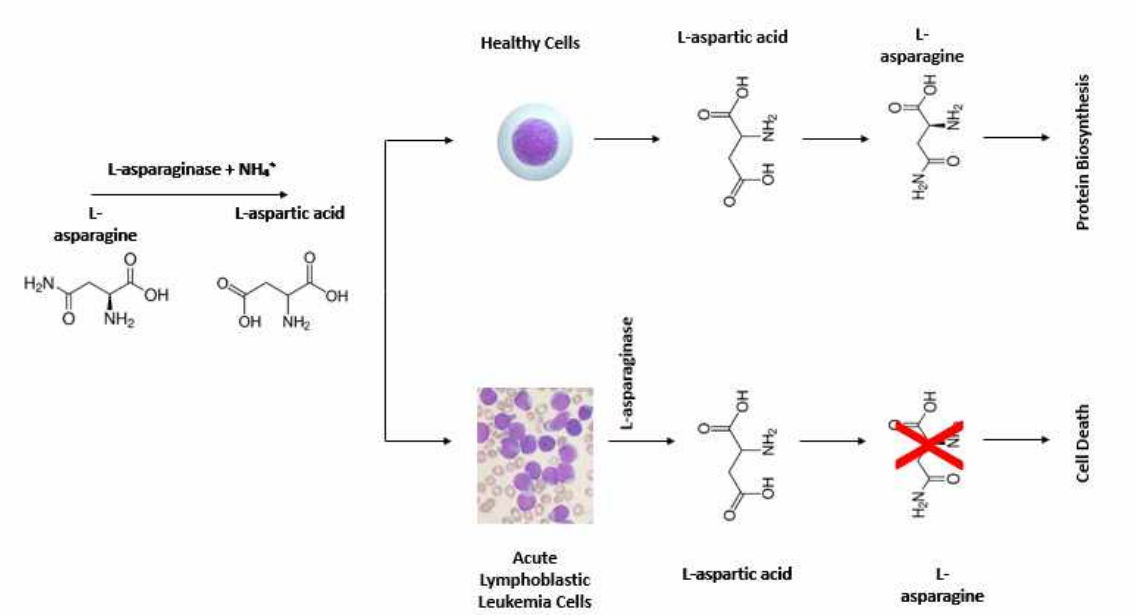

Fig. 1 Metabolic mechanism for L-asparagine synthesis (Adapted from Pieters et al., 2011 and Batool et al., 2016)

It should be noted, however, that there are tumor cells that also have the L-asparagine synthase enzyme and are resistant to treatment with L-asparaginase, since they convert L-aspartic acid into L-asparagine and do not suffer from the absence of this amino acid

(Van Den Berg 2011; Verma et al. 2007; Pieters et al. 2011). The use of L-asparaginase as a drug is currently only standardized for the treatment of pediatric patients with acute lymphoblastic leukemia (ALL) and for some types of lymphomas, since tumor cells from these leukemias are the most susceptible to treatment with this enzyme (Pieters et al. 2011).

L - asparaginase – Enzyme for food use

Enzymes have been used in food production and processing for centuries. Examples include rennet for cheese making, diastase for hydrolysis of starch, pectinase for hydrolysis of pectin, amylases and proteases in the production of soy foods (Gurung et al. 2013). Enzyme applications in the food industry are not only related to catalytic specificity, the ability to operate in mild conditions or biodegradability, but also to the label generally considered as safe - GRAS. Specific enzymes or enzyme formulation must fulfill a complex set of regulatory restrictions established by the Federal Food and Drug Administration - FDA (Agarwal 2014) to achieve food grade status. These enzymes are used in certain kinds of foods to favor and improve specific characteristics, such as taste, nutritional value, aroma, digestibility, texture, promotion of coagulation and preservation (Li et al. 2012). They are widely used in bakery, brewing, wine and fruit juices industries. The addition of enzymes must be in accordance with the national regulation of each country, which defines them as additives and processing aids (Fernandes 2010).

Food enzymes account for a significant share of the industrial enzyme market. In 2018, the food enzyme market was valued at $ 1,944.8 million and a value of $ 3,056.9 million is estimated by 2026, which represents an annual increase of 5.6% from 2019 to 2026. Enzymes belonging to the classes of carbohydrases, lipases and proteases are those that tend to contribute more to the growth of this segment (BCC Research 2020).

The search for high quality food in terms of food technology coupled with food security in developing countries has created a shift towards innovation and production of new foods. This demand triggered the need for transformation within the food industry (Li et al. 2012).

Within this context, the enzyme L'asparaginase of microbial origin gained prominence (Krishnapura et al. 2016; Krishnapura and Belur 2016a; Kumar et al. 2014). In recent years, L-asparaginase has been used as a food enzyme due to its application in

reducing the formation of acrylamide. Asparagine is present in most starchy foods. This amino acid reacts with reducing sugars during thermal processing, leading to the formation of an undesirable compound called acrylamide (Figure 2), which is one by-product of the Maillard reaction (Mottram et al. 2002).

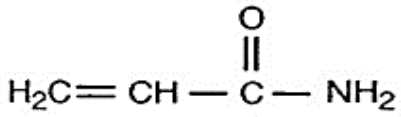

Fig. 2 Chemical structure of acrylamide (Adapted from Mottram et al., 2002**)**

In 1994, acrylamide was classified as "potentially carcinogenic to humans" by the International Agency for Research on Cancer (IARC 1994). In 2001, the European Committee on Toxicity, Ecotoxicity and Environment demonstrated its toxic effects in animals, such as neurotoxicity, genotoxicity, carcinogenesis and reproductive toxicity. According to Claus et al. (2008), the results of a study with several rodent models showed that acrylamide produces tumorigenesis in multiple hormonally regulated organs, such as the mammary gland, thyroid and peritesticular mesothelium. Therefore, it is a potent carcinogen and neurotoxic compound that forms an adduct with hemoglobin when consumed in high doses, and causes reproductive disability (Tyl and Friedman 2003).

To prevent risks to human health, the toxicity of acrylamide and reduction of its content in food have been extensively studied. International agencies food-related, such as the US Federal Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), have established measures to prevent or reduce the content of acrylamide formed during thermal food processing of fried or cooked food products (Tareke et al. 2002).

The first stage of acrylamide formation mechanism is a reaction between the free asparagine with a carbonyl source (amino-carbonyl reaction), resulting in the compound N-glycosylated asparagine, which undergoes dehydration at high temperature to give rise to the Schiff base (imine). The Schiff base undergoes additional decarboxylation and, after tautomerization, the decarboxylated Amadori compound is formed. Subsequently, this compound reacts by breaking the carbon-nitrogen covalent bond resulting in the formation of acrylamide, together with an amino ketone. In another reaction route, the decarboxylated Schiff base is decarboxylized again to form a new compound, 3-

aminopropionamide (3-APA), which in turn, forms acrylamide with the removal of ammonia (Granvogl and Schieberle 2006). The decarboxylation of free asparagine at high temperatures (100-180 °C) forms the compound 3-aminopropionamide, which gives rise to acrylamide in later steps. Therefore, the presence of reducing sugars is not always necessary for the production of acrylamide. The main steps of the reaction are shown in Figure 3 (Mustafa, 2008).

Some agronomic characteristics and processing strategies directly affect the formation and degradation of acrylamide in foods (Elmore et al. 2005). Selection of food varieties with low reducing sugar content, storage under controlled conditions, immersion and bleaching, addition of sodium diphosphate, reduced frying temperature, control of final moisture content, among others strategies have contributed to reduce acrylamide formation (Mesías and Morales 2016; Food Drink Europe 2013; Sanny et al. 2013). Variable and process parameters that must be controlled because of their effect during the reaction of acrylamide formation can be classified into four groups. Group A variables are related to food chemical composition: reducing sugar content, total sugar content, asparagine content, color, and humidity parameters. Group B variables are related to frying process, such as type of frying oil, content of polar compounds in the frying oil, use of immersion or not, and type of equipment. In group C there are variables that involve roasting of food, during which temperature and humidity directly influence acrylamide formation. And finally, group D variables include food-related properties, such as color and thickness parameters. According to studies carried out with food models, all of these parameters directly interfere on the formation of acrylamide (Mesías and Morales 2016).

Continued efforts to understand the pathway of acrylamide formation have demonstrated that other carbonyls besides α-hydroxycabonyl can react with asparagine to produce acrylamide. Only one carbonyl compound is needed to convert asparagine to acrylamide, and carbonyl can come from several sources (Jin et al. 2013). One example is the reaction with sucrose that produces 5-hydroxymethylfurfural (5-(hydroxymethyl) furan-2-carbaldehyde) (HMF), a furan compound that is formed by direct dehydration of sugars in acidic conditions (caramelization) and is also an intermediate in Maillard reaction (Kennedy and Knill 2003). HMF contains α, β, γ, δ-di-unsaturated carbonyl groups that can efficiently convert free asparagine to acrylamide during heating (Gökmen et al. 2012). As mentioned above, intermediates in the Maillard reaction can be a source of reactive carbonyls that, when heated with asparagine, form a series of intermediates

such as specific flavonoids that lead to the formation of acrylamide (Jin et al. 2013). Nonetheless, there are also lipid oxidation reactions that contribute to the formation of acrylamide. It has been demonstrated that lipid oxidation products are capable of converting asparagine to acrylamide. This conversion is favored by oxidized lipids with an α, β, γ, δ-di-unsaturated carbonyl group, which comes from the oxidation of unsaturated fatty acids and their corresponding esters (Zamora and Hidalgo 2008). Other reaction pathways can also contribute to the formation of the acrylamide compound, such reaction pathways not involving asparagine that will not be discussed in this review.

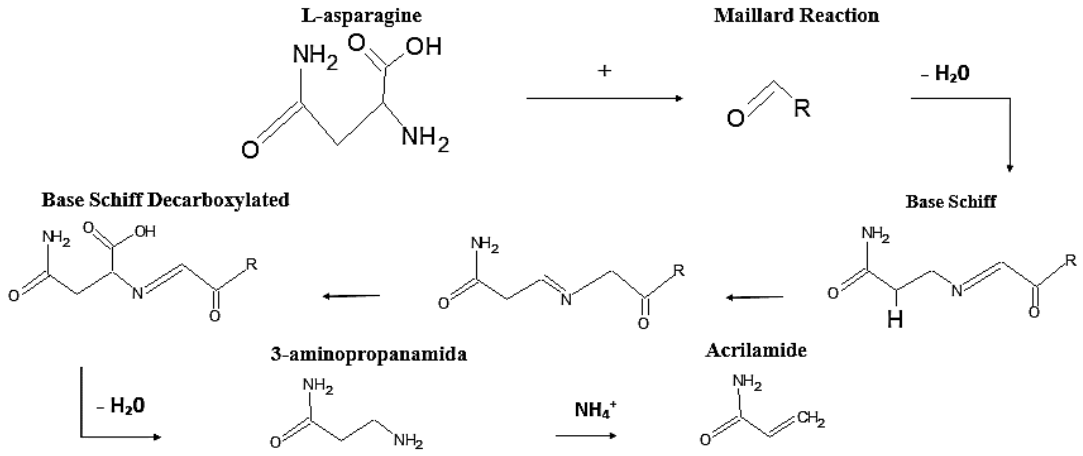

Fig. 3 Mechanism of acrylamide formation in foods processed under high temperatures (Adapted from Granvogl and Schieberle, 2006 and Mustafa, 2008)

The use of enzymes to reduce the formation of acrylamide was first proposed by Amrein et al. (2004), who used asparaginase to hydrolyze asparagine into aspartic acid and ammonia. This approach is considered efficient because asparagine is not a major contributor to taste and color of cooked foods, so desirable sensory properties are maintained (Parker et al. 2012).

Second Meghavarnam and Janakiraman (2018a), claim that studies have been carried out regarding reduction of acrylamide formation in foods, which made it possible to establish guidelines for the thermal processing of food, and on your study demonstrated that enzymatic pretreatment of foods with the enzyme asparaginase leads to significant reduction of acrylamide content, in addition to other treatment procedures. Different food products have been subjected to pretreatment with asparaginase, clearly demonstrating a reduction of acrylamide.

Sources of L-asparaginase

The presence of L-asparaginase has been reported in several organisms, including animals, plants and microorganisms, except humans. Although it is present in several groups of animals and plants, other sources such as microorganisms (bacteria, fungi, algae, yeasts and actinomycetes) are considered more appropriate for use due to the complexity of extraction procedures (Batool et al. 2016).

Enzymatic preparations obtained from plant extracts or animal tissue have been commonly used, although microorganisms are by far the main suppliers of commercial enzymes for food applications. They are easier to cultivate and manipulate than mammalian or vegetable cells, which allows large-scale production. In addition, they are considered reliable sources due to selectivity in biosynthesis. Microorganism producers of commercial enzymes are specifically selected or genetically modified in order to maximize enzyme production and reduce contaminants. Enzymes of animal origin bring a considerable risk both in handling and in manufacturing such as contamination by disease, which is much more complex to handle and expensive than microbial production (Agarwal 2014). Table 1 shows studies of asparaginase production from different origins.

Plant sources

A variety of plant species contain a significant amount of asparaginase, such as green peppers (*Capsicum annum* L.) and tamarind (*Tamarindus indica*). Inactive precursor molecules produce plant-type asparaginase. High amounts of asparaginase have been detected in plants, mainly during the development of leaves or roots. Plants contain two different types of asparaginase classified based on reaction catalysis and potassium dependence (Michalska and Jaskolski 2006). Comparatively, potassium-independent enzymes are more stable than potassium-dependent enzymes, but potassium-dependent enzymes have higher affinity for the substrate (Chagas and Sodek 2001; Michalska et al. 2008; Michalska and Jaskolski 2006). Despite being classified into different groups, the two types of plant enzymes share significant levels of sequence similarity. On the other hand, they have no relationship with sequence or mode of action of microbial origin L-asparaginases (Izadpanah et al. 2018).

Microbial Sources

Fungi and yeasts

Fungi are a potential source of L-asparaginase along with bacteria. Due to the ease of being produced extracellularly and being an enzyme considered easier to purify, asparaginase of fungal origin acquired greater importance when compared to other sources of this enzyme. Its production from different strains of fungi is extremely influenced by the composition of the fermentation medium, especially regarding sources of carbon and nitrogen, and physical factors, such as temperature, pH, agitation, inoculum concentration and fermentation time. Each species has specific conditions for maximum enzyme production. Genera of mitosporic fungi such as *Aspergillus, Penicillium* and *Fusarium* are commonly used for synthesis of this enzyme (Souza et al. 2017; Sarquis et al. 2004).

Yeasts have also been reported as an interesting alternative for the production of L-asparaginase, including *Saccharomyces* spp, *Candida sp*., *Pichia* spp, *Rhodotorula* spp, *Hansenula* spp and *Spobolomyces* spp (Nagarethinam et al. 2012).

Bacteria

L-asparaginase production has been reported for Gram-positive and Gram-negative bacterial species in terrestrial and marine environments. Gram-positive bacteria gained less consideration than gram-negative bacteria (Izadpanah qeshmi et al. 2014). L-asparaginases from Gram-negative bacteria can be categorized into two main L-asparaginase types: type I and type II. L-asparaginase type I has enzymatic activity in the amino acids L-glutamine and L-asparagine, while L-asparaginase type II has high specific activity in L-asparagine, and is only induced in anaerobic condition. Currently, L-asparaginase type II produced from *Escherichia coli* (Ec AII), *Erwinia chrysanthemi* (ErA) and *Erwinia carotovora* have been used in the treatment of malignant tumors. They are used in the treatment of acute lymphoblastic leukemia due to its higher specific affinity for the amino acid L-asparagine, which is an essential nutrient for tumor cells (Narayana and Vijayalakshmi 2008).

Actinomycetes

Actinomycetes are widespread in soil, water and nature worldwide. They are also found in live animals, especially in fish, and have good enzymatic activity. Actinomycetes are considered a better source of L-asparaginase when compared to bacteria and fungi (Sahu et al. 2007). Studies report hyperproductive marine actinomycetes capable of producing L-asparaginase with high activity, even when using a cheap low-cost substrate, such as soy flour, indicating they are a new promising source of L-asparaginase (Saleem basha et al. 2009).

Table 1. Studies addressing the synthesis of L-asparaginase from microbial sources and plants demonstrating its operational stability, such as pH, Vmax, Km and temperature.

Source Microorganism	Production Methods	pH	Temperature (ºC)	Vmax	Km	Operat. Stability	Applications	References
Bacteria, Fungi and yeasts								
Trichoderma viride	SmF	7	37	270.27 U/mL	2.56 µM	–	Reduced acrylamide level in food	(Lincoln et al. 2019)
A. niger AKV-MKBU	SmF	4	28	46.75 IU/mg	0.8141 mM	< 10	Activity against various cancer cell lines	(Vala et al. 2018)
E. aerogenes	SmF	6	33	18.35 IU/mL	_	4 cycles	_	(Erva etal. 2017)
B. subtilis WB600	SmF	7.5	65	102.41 U/mL	5.29 mM	30 cycles < 38.1%	Uses in the food and medicine industry	(Feng et al. 2017)
B. subtilis KDPS1	SSF	5	37	47 IU/ml	_	< 40%	Agricultural residues	(Sanghvi et al. 2016)
B. tequilensis PV9W	SmF	8.5	35	0.036 IU/ml	0.045 mM	–	Anticancer	(Shakambari et al. 2016)
Enterobacter cloacae	SmF	7-8	35-40	–	–	–	–	(Husain et al. 2016[a])
Pseudomonas otitidis	SmF	7.5	40	–	–	–	–	(Husain et al., 2016b)
Aspergillus oryzae CCT 3940	SmF	8	50	313 IU/mL	0.66 mmol/L	< 50%	High potential for use pharmaceutical	(Dias et al. 2016)
P. aeruginosa strain EGYII DSM 101801	SmF	8.5	45	1121 µmol/mg	63.11 mM	5 cycles < 90%	–	(El-Sharkawy et al. 2016)

Source Microorganism	Production Methods	pH	Temperature (ºC)	Vmax	Km	Operat. Stability	Applications	References
Bacteria, Fungi and yeasts								
Talaromyces pinophilus	SmF	8	28	204 µmol/mL	6.4 mM	< 95%	Clinical and industrial trials	(Krishnapura e Belur 2016b)
B. megaterium	SmF	7	40	10 U/mL	6.08 mM	< 80.5%	Reduction acrylamide.	(Zuo et al. 2015)
Actinomycites								
Actinomycete Algerian Mill CA01	–	7.2	27.83	8.03 IU/ml	–	–	Producers of antibiotics	(Chergui et al. 2018)
Streptomyces sp.	SSF	6	35	20.80 IU/ml	0.065 mM	–	Producers of antibiotics e Bioactive Compounds	(Desai e Hungund 2018)
S. fradiae NEAE-82	SmF	8.5	40	95.08 U/ml	0.01007M	86%	Activity anticancer	(El-Naggar et al. 2016)
Actinobacterial sp.	SmF	8	35	670.04 IU/mg protein,	–	–	–	(Varma, et al. 2016)
Nocardiopsis alba NIOT-VKMA08	SmF	8	37	5.50 U	0.127 mM	30 cycles < 20%	Antineoplastic agent	(Meena et al. 2015)
S. brollosae NEAE-115	SSF	–	–	–	–	–	–	(El-Naggar et al. 2015)

* SmF- Submerged fermentation

* SsF - Solid state fermentation

Enzyme immobilization

Enzymes are catalysts with excellent properties that can allow the execution of the most complex chemical processes, under various experimental and environmental conditions (Koeller et al. 2001).

Despite this and other advantages of using enzymes over traditional chemical catalysts, there are still some practical problems associated with their use in industrial applications, such as being proteins highly sensitive to various denaturation conditions. When enzymes are isolated from natural environments, their sensitivity to process conditions, such as temperature, pH and substances present in the solution that can act as inhibitors, can increase operational cost. On the other hand, unlike conventional heterogeneous chemical catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems, which can lead to contamination of the product, limiting its recovery and reuse. Thus, one of the successful methods proposed to overcome these limitations is the use of an immobilization process (Van de Velde et al. 2002).

In recent years, a wide range of interest has been directed to explore the potential of immobilized enzymes. Immobilized enzymes are generally more stable and easier to manipulate than their free forms; in addition, products of their reaction are not contaminated with the enzyme (especially useful in food and pharmaceutical industries) (Massolini e Calleri 2005). Immobilization technique have been revealed as a tool to improve almost all enzymatic properties, such as stability, activity, specificity, selectivity and reduction of inhibition, if properly prepared. However, to allow reuse of the enzyme, stability of the final preparation of these biocatalysts must be high; therefore, the enzyme must be very stable or highly stabilized during the immobilization process to be suitable (Mateo et al. 2007).

In the literature, different protocols for the immobilization of the enzyme have been reported. Immobilization occurs because the enzymes can be linked to different supports through various chemical interactions, such as physical adsorption, ionic bonds, covalent bonds and trapping. Enzymatic immobilization methods can be classified in several forms, such as reversible or irreversible methods, or based on their chemical reaction (Figure 4). All immobilization methods have certain advantages and disadvantages. The choice of the appropriate method depends on the characteristics of the enzyme, reaction medium and carrier material (Brena et al. 2013).

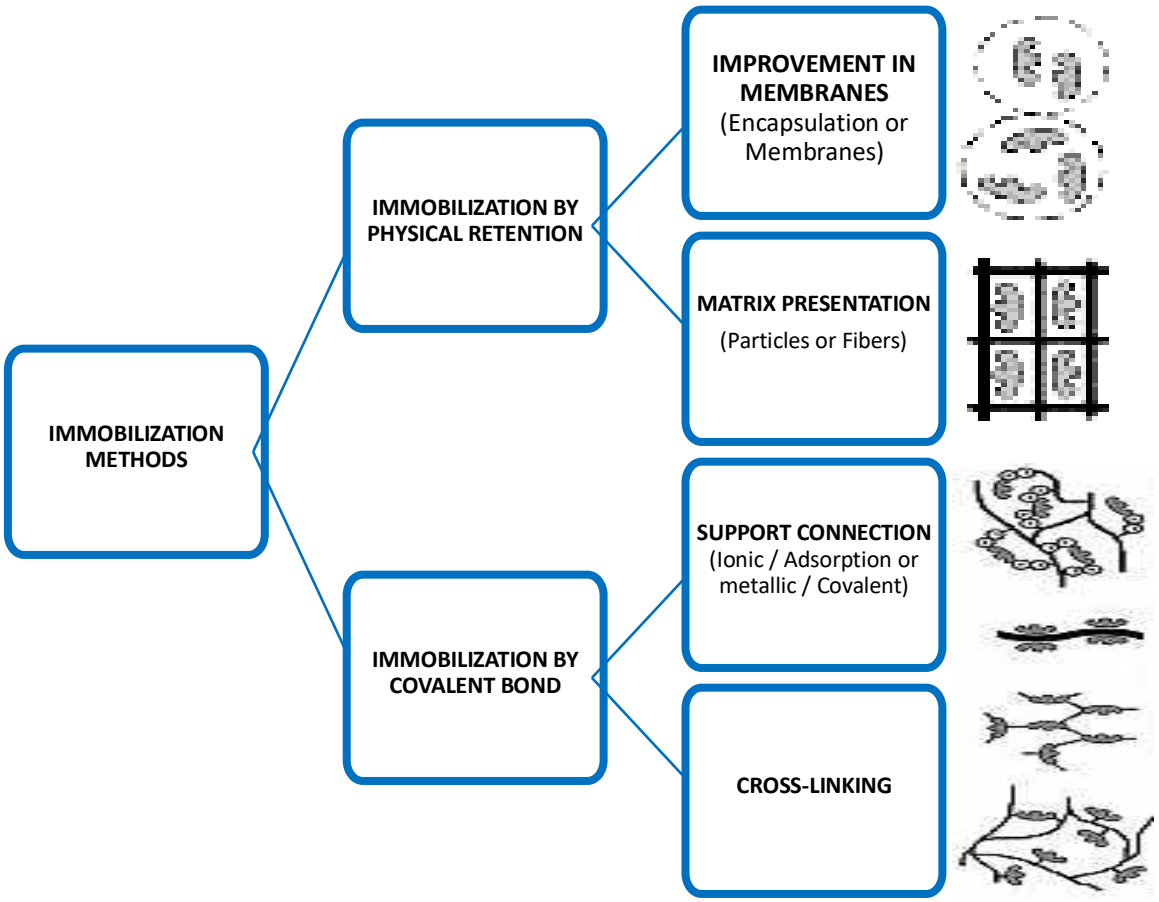

Fig. 4 Classification of immobilization methods according to physical and chemical methods (Adapted from Brena et al., 2013 and Owner Author)

Irreversible enzyme immobilization methods

When the biocatalyst is carried to the support, it cannot be isolated without destroying biological activity of the enzyme or the support itself. So, it is considered an irreversible immobilization method. The most common procedures for irreversible enzyme immobilization are covalent coupling, trapping or microencapsulation and cross-linking (Brena et al. 2013).

Covalent bond method

This method relies mainly on the formation of a covalent bond between an enzyme and a support material. In the side chain amino acids present in the enzyme, such as histidine, arginine, aspartic acid and other amino acids, covalent bonds between the

enzyme and its matrix will form. Reactivity depends on the presence of different functional groups, such carboxyl, amino, indole and phenolic, sulfhydryl, thiol, imidazole and hydroxyl groups. By preventing denaturation of amino acid residues from the active site, efficient enzymatic activity can be achieved (Fu et al. 2014). There are many commercially available supports for immobilization by the covalent bonding method. The best choice in each case requires careful consideration of relevant properties of the catalyst and the intended use. It is usually necessary to try more than one approach and adapt the method to specific circumstances. Commonly used covalent reactions give rise to enzymes linked to the support through amide, ether, thioether or carbamate bonds, so the enzyme is strongly linked to the matrix and, as in many cases, it is able to maintain its stability in the immobilization support (Brena et al. 2013).

Glutaraldehyde is a crosslinking agent and the most used substance in immobilization by covalent bonds, due to its solubility in aqueous solvents, that allows formation of stable covalent bonds between subunits, being popularly used as a bifunctional crosslinker (Sirisha et al. 2016). This cross-linking agent acts directly on the binding of the enzyme's amino acid to a chemical group on the support, promoting fixation of one over the other. The maintenance of the structural and functional properties of the immobilized enzymes is especially important and can be performed by this cross-linking agent (Adamiak et al. 2020).

Matrix and membrane entrapped method

The entrapped method in matrices or polymeric membranes is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass but with retention of the enzyme. This method differs from the coupling methods described above, in which the enzyme is not bound to the matrix or membrane. There are different entrapment approaches, such as gel or fiber entrapment or microencapsulation. The practical use of these methods is limited by the mass transfer (Brena et al. 2013).

Reversible enzymatic immobilization methods

Reversibly immobilized enzymes can be isolated from the support under mild conditions due to the type of binding to the enzyme support. The use of reversible methods for enzymatic immobilization is highly attractive, mainly for economic reasons, because the support can be regenerated and recharged with new enzyme when the enzyme

activity declines. In fact, the cost of support is often a significant (or preponderant, if primary) factor in the total cost of the immobilized catalyst. Reversible enzyme immobilization is particularly important for application in bioanalytical systems (Brena et al. 2013).

Chemical Methods (Adsorption, ionic and affinity)

Adsorption

In this method, enzyme molecules adhere to the surface of the support through a combination of hydrophobic interactions. This process involves preparing a suspension of the matrix with the enzyme or drying the enzyme's molecules on the electrode surfaces. The most used matrix materials are glass, synthetic polymers and water-soluble supports, such as polysaccharide derivatives. (Cordeiro et al. 2011). There is a strong interaction between the enzyme and support matrix, although some physiological conditions, such as high temperature, pH change, or the addition of substrate can weaken the interaction. Adsorbed enzymes are generally resistant to proteolysis and aggregation due to their hydrophobic interaction with interfaces (Spahn and Minteer 2008).

Ionic bond

The ionic bond method is based on the ionic bonding of the enzymatic protein and water-insoluble supports that contain ionizable groups. This type of non-covalent immobilization can be reversed by changing ionic strength, polarity or temperature; this principle is similar to the principles of protein and ligand interactions used in chromatography (Bickerstaff et al. 2003). This method is similar to the physical adsorption method. The difference is in the binding force, since there is formation of an ionic bond between the enzyme and support in this technique, with residues exchanging ions. Thus, as with physical adsorption, enzyme desorption may occur in this method (Bon et al. 2008).

Support materials such as polysaccharides and synthetic polymers have ion exchange centers that are generally used in this type of enzyme immobilization. The enzyme binding to the support is simpler, and the immobilization conditions used are milder than immobilization by covalent bonding, both being advantages of this method. In addition, ionic binding causes minor changes in conformation and on the active site of the enzyme, leading to high enzyme activity most of the time. However, enzyme

desorption in the support can occur when substrate solution with high ionic strength or solution with inadequate pH is used, since they favor reduction of the number of groups available for ion exchange due to alteration of the enzyme charge (desorption) and support. The support, too, can change the number of ionizable groups available depending on pH. This is due to the weak link between enzyme and support. In the case of ionic bonding, the bond between enzymes and support is much stronger than physical adsorption but weaker than covalent bonding (Datta at al. 2013).

Affinity immobilization

Affinity binding is immobilization of the enzyme in the support matrix by specific interactions. This method uses the specificity of the enzyme with its support under various physiological conditions. This can be achieved by the following two methods. The first method is activation of the support material that contains the attached affinity binder so that the enzyme is added. In this method, the enzyme is not exposed to adverse chemical conditions. The second method consists of enzyme modification or conjugation to another molecule that develops affinity with the matrix (Nisha and Gobi 2012). This technique exponentially increases the capacity of binding enzymes and their reuse, due to the presence of non-covalent forces, such as coulombic, hydrogen bonding, van der Waals forces, etc (Sardar e Gupta 2005; Haider e Husain 2008; Datta et al. 2013).

Metal bond immobilization

The metal bond method is a simple, easy and reversible process. It involves precipitation of metal salts on the surface of the support matrix. The metals have the ability to bind to nucleophilic groups, and the precipitation of the metal ion in the support matrix can be obtained by heating. The enzyme immobilized by this method shows a relatively 30 to 80% higher enzyme activity. By decreasing the pH of the solution, the enzyme and the carrier molecule can be separated, and both the enzyme and the matrix can be regenerated in this method (Yücel 2011).

L-asparaginase immobilization

Although L-asparaginase has been produced from a variety of sources, such as bacteria, yeasts, fungi, plants and actinomycetes, L-asparaginase of bacterial origin has a high molecular weight and, when exposed to the body's immune system for medication,

its half-life decreases. Another restrictive factor is that when L-asparaginase is injected into the patient; it can cause severe hypersensitivity reactions, such as fever, skin rashes, allergic reactions and even anaphylactic shock (Naveed et al. 2016). In addition, it is a drug with a high cost, but it is necessary in therapy, especially in children. When directed to food applications such as acrylamide reduction, food preparation conditions, such as temperature and pH, can negatively influence its performance in reducing the amino acid asparagine. To overcome these limitations, enzymatic immobilization is a promising strategy (Verma et al. 2007). Table 2 presents some studies that show in detail the immobilization process of the L-asparaginase enzyme.

To stabilize the quaternary structure of multimeric enzymes, a strategy based on covalent immobilization of several units of the enzyme on activated supports (agarose-glutaraldehyde) was developed. Activated supports have different densities of reactive groups; the higher the density of the groups, the greater the stabilization achieved. Nevertheless, when targeting complex enzymes, even with the use of higher densities of reactive groups, it is not possible to cover all four subunits in the immobilization process, demanding additional chemical intersubunit cross-links with aldehyde-dextran. With this procedure, Balcão et al. (2001) managed to stabilize the quaternary structure of L-asparaginase from *E. coli* by covalent immobilization with several subunits of the enzyme. The support used was agarose, which was subjected to crosslinking with glutaraldehyde, and chemical crosslinking of additional intersubunit with aldehyde-dextran to obtain a multimeric structure, fully stabilized, preventing inactivation of the enzyme by the dissociation subunit, and improving its thermostability.

Zhang et al. (2004) immobilized L-asparaginase covalently by polar groups on the surfaces of sericin microparticles (a globular protein derived from the *Bombyx mori* cocoon) using glutaraldehyde. The immobilized enzyme showed an affinity for the substrate eight times greater than native asparaginase. Ghosh et al. (2012) immobilized L-asparaginase with polyaniline nanofibers and observed greater stability and activity, which may have been due to conformational changes in the enzyme through ionic interactions with polyaniline nanofibers. The L-asparagine microplate biosensor project was developed by Labrou and Muharram (2016) presenting high sensitivity, low cost and good stability and selectivity, which contribute considerably to applications in food and clinical chemistry, providing a new approach for measurement and also for specific, economically viable and fast analyses of L-asparagine. The L-asparaginase enzyme from *E, carotovora* was immobilized by crosslinking with glutaraldehyde and bovine serum

albumin (BSA). While the free enzyme completely lost its activity after 8 days of incubation at 4 °C, the immobilized enzyme maintained about 50% of its intrinsic activity after about 30 days of storage. The biosensor showed high sensitivity for the detection of L-asparagine with a linear range of 10 to 200 μM. The method's reproducibility was in the order of ±3–6%, and L-asparagine mean recovery was 101.5%.

El-Refai et al. (2016) reported successful immobilization and improved stabilization of L-asparaginase in Amberlite IR-120 by ionic bond. Immobilization did not change the ideal pH (8.0) or temperature (40 °C) compared to the free enzyme, but it showed greater thermostability as a result of the immobilization. The immobilized enzyme retained 100% of activity when exposed to 60 °C, and also retained 70% of its activity relative to pH 9.0 for 50 min. Da Rocha Junior and Caseli (2017) immobilized L-asparaginase in monolayers of the phospholipid dipalmitoylphosphatidylcholine (DPPC) chosen based on abundance in several cells. The enzyme immobilized in the ultrathin lipid films preserved more than 78% of the enzymatic activity after 30 days while the homogeneous medium preserved less than 13%. Therefore, lipid films are an adequate carrier matrix to protect L-asparaginase activity and can also be considered for the detection of asparagine, hence revealing that ultrathin lipid films (Langmuir e Langmuir-Blodgett) can be used to immobilize L-asparaginase by adsorption.

Recently, studies on permeable polymers such as polyethylene glycol (PEG) as nanoreactors from ASNA (L-asparaginase) have been developed by Blackman et al. (2018). PEG is a non-immunogenic polymer commonly conjugated to proteins (PEGylation) to increase its hydrodynamic volume that reduces glomerular filtration and improves biological half-life. Polymerization-induced self-assembly (PISA) used for encapsulation of clinical biological ASNS inside inert vesicles and selectively permeable in size protects it from proteases and recognition by antibodies. These polymersome materials lead to depletion of L-asparagine without releasing ASNS from the nanostructures into the bloodstream, consequently reducing proteolytic degradation of the enzyme and recognition of antibodies in comparison with the free protein or protein conjugated with PEGylation. ULU (2020) analyzed metal–organic frameworks (MOFs), verifying that these materials have a huge surface area, large porosity and tunable functionality, aspects that are positive for carrying out enzyme immobilization tests, specifically for L-asparaginase. After enzymatic immobilization, the author found that the ideal temperature changed from 50 to 60 °C, while the ideal pH remained unchanged

at 9.0. ASNase @ ZIF-8 exhibited excellent long-term storage stability (56% of initial activity at 25 °C for 4 weeks).

Based on the studies presented in this work, it appears that processes that use the immobilization of L-asparaginase against different methods such as adsorption, covalent, ionic bonding, encapsulation and entrapment, presented better enzymatic properties when compared to the free enzyme. Properties are how much it increased half-life, significantly greater stability to allergic reactions compared to the free enzyme for clinical use, and better stability of enzymatic activity when directed to reduce acrylamide for food use.

Table 2. Studies addressing different methods (reversible and irreversible) of L-asparaginase immobilization from different sources (microbial, soil and plant), including operational stability such as pH, Vmax, Km and temperature.

Methods Irreversible										
Source Microorganism	**Carrier**	**Status**	**pH**	**Temp.**	**Vmax**	**Km**	**Operat. Stability**	**Advantage**	**Application**	**Reference**
Covalent binding										
Erwinia carotovora	Glutaraldehyde and bovine serum albumin	Immobilized	7	60 ºC	0.07 mM	–	20 cycles < 60%	Immobilized enzyme showed good stability upon storage	Development of microplate-based biosensor for L-Asn determination	(Labrou e Muharram 2016)
Escherichia coli	PVDMA (poly(2-vinyl-4,4-dimethylazlactone)) nanoparticles	Free	8.6	37 ºC	0.11 mM /min	0.70 mM	10 cycle <95.7%	Affinity of the immobilized increased compared with that of free enzyme	Fabrication of various efficient enzyme reactors	(Mu et al. 2014)
		Immobilized	8.6	37 ºC	1.45 mM /min	0.56 mM				
Escherichia coli	Fatty acid bioconjugates	Immobilized	8.5	37 ºC	–	–	10h < 90%	More resistant to proteolysis, more stable at different pH	Clinical use: Strategy for intravenous delivery of L-asparaginase.	(Ashrafi et al. 2013)
Bacillus licheniformis - Strain HSA3-1[a]	Activated Glutaraldehyde-Carbon	Free	8	50 ºC	616.26 UI / mg	–	2 cycle < 84.79%	–	–	(Ahmad et al. 2013)
		Immobilized	7	60 ºC	499.27 IU/mg	–				
Encapsulation and Entrapment										
Escherichia coli	Metal–organic frameworks (MOFs) - zinc nitrate hexahydrate (ZIF-8)	Free	9	50 ºC	68.0 lmol / min	0.40 mM	10 cycles < 45%	High enzyme catalytic activity	Industrial applications, especially in the food and sensor fiel	(Ulu 2020)
		Immobilized	9	60 ºC	64.5 µmol / min	0.18 mM				

Methods Irreversible										
Source Microorganism	**Carrier**	**Status**	**pH**	**Temp.**	**Vmax**	**Km**	**Operat. Stability**	**Advantage**	**Application**	**Reference**
Encapsulation and Entrapment										
–	Poly (2-hydroxypropyl methacrylate)	Immobilize	–	–	–	–	61%	Increased the proteolytic stability and reduced antibody recognition	Clinically used for leukemia treatment	(Blackman et al. 2018)
Escherichia coli	P(MAA-coMMA)-S - poly- (methacrylic acid-co-methyl methacrylate);	Free Immobilized	8;5	35−40 °C	301 U/mg 609.8 U/mg	0.216 mM 0.0378 mM	30 d: 80%	Showed better thermal and pH stability	Chemotherapeutic agent in acute lymphoblastic leukemia	(Ulu et al. 2016a)
Escherichia coli	pHEMA-S poly(2-hydroxyethyl methacrylate);	Free Immobilized	8.5 10	60 ºC	301 U/mg 253 U/mg	0.216 mM 0.0256 mM	15 D < 60%	High thermal stability and biodegradability	–	(Ulu et al. 2016b)
Escherichia coli	PMMA-S; pHEMA; P(MAA-co-MMA)	Free Immobilized	8;5	45 - 50 ºC	5882 U/ mg 3610 U/ mg	1.58 mM 0.56 mM	30 cycles < 60%	More advantageous in terms of enzymatic affinity, thermal, pH and storage stability	–	(Ulu et al. 2016c)
Erwinia carotovora	PEG-chitosan and glycol-chitosan conjugation	Free Immobilized	7;5	37 ºC	2940 IU/mg 1460 IU/mg	0.10 mM 0.05 mM	–	Improve the therapeutic properties of L-asparaginase	Clinical use: Antitumor activity	(Sukhoverkov e Kudryashova 2015)

Methods Irreversible										
Source Microorganism	**Carrier**	**Status**	**pH**	**Temp.**	**Vmax**	**Km**	**Operat. Stability**	**Advantage**	**Application**	**Reference**
Encapsulation and Entrapment										
Escherichia coli	Polyimide (pyromellitic dianhydride and 4,40-oxydianiline (ODA)	Free	8.7	30 ºC	–	–	–	Strong adherence to the electrode surface, easy preparation, chemical stability e selectivity	Amperometric biosensor	(Erdogan et al. 2014)
Escherichia coli	Poly(dextran/L-arginine)-CaCO3	Free Immobilized	7.5	51.6 ºC 54.6 ºC	– –	120 mM 10 mM	–	Thermal improvement, physiological temperature and storage	Antileukemic therapy	(Karamitros et al. 2013)
Methods Reversible										
Ionic binding										
Escherichia coli	Aluminum oxide pellets (AlOPs)	Immobilized	7.5	37 ºC	8.2 µM/min	0.00539 mM	85%	Decrease L-asparagine level in blanched potato chips	Blanched potato chips	(Agrawal et al. 2018)
Penicillium cyclopium	Amberlite IR-120	Free Immobilized	8 9	40 ºC 60 ºC	– 757.6 U/mg	– 0.0259 mM	70%	Optimal activity to several pH values, higher thermostability and affinity to the substrate.	–	(El-Refai et al. 2016)
Adorption										
Escherichia coli	DPPC (phospholipid dipalmitoylphosphatidylcholine)	Free	6	26 ºC	–	–	–	Preserving the catalytic activity	Lipid monolayers	(da Rocha Junior e Caseli 2017)

Methods Reversible										
Source Microorganism	**Carrier**	**Status**	**pH**	**Temp.**	**Vmax**	**Km**	**Operat. Stability**	**Advantage**	**Application**	**Reference**
Adorption										
Acinetobacter Baumannii	Silica gel - Sephadex G-50 led	Free	8.5-9	–	–	–	30 cycle, < 95%	Increased of activity and of stability of of L-asparaginase	Food additive in processed foods	(Muslim et al, 2015)
Escherichia coli - BL21pLysS	Chitosan-tripolyphosphate – TPP	Free Immobilized	6.5	37-80 ºC	18.6 U/mg 111.5 U/mg	–	–	Increased in vitro half-life in comparison with the free enzyme	Antineoplastic agent	(Bahreini et al. 2014)

* PMMA-S poly(methyl meth-acrylate); pHEMA, poly(2-hydroxyethyl methacrylate); P(MAA-co-MMA), poly- (methacrylic acid-co-methyl methacrylate)

L-asparaginase Food Industry Application: Acrylamide Reduction

In most studies involving application of enzymes in foods, it is easy to notice the recurrent application of free enzymes is more disseminated than its immobilized form. This fact happens because industrial enzymes such as proteases, amylases, cellulases are often used to modify macromolecules, surfaces and complex mixtures. Therefore, the effectiveness of immobilized enzymes is often lower than that of their soluble equivalents. This is mainly due to diffusion restrictions imposed by carried supports that affects the ability of the substrates to access active sites of the enzyme and of the products leaving the solution (Di Cosimo et al. 2013).

Products from coffee, potatoes and bread are sources of the highest acrylamide intake concentrations. Synthesis of acrylamide occurs due to the presence of the amino acid asparagine that is a limiting substrate for starch and cereal based systems (Bråthen and Knutsen 2005; Elmore et al. 2005). It was also found that acrylamide in baked wheat bread is correlated with the amount of asparagine added to the dough, while the addition of sugar-reducing fructose did not result in a similar correlation (Surdyk et al. 2004). According to EFSA Journal (2015), the intake of acrylamide in global diet is estimated to be between 0.3 and 1.9 μg/kg orally; however, foods with the greatest contribution to this intake vary from country to country, according to food standards and preparation methods (Claus et al. 2008). Among factors that influence the formation of acrylamide in food are processing conditions (temperature, humidity, cooking time and product matrix) and its precursors, such as reducing sugars and free amino acids like L-asparagine (Zuo et al. 2015).

The Federal Food and Drug Administration (FDA) together with the European Food Safety Authority (EFSA) have carried out extensive research on the content of acrylamide present in different food products, analyzing levels of toxicity in humans. Results were published as various review and scientific manuscripts regarding the amount of acrylamide formation in different types of food products, and also the formation of other contaminants in the heat-processed food system. The observed results showed that the main food groups, the ones that contribute the most to acrylamide exposure, are French fries, bakery products and coffee, that result in an estimated of global daily acrylamide intake from diet between 0.3 and 1.9 μg/kg orally (EFSA Journal 2015); Robin and Clanci 2007). Studies carried out in India have found high levels of acrylamide present in chips and French fries, with values above 3020 μg/kg and 4475 μg/kg,

respectively (Meghavarnam and Janakiraman 2018a). In 2013, the Brazilian Consumer Protection Association (Proteste, 2013) evaluated that French bread, sweets and cookies had the highest values in acrylamide content. Sweet cookies, for example, showed values 443 μg/kg according to results from the EFSA at Finland (2015). The Contaminant Panel in the Food Chain sector (CONTAM) analyzed 1500 coffee-based products between 2010 and 2013 and found an average acrylamide concentration of 578 mg/g in roasted coffee (EFSA Journal. 2015).

Since then, studies have been carried out to understand the mechanisms of acrylamide formation and to develop reduction strategies. Most of the developed methods seek to remove its precursors (amino acids) to inhibit or reduce the intensity of the Maillard reaction to attenuate its formation (Pedreschi 2009). Among used methods, silencing of the asparagine synthase-1 (Asn1) gene present in tubers can be highlighted. This method results in lower levels of asparagine, use of additives such as vegetable antioxidants, addition of amino acids before thermal processing of food to provide a competition with asparagine in the Maillard reaction, and incorporation of acid to reduce the pH that influences the formation of this compound. These and other strategies result in adverse effects on the final product as negative impact on flavor and appearance of the product (Rifai and Saleh 2020). Potato-derived products (*Solanum tuberosum*) such as French fries are widely consumed in many countries (Mesias et al. 2018). Potential acrylamide formation in potatoes is related to several factors, such as L-asparagine and reducing sugars content, color and moisture (of fresh potatoes), oil, utensils used in frying, content of polar compounds in frying oil (during the frying process), and thickness (after frying).

The reduction of acrylamide in French fries has been tested by different authors. A 90% reduction was achieved when bleaching (85 °C for 3.5 min) was applied, followed by immersion in solution of L-asparaginase from *Aspergillus oryzae* (50 °C for 20 min). The heat treatment was able to cause changes in structure, facilitating diffusion of the enzyme in tissues, and consequently favoring and improving its action (Pedreschi et al. 2011). Another research was used L-asparaginase synthesized by *Fusarium culmorum* (ASP-87) used to reduce acrylamide in potato-based products. Fries were treated with the enzyme at 40 °C for 30 min, followed by frying at temperatures of 170-180 °C for 90 s. It was observed that 300 U/L L-asparaginase was needed to reduce 85% to 94% of acrylamide levels in French fries (Meghavarnam and Janakiraman 2018a). Kumar et al.

(2013) produced, purified and characterized a new L-asparaginase *Cladosporium sp*., which was able to reduce levels of acrylamide in potato snacks by up to 96%.

Anese et al. (2011) found that the use of intermediate concentrations of L-asparaginase of 500 U/Kg resulted in minimal acrylamide formation in shorter time and lower incubation temperature, while reporting that the color parameter of the cookie was not affected by the application of the enzyme. The L-asparaginase gene from *Rhizomucor miehei* has been cloned and expressed in *E. coli* (Huang et al. 2014), and the synthesized and purified enzyme (10 U/mg of flour) was used to reduce the level of acrylamide in cookies, reaching a reduction of approximately 80%, being also useful in reducing acrylamide present in the breads. Kumar et al. (2014) applied different concentrations of L-asparaginase produced from *Cladosporium* sp. on sweet bread. The authors did not observe changes in the physical-sensory characteristics of bread with treatment with L-asparaginase. Specifically, there was a reduction of 97% and 73% in the formation of acrylamide in the regions of dough and on the outside of the bread (crust), respectively in the treatment with 300 U. According to the authors, these results indicate the potential of L-asparaginase for reducing acrylamide with industrial and domestic application. The same enzyme was tested to reduce levels of acrylamide in loaf bread, being able to reduce by up to 97% of this compound in the bread crust, where the Maillard reaction occurs with greater prominence.

Along with potatoes and cereal products, roasted coffee is one of the products with high concentration of acrylamide (Mesias et al. 2018), being present in the two main coffee species most consumed worldwide, *Coffea arabica* and *Coffea canefhora*. Coffee has high levels of acrylamide due to the roasting process (105 ºC), and storage conditions can also contribute to the formation of this compound (Mesías and Morales 2016). Exposure to acrylamide resulting from roasted coffee consumption varies from country to country, age and sex of the consumer, volume of coffee ingested, degree of roasting, etc. (Anese 2016). Consequently, daily intake of this product represents a significant source of exposure to acrylamide (Şenyuva and Gökmen 2005). Hendriksen et al. (2013) evaluated the effect of L-asparaginase on reducing acrylamide in coffee. Green grains of *Arabica* species were steamed with water for 45 minutes at 100 °C, and subsequently treated with L-asparaginase at 60 °C for 60 min. The authors observed the greatest reduction when they used an enzyme concentration of 6000 U/kg that resulted in a reduction of 70 to 80% of L-asparagine content, and 55-74% in the formation of acrylamide. Another work developed by Xu et al. (2015) showed a reduction in free

asparagine during the treatment stage with asparaginase when coffee was subjected to a roasting stage, reflecting losses of the acrylamide compound from 69% to 86% by using dosages of 2,600 to 20,000 U, respectively.

The enzymatic treatment was able to reduce levels of acrylamide in all tested products that have high amounts of this compound. In all the various studies presented above (Table 3), authors concluded that thermal bleaching was able to improve the effect on the reduction of acrylamide. Thermal bleaching can occur in two different stages: the first is thermal treatment (heated solution) without the enzyme and a second thermal treatment with the enzyme (heat treatment together with the enzyme). The strategy of using L-asparaginase is relatively new, promising, and an excellent technique for reducing acrylamide in food. This enzyme helps in two-way mitigation strategies, interference with the Maillard reaction or removal of precursors by converting L-asparagine to L-aspartic acid (non-toxic) without changing nutritional value, appearance or taste of the final product (Batool et al. 2016; Hendriksen et al. 2009).

Table 3. Studies that address a reduction of acrylamide present in different products through the immobilized enzyme L-asparaginase, from different sources (microbial, soil and vegetable)

Enzyme Source - Microorganism	Food source	Optimized conditions - Temperature (ºC), Time (min) e Enzimatic Charge (U/L or U/mL)	Acrylamide Reduction	Reference
Escherichia coli	Potato chips	40 ºC / 30 min / 400 U/L	55.9%	(Jiao et al. 2020)
Fusarium culmorum	Sweet bread	37 ºC / 30 min / 300 U/L	78%	(Meghavarnam and Janakiraman 2018b)
Fusarium culmorum	Potato chips	40 ºC / 30 min / 300 U/L	85%	(Meghavarnam and Janakiraman 2018a)
Aspergillus terreus	Carrot slices	170 °C / 10 min / 3 U/mL	88%	(Aiswarya and Baskar 2018a)
Aspergillus terreus	Fried potatoes	60 °C / 20 min / 5 U/mL	55%	(Aiswarya and Baskar 2018b)
Aspergillus terreus	Sliced potatoes	170 °C / 15 min / 4 U/mL	62 to 82%	(Aiswarya and Baskar 2018b)
Aspergillus terreus	Banana slices (Kochchi kesel)	180 °C / 25 min / 5U/ml	85%	(Ravi and Gurunathan 2018)
Aspergillus oryzae	French fries	180 ºC / 7 min / 50 U/mL	72%	(Dias et al. 2017)
Thermococcus zilligii	French fries	80 ºC / 4 min / 0–20 U	80.5%	(Zuo et al. 2015)
Aspergillus. subtilis	Sliced potatoes	50 ºC / 90 min / 0–40 U	90%	(Onishi et al. 2015)
Cladosporium **sp**	Sweet bread	30 ºC / 90 min / 50-300 U	97%	(Mohan Kumar et al. 2014)

Conclusion

L-asparaginase has been targeted for therapeutic use and studied for decades as a limiter of the amino acid asparagine, inhibiting the formation of carcinogenic compounds. In the food industry, the use of the biocatalyst L-asparaginase is related to a reduction of the acrylamide compound in thermal processed foods since the availability of free amino acids such as asparagine together with reducing sugars (carbonyl groups) during the Maillard reaction, contributes to the synthesis of acrylamide. However, the process of synthesizing L-asparaginase and its purification is one of the biggest challenges regarding its application. Processing the enzyme has a high cost, consequently making the product less accessible. Another issue that hinders its use for application purposes (food/therapeutic) is that the enzyme is limited in terms of stability in the reaction medium and, in many cases, there is no possibility of reuse. However, using immobilized enzymes is a method that efficiently solves this situation. Hence, this method has been increasingly used and is replacing conventional methods in several sectors of the industry, for presenting efficiency and faster performance in comparison the use of chemical catalysts and free enzyme. Thus, L-asparaginase is an important method used in the treatment of ALL (Acute Lymphoblastic Leukemia), and the immobilization of this biocatalyst is an alternative to reduce adverse effects in the body. It is also used to reduce carcinogenic compounds (acrylamide), which together with the immobilization of the enzyme is important for increasing or maintaining its stability. This review aimed to summarize the development of immobilization processes, aiming to expand this segment, while reducing costs and improving stability of the biocatalyst.

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