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Review Paper

A concise review on source, production, purification and characterization of L-asparaginase and its application in food industries

R.M. Dhingani[1*] and G. Shah[2]
[1]Department of FQA, College of FPTBE, Anand Agricultural University, Anand, Gujarat, India
[2]Department of Biotechnology, Veer Narmad South Gujarat University, Surat, Gujarat, India
rashmin17@gmail.com

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Abstract

L-asparaginase enzyme belongs to amidase group which carry out the deamination of L-asparagines to ammonia and aspartic acid. It has been using as a drug for the treatment of disorders like acute lymphoblastic leukemia. Another significant use of L-asparaginase enzyme is in the food manufacturing industry, where it reduces acrylamide formation in fried or baked foods while maintaining their sensory attributes like flavor, odor, taste, etc. The generation of the acrylamide during baking and frying relies on the Maillard reactions, within that acrylamide formation occur from the reaction between L-asparagine amino acid and reducing sugars during baking or frying condition. At the same time, this gives good sensory characteristics to the final product. The application of L-asparaginase can control the acrylamide production without affecting the sensory properties of baked or fried food. During processing a balance of contact time and reaction conditions is very much desired for enzyme effectiveness. Thus, this review represents production, purification, and application of L-asparaginase in the food manufacturing industry.

Keywords: Maillard reaction, L-asparaginase, Acrylamide, Leukemia.

Introduction

L-asparaginases are important group of enzymes present in bacteria, fungi, animal, plant etc. It hydrolyzes the L-asparagines amino acid into ammonia and aspartic acid by an act on the amide group side chain of L-asparagine amino acid[1]. Mostly, the forms of the enzyme used in the industry as a drug are mainly obtained from the bacteria and other microbes like yeast and fungi. An L-asparagine amino acid is vital for the survival of certain malignant cell, while, the non malignant cells are self-sufficient to produce it even in a lower concentration. The major application of this enzyme is for the treatment of childhood acute lymphocytic leukemia, in which uncontrolled cell division process occur in the blood and bone marrow cells which leads to accumulation of abnormal blood cells and it may leads to death. Mortality rate of leukemia is very higher as compared to other cancer particularly in children of age around twenty[2]. Bacterial L-asparaginase have been using as a one of the popular chemotherapeutic agent among all for the control of acute lymphoblastic leukemia.

Besides therapeutic application, L-asparaginase is also used in food manufacturing industry to mitigate the acrylamide formation in baked and fried foods. Acrylamide is considered as a suspected carcinogen produces during baking and frying process (at more than 120°C) from the free acrylamide amino acid and reducing sugar residues[3]. L-asparaginase reduces acrylamide formation by hydrolyses L-asparagines from food into ammonia and L-aspartic acid. Acrylamide mitigations have been deeply investigated during the last few decades. Zyzaket al.[4] reported that free L-asparagines amino acid residues plays crucial role in acrylamide formation during the Maillard reaction.

Most of the L-asparaginase is highly specific for L-asparagine amino acids, however, few L-asparaginase also shows affinity for L-glutamine amino acid because of the structural similarity of the amino acid. A similar enzyme called glutaminase-asparaginase has affinity for both the amino acids but it prefers L-glutamine as its substrate[5]. It is believed this multi affinity enzyme causes multiple side effects in human like pancreatitis, liver problem and leucopenia[6].

So, there is great scope for the isolation of efficient or novel L-asparaginase enzyme from the bacteria and other microbes and one can also explore thermophilic and halophilic microbial sources so it can have other beneficial aspects which are important for food industries. Without affecting the sensory attributes, maximum acrylamide diminution in the fried and baked food needed to be exploited as much as possible using different microbial source.

Sources of L-asparaginase

L-asparaginases are distributed throughout the birds, mammals, plants, fungi, actinomycetes and diverse bacterial species. Even

though there are various sources for this enzyme but microbial source preferred over others because of its huge diversity, high reproduction rate and better economics. The yield of this enzyme using different microbial source is mainly depend upon microbial strain and fermentation conditions[7]. All the metabolic reactions including L-asparaginase production are influenced by many factors like nutritional factors, environmental factors, fermentation process parameters, etc.[8]. Microbial source is dominating in both the pharmaceutical and food sector industries. L-asparaginase is an intracellular (cytoplasmic) and extracellular (periplasmic) enzyme that is acquired from the various bacterial sp. Like *Erwiniacarotovora, E. coli, Bacillus* sp., *C. glutamicum, E. aerogenes, etc*[9]. Different enzymes collected from the diverse source have different abilities and not all of them have anti malignant characteristics. For them, clearance rate and substrate affinity are the deciding factors for the enzyme activity[10]. So, the selection of enzymes with the desired characteristics is a very important criterion during the screening process.

Some of the bacterial enzymes show a toxic effect on humans due to its prokaryotic origin. Being eukaryotic in nature fungal L-asparaginasesare a significant alternative for bacterial L-asparaginases to decrease the side effects. Fungi are evolutionarily closer to humans, so, chances of immunological reactions against fungal L-asparaginase are less as compared to bacterial L-asparaginase[11]. So, for the cure of childhood acute lymphocytic leukemia using this enzyme, less allergic bacterial enzyme source needs to be explored or other alternatives like fungal source can also be a better option for the bacterial enzyme. Recombinant DNA technology can also be exploited to manipulate the gene of the L-asparaginase enzyme in such a way that it has less allergic conditions in humans. For another application of this enzyme in the food manufacturing industries to mitigate acrylamide formation, bacteria can be an important source for isolation and purification of efficient enzymes.

Production of L-Asparaginase

There are wide sources of L-asparaginase, among them the most popular is bacterial L-asparaginase because of its easy handling, fast cultivation rate and a huge source of diversity. Although different strains have different ability to produce L-asparaginase, efficient production of microbial L-asparaginase depends on nutritional components like carbon source, nitrogen source, metallic ion, micronutrients, macronutrients, growth factors, etc. and different process parameters like pH, temperature, gaseous content, inoculum size, fermentation time, etc. Hence, these parameters must be optimized by using a proper statistical tool to achieve maximum yields of L-asparaginase. These aspects need to be considered for economic viability of industrial L-asparaginase production.

For the production of industrially important bioactive components, two broad fermentation techniques have been using widely, solid state fermentation and submerged fermentation technique. Solid state fermentation technique utilizes solid substrate and thus nutrients releases slowly and steadily from that so it is most suitable for organisms that require less moisture like fungi. Various bacterial species also able to produce metabolites under solid state fermentation conditions but the productivity remains lower related to the submerged fermentation method[12]. Various solid substrates have been explored for the production of L-asparaginase enzyme like, wheat bran, Groundnut oil cake, soybean meal, coconut oil cake, cottonseed meal etc. In solid state fermentation, maximum 94.21U/g enzyme productions achieved using *Aspergillus niger* with soybean meal, wheat bran, orange peel and cottonseed meal after 96 h of fermentation[13].

The submerged fermentation technique uses liquid broth containing free-flowing substrates such as soluble sugars, molasses, etc. Here, the substrate utilizes rapidly and required higher moisture, so it is suitable for fast-growing microbes like bacteria[14]. The submerged fermentation technique has been a method of choice for the production of this enzyme. Various microorganisms found to produce L-asparaginase like, *E. coli*, *E.carotovora*, *Pseudomonas fluorescens*, *Serratiamarcescens*, *S. cerevisiae*, *Penicillium*, *Aspergillus*, *Fusarium spp.etc.*[15]

Each organism has its ideal conditions for the growth by optimizing these conditions and evaluating it using appropriate statistical design, enzyme production can be increased up to many folds. Medium optimization by choosing appropriate statistical tools instead of traditional method like one-factor-at-a-time gives a significant difference in enzyme production. Kenari, et al.[16] reported that, 10 fold increases in L-asparaginase production achieved in *E. coli* using response surface methodology. Arastoo, B. D.[17] mentioned that L-asparaginase production was accomplished by optimizing different parameters like glucose, yeast extract, L-asparagines, NaCl, K_2HPO_4, $MgSO_4$ and pH. Maximum 785 U/ml L-asparaginase production achieved using glucose (0.2%), L-asparagine amino acid (0.5%), K_2HPO_4 (0.045%) and NaCl salt (0.045%).

Prakasham et al.[18] mentioned that significant L-asparaginase production achieved by Staphylococcus sp.-6A using different nutritional factors such as carbon sources and nitrogen sources and physiological conditions like, pH, incubation temperature, agitation and aeration. From the fractional factorial design method, it has resulted than more than 60% L-asparaginase production was contributed by pH, inoculum level and incubation temperature only from the rest of the factors. Agitation and aeration are less significant at an individual level but highly significant at an interactive level. Around 9 fold enzyme production achieved using factorial design experiment during medium optimization.

Purification and Characterization of L-Asparaginase

L-asparaginase has been purified from several different bacteria such as *E. coli*, *Corynebacterium glutamicum, Pseudomonas aeruginosa* and many more. Various techniques have been

explored by the many scientists for the purification of enzymes like, alkaline lysis, liquid-liquid extraction, ammonium sulfate precipitation, dialysis, ion-exchange chromatography, gel filtration chromatography, affinity chromatography and crystallization. Purification of some important enzymes was attained by the cation-exchange chromatography technique because enzymes of bacteria are acidic in nature[19].

L-asparaginase enzyme purification from diverse microbial sources usually carried out by precipitation. It is a classical technique that is used for the recovery and purification of biomolecules from a liquid solution. Precipitation process reversibly disrupts secondary and tertiary structures of protein which cause protein precipitation. Along with the precipitation and other traditional techniques, some advanced techniques collectively used to enhance process yield and purification fold of the desired biomolecules[20].

Liquid-liquid extraction (LLE) is one of the alternates that can be used for purification of different enzymes by using two-phase aqueous systems. Removal of a solute from a liquid mixture after the interaction with another non-soluble or partially soluble liquid mixture at which solute is differently soluble is known as liquid-liquid extraction. Qin and Zhao[21] have reported micellar two-phase aqueous system for L-asparaginase isolated from the *Escherichia coli* ATCC 11303. Around 80% L-asparaginase was released from *E. coli* cells when it was treated with the triton X-100 (15% w/v) and potassium hydrogen phosphate (9.4% w/v) for 15-20 h at 25°*C*.

Gel filtration, ion-exchange chromatography and precipitation with $(NH_4)_2SO_4$ are the most widely used method for the purification of enzymes. Lopes et al. reported that extraction and purification of enzyme from the media share around 50-80% of the total production costs[22]. In one of the study carried out with the precipitation using $(NH_4)_2SO_4$, gel filtration using Sephadex G-100 and ionic exchange using CM-Sephadex C50, specific activity of L-asparaginase was improved from 17.90 IU/mg of crude extract to 1900 IU/mg[19].

Dash, et al.[23] worked on the optimization of L-asparaginase production from *A. bacterium* using submerged fermentation. The purification of crude enzyme was carried out using ammonium sulfate precipitation technique followed by dialysis, ion-exchange chromatography and lastly by gel filtration technique. The enzyme was purified with a 95.06 fold and purified enzyme showed a 3.49% yield and 204.37U/mg specific activity.

Even though *E. coli* L-asparaginase is more popular as a drug, because of the distant origin it is responsible for the hypersensitivity reaction in humans. It also causes other side effects, such as hyperglycemia, hepatotoxicity, pancreatitis, etc. PEG-asparaginase produces prolonged depletion of L-asparagine and which causes fever hypersensitivity reactions and thus it is preferable as a chemotherapeutics agentas compared with the *E. coli* asparaginase[24]. Thereby for the medical application of L-asparaginase, conjugation of enzyme with PEG has been used to improve the bioavailability and biostability of an enzyme, it reduces the immunological response against this compound. However, it results in loss of its biological activity compared with the native enzyme.

Application of L-Asparaginase in the Food Industry

Acrylamide formation: Tareke, et al.[25] had confirmed the acrylamide in a fried and baked foods. Acrylamide considered as a carcinogenic agent which is act like neurotoxin and therefore it is harmful to human. As per the epidemiology study conducted by the Maastricht University and Food Safety Agency of Dutch, risk of ovarian and endometrial cancer increases by intake of dietary acrylamide[26].

L-asparagine as a substrate for acrylamide production: Many scientists have reported that reducing sugar and L-asparagines are responsible for acrylamide formation during baking and frying process[27]. L-asparagine and reducing sugars interact with each other and form *N*-glycosyl L-asparagine. Under the high-temperature treatment, it will further convert into Schiff base (decarboxylated) and finally converted into acrylamide[28]. In the Maillard reaction, L-asparagine is the significant molecule for the synthesis of acrylamide. Carbonyl containing molecules like starch, lipid-derived aldehydes like molecules can form acrylamide by reacting with the L-asparagine. Very less or no acrylamide formation occurs if L-asparagine substrate is unavailable in the food matrix[29,30].

Use of L-asparaginase to prevent the acrylamide formation: From the last decade, food industries have been using different strategies like a selection of raw materials, processed based mitigation, use of additives, fermentation-based approach and enzymatic approach for the mitigation of acrylamide in fried and baked food products. Out of this enzymatic approach has been a remaining choice of the method. L-asparaginase has a capability to convert L-asparagine amino acid into aspartate because of this reason L-asparaginase enzyme can be used in the food processing industry to decrease acrylamide formation in starchy foods. To avoid acrylamide production, pre-treatment of starchy foods likebread and potato dough is done with L-asparaginase[31].

The US government has declared L-asparaginase as a GRAS (Generally Recognized as Safe) molecule. Expert committee of FAO/WHO on food additives in 2007 has declared L-asparaginase as a food additive and has been used in many countries. Amrein et al. had given the first idea about the use of this enzyme to change the reaction pathways by using L-asparaginase enzyme to lower down the precursor for acrylamide formation[32]. As it is not affecting the sensory attributes of the final food product this treatment can be one of the promising area for the acrylamide mitigation in baked and fried food products[28].

The effect of L-asparaginase on the reduction of acrylamide production in potato products like French fries and potato snack pellets have been studied using Acrylaway enzyme by Hendriksen et al.[33]. Around 43% reduction of acrylamide was achieved in potato products. Pedreschi et al.[31] have also used the commercial enzyme Acrylaway for the reduction acrylamide production in the French fries. They have optimized 7.0 pH and 60°C temperature and a reduction of around 67% was achieved.

Different ways and means have been carried out by different scientists for the mitigation of acrylamide formation. Amreinet al.[32] worked on gingerbread and commented that acrylamide formation is also affected by the amount of ammonium bicarbonate added during preparation. They have achieved around 60% reduction in acrylamide formation by replacing ammonium bicarbonate with the sodium hydrogen carbonate. They have also attempted the use of organic acid or sucrose instead of reducing sugar and it resulted in a satisfactory reduction of acrylamide production. Vass et al.[34] report that the presence of ammonium hydrogen carbonate baking agent increases the acrylamide formation. By replacing ammonium hydrogen carbonate with equivalent functional components can decrease the acrylamide formation in the food. Secondly, by lowering the percentage of reducing sugar or replacing it with the sucrose also reduces acrylamide formation. They have also reported that around 70% acrylamide formation can be reduced using L-asparaginase in dough preparation. Application of L-asparaginase is the matter of choice for the reduction of acrylamide formation without changing the formulation or process parameters and thus it maintains sensory attributes of the final product.

As per the FDA guidelines for industries, potato dough can be treated with L-asparaginase, calcium or acidulants during potato chips and other potato snack production which may produce less acrylamide content products. L-asparaginase can also be used to decrease acrylamide formation in cereal-based products with considering other significant factors like contact time, enzyme dose, pH, the water content in dough, etc. L-asparaginase has been also helpful to decrease the formation of acrylamide in French fries and other cooked potato products. L-asparaginase can significantly reduce acrylamide formation in potato-based products but some pretreatment is required to penetrate enzyme through the cell wall of potato. Pre-treatments like soaking, blanching, ultrasound treatments and use of acidulants increase the diffusion of the enzyme into the potato slice but it has to be properly controlled otherwise it affect the flavor and texture of the final product[35].

Although it has significant applications in medical and food manufacturing industries, it has several limitations that need to be investigated and solved out for the betterment of human being. More research needs to be carried out at large scale level particularly for the use in food manufacturing industries to lower down the acrylamide formation. Along with the enzymatic approach for acrylamide reduction, other pre-treatments like blanching and post-treatments like modification of cooking conditions such as time and temperature also required to be encompassed. More efficient or novel enzymes need to be explored for the better economic enzymatic treatments.

Conclusion

L-asparaginase has already shown its potential application in pharmaceutical industries as a chemotherapeutic agent and explored in depth. However, recently it has emerged as a promising treatment for the acrylamide reduction in the food manufacturing industries. Presently, formation of acrylamide in fried and baked products is serious concern. Production of novel L-asparaginase with desired characteristics particularly, for the mitigation of acrylamide formation is an area of investigation in the present context. Various biotechnological interventions have been adopted for the search of novel and cost-effective L-asparaginase production. Recombinant technological approaches could play an important role to increase efficiency, reduce immunogenicity and increase shelf life of L-asparaginase. Moreover, the possible side effect of treated fried or cooked food needs to be explored. It needs to be analyzed systematically and scientifically about the relation between processing conditions, ingredients and acrylamide level formation in food. More scientific model has to be undertaken to understand the fate and mechanism of acrylamide formation. As fried and baked food becomes the popular and routinely consumed processed food for the current generation, more and more research is required to optimize formulation and process parameters to maximally decrease the acrylamide formation during frying and baking process.

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