Chapter 5
Recent Development in the Uses of Asparaginase as Food Enzyme

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Abstract Asparaginase (E.C.3.5.1.1), an important enzyme, is broadly disseminated in all the three domains of life and responsible for catalysing conversion of asparagine into aspartic acid and ammonia. It is one of the most utilized clinical enzymes used in the treatment of different types of cancers. However, there has been a renewed interest in other application of this enzyme especially for minimizing the acrylamide content in baked/fried starchy food products. Acrylamide is generated as a by-product of Maillard reactions between asparagine and reducing sugars. The reactions usually occur at temperature above 100 °C and account for colour and flavour developments in fried/baked starchy foods. In the year 1994, Acrylamide was first time classified in Group B2, i.e. as probably carcinogenic to humans by the International agency for research on cancer. Significant contents of acrylamide have been detected in range of food products including roasted potatoes, root vegetables, chips, crisps, toasts, cakes, biscuits, cereals and coffee. Extensive efforts have been made to reduce the formation of acrylamide during baking/toasting or frying by incorporating asparaginase enzyme as pretreatment. The present chapter encompasses all these aspects and sources of asparaginases, their enzymatic properties, engineered thermostable asparaginase and their various applications in food processing.

5.1 Introduction

Asparaginase (asparagine aminohydrolase; E.C.3.5.1.1) belongs to the amino acid amidohydrolase that catalyses the conversion of L-asparagine to L-aspartate and ammonia. This enzymatic reaction played a central role in a number of metabolic processes in diverse living organisms. Asparaginases have remarkable considerable attention from last few decades due to its application in amino acid metabolism,

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B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_5

human health and food industry. The global demand of asparaginase was 380 million USD in 2017 and market will reach upto 420 million USD by 2025 (Global Asparaginase Sales Market Report 2018). Hence, it is important to deal with the limitation to meet this huge global demand. Although, the enzyme is widely distributed in almost all clade of life including plant, animal, bacteria and archaea, however, increasing attention has been drawn to microbial asparaginases particularly from bacterial sources due to high yield, easy maintenance, versatile characteristics and suitability for the industrial application.

For last more than 4 decades, asparaginase enzyme was widely studied and used in the treatment of different type of cancer mainly non-Hodgkin's lymphoma, acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in children. It is one of the leading medically important enzymes because of its high efficiency and efficacy against leukemia and lymphoblastic leukemia (Swain et al. 1993). Asparagine (a free amino acid) found in foods and taken up by the lymphoma or myeloid cells as a nutrient. Asparaginases convert asparagine into aspartate and ammonia (Krishnapura and Belur 2016), which reduces the asparagine availability to myeloid cells (Fig. 5.1). Generally, myeloid cells do not have asparagine synthetase enzyme which is responsible for asparagine production and due to unavailability of asparagine, it leads to starvation of cell (Asselin and Rizzari 2015).

Apart from its medicinal use, in recent years, asparaginase was also explored in the food industry for the reduction of acrylamide formation in finished products. The International agency for research on cancer classified acrylamide in Group B2, i.e. as probably carcinogenic to humans (IARC 1994). Acrylamide is mainly

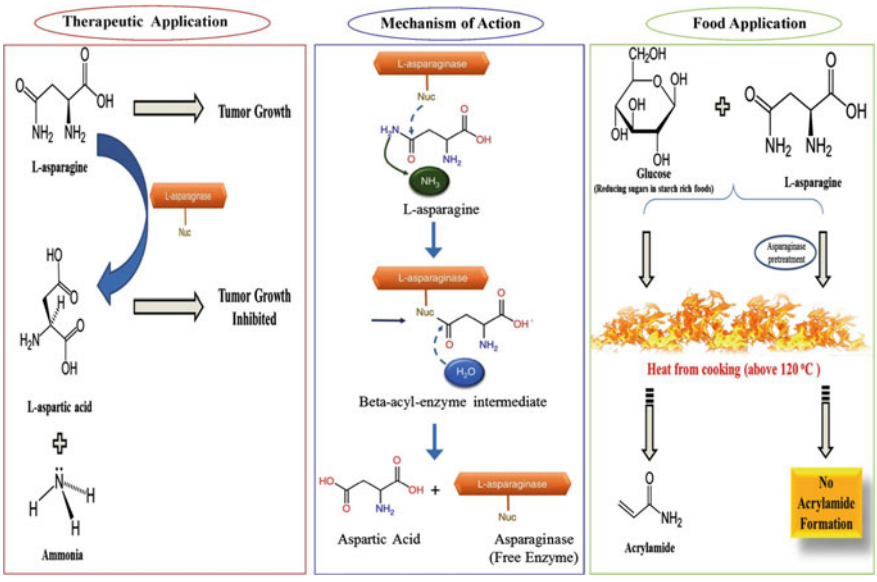

Fig. 5.1 Mechanism of action of asparaginase and its role in different applications

formed during baking/frying of starchy/high carbohydrate food items, when reducing sugar and asparagine reacts at high temperature more than 120 °C and with low water content. The reference dose (RfD) established for acrylamide is around 25–35 μg of acrylamide per day for a person weighing 68 kg by different agencies like the US EPA, United Kingdom (British Food Standard Agency; www.foodstandards.gov.uk), Norway (www.snt.no), Switzerland (Swiss Federal Office of Public Health 2002) and Germany (www.bfr.bund.de). Hence, the food industry is under pressure to reduce the formation of acrylamide in different food products. Various mitigation strategies like physicochemical methods, asparaginase pretreatment, etc were reported by several researchers. Physicochemical methods were not well accepted due to the formation of different secondary attributes in the finished food. Asparaginase played a major role in the food industry for reducing the acrylamide content without forming any secondary attributes like odour, souring etc (Fig. 5.1). Asparaginases enzyme hydrolysed the asparagine during pretreatment which leads to the inhibition of Maillard reaction and the amount of acrylamide was reduced significantly (Pedreschi et al. 2008; Ravi and Gurunathan 2018). Although the use of asparaginase in food industry has several limitations like high cost, low productivity and low thermal stability still remain a matter of concern.

Some researchers have worked upon thermal stability of enzyme by screening asparaginase from thermostable microbial sources (Pradhan et al. 2013). Moreover, engineering of asparaginase enzyme using different techniques like immobilization of asparaginase (Balcao et al. 2001) and specific site saturation mutagenesis (Feng et al. 2018) for thermal stability and high yield were also studied.

There are commercially available asparaginases which were efficiently utilized for acrylamide reduction, i.e. PreventASe^{TM} from DSM (Heerlen, The Netherlands) and Acrylaway® from Novozymes A/S (Bagsvaerd, Denmark). For better enzymatic properties, and thermal stability, enzymes need to be isolated from novel sources with enhanced properties.

This book chapter highlights different microbial sources of asparaginase and their enzymatic characteristics. Asparaginase mechanism of action, sequence-based structural and functional relationship, chemical and molecular modifications of enzyme for enhancing enzymatic properties is also elaborated.

5.1.1 History of Asparaginase

The discovery of asparaginase as anticancer drug started first in the year 1953, when J. G. Kidd showed the regressive effect of guinea pig serum on transplanted lymphomas in rat and mice model. They had proposed certain active constituents in serum leads to the regression of lymphomas (Kidd 1953). Earlier to this, Clementi in 1922 had suggested the characteristics of guinea pig serum and claimed that they were enriched with asparaginase enzyme (Clementi 1922). Progressive studies by Broome in 1961 also showed the inhibitory effect of asparaginase present in guinea

pig serum on lymphoma 6C3HED cells (Broome 1963). Further, advanced studies by Mashburn and Wriston (1963) indicated the potential of microbial asparaginase as a mean of chemotherapeutic agent to inhibit tumour growth. Tumour cells require asparagine as nutrient source and the ability of asparaginase to deplete asparagine made it possible in the lost battle of fighting against malignant tumour and lymphoblastic lymphomas. Asparaginase from microbial sources came out as flawless weapon and explored widely for its medical application. Several trials were tested to treat Acute Lymphoblastic leukemia (ALL) and later it was proposed as an excellent approach for absolution in leukemic patients (Hill et al. 1967).

5.1.2 Types of Asparaginase

L-asparaginase of two types, i.e. type I and type II asparaginase, which are widely distributed in microorganisms, plants, vertebrates and animal tissues (Qeshmi et al. 2018). Type I asparaginases are found in the cytosol, and hence, these enzymes are named as cytosolic asparaginase which shows a low affinity towards L-asparagine and greater affinity towards L-glutamine. Hence, these enzymes are considered confound as they showed an affinity towards the unspecific substrate. Type II asparaginases are better studied and found at the vicinities of periplasmic spaces. They are produced extracellularly. They showed high affinity towards L-asparagine and less specific towards L-glutamine (Swain et al. 1993). Both enzymes were substrate specific but Type II enzymes were less immunogenic. Due to this, Type II asparaginase has been comprehensively used for medical applications, mainly in treatment of acute lymphoblastic leukemia (ALL) (Zuo et al. 2015). Type II asparaginase has also been implicated in the food industry for removal of asparagine from the primary ingredients before processing to reduce the acrylamide formation in the finished food items (Hendriksen et al. 2009).

5.1.3 Classification of Asparaginases

L-asparaginase is produced by all kingdoms of life: Plants, microorganisms and animals. Hence, enzymes are classified based on the sequences of amino acids and biochemical characteristics of the enzyme. It is classified and grouped into mainly three types: bacterial, plant and rhizobial type. Bacterial enzymes mainly from *E. coli* and *Erwinia chrysanthemi* were studied extensively because of their varied properties and has been used vigorously as chemotherapeutic agent in ALL treatment from last two decades (Duval et al. 2002; Shi et al. 2017).

(a) **Bacterial type asparaginase**

Most of the Type II enzymes are isolated from mesophilic sources consisting tetra dimers and their molecular masses were analysed in the range of 140–170 KDa, whereas Type I enzymes are mainly isolated from thermophilic sources and are homodimer structurally. The molecular mass of this enzyme was up to 70 KDa. Some of the microbes like *E. coli* produced both isozymes in which Type I enzymes are cytosolic and have a low affinity for L-asparagine and considered to be a constitutive enzyme. Type II asparaginase showed higher affinity for L-asparagine and considered periplasmic. These enzymes are expressed subject to stress conditions, aeration and changes in the media or nutrient sources like carbon and nitrogen sources, etc. One of the two types of asparaginase showed antitumour activity and these properties vary from strain to strain and culture conditions. *E. coli* and *P. geniculate* comprise of type II asparaginase and known for their antitumour properties. Whereas in *Mycobacterium tuberculosis*, it has both isozymes but only one enzyme has shown antitumour activity. These unlike activities differ due to changes in pH, temperature, clearance rate (Half-life inside serum), activity profile and significant activity towards L-asparagine (Qeshmi et al. 2018; Badoei-Dalfard 2015).

Asparaginase from *E. coli* and *E. chrysanthemi* was studied extensively, and hence, asparaginase enzymes from different species were always harmonized with these microbes. Genus *Bacillus* contains both isozymes and genome sequencing of ansZ gene showed functional asparaginase, with 59% similarity with *Erwinia* asparaginase and 53% similarity with *E. coli* Type II asparaginase. Also, Gene ansA in *Bacillus* sp. encodes Type I asparaginase. Asparaginase from *Bacillus* sp. was utilized expeditiously for removal of acrylamide content in food processing but its role in antitumour activity is a matter of research.

(b) **Plant-type asparaginase**

Asparagine hydrolysis in higher plants is done utilizing two different kinds of routes. In the first catabolic pathway, amino nitrogen was transaminated by the enzyme asparagine oxo acid amino transferase and produces 2-oxosuccinamic acid and an amino acid. The second route utilized L-asparaginase which hydrolysed the asparagine into aspartate and ammonia found mainly in temperate legumes. Plant-type asparaginase is produced as inactive precursor molecules and belongs to N-terminal hydrolases. High level of asparaginase was detected mainly in developing leaves or roots (Michalska et al. 2008). Plants contain two different kind of asparaginase—classified on the basis of reaction catalysis and their potassium dependence. These are immunological significant and separate from each other (Lea et al. 1984; Michalska and Jaskolski 2006).

Plants like *Sphagnum fallax* have two types of cytosolic L-asparaginase. Potassium ions have no effect on the overall stability of the enzyme but required for the synergistic higher activity of the enzyme (Heeschen et al. 1996). Comparatively, potassium-independent enzymes are highly stable than potassium dependent but potassium dependent enzymes have higher affinity for substrate (Chagas and Sodek

2001). L-asparaginase extracted from *Phaseolus vulgaris* has a low glutamine activity which removes unwanted side effects and the purpose of chemotherapy could be sorted in the treatment of ALL (Mohamed et al. 2016).

Plant asparaginases were studied exclusively on the basis of homology modeling comparison with the crystal structure of *E. coli* asparaginase (Michalska et al. 2008). *Sphagnum fallax* asparaginase has intermediate properties of microbial as well as plants of higher origin (Heeschen et al. 1996). Marine algae *Chlamydomonas* and *Pisum* reported to have better enzymatic activity in the light region. Two group of plant asparaginase showed higher sequence similarity with each other and it is unlike to the microbial asparaginase where they are distinct from each other (EcA I cytosolic and EcAII periplasmic).

(c) **Rhizobial-type asparaginase**

Rhizobial-type asparaginase has shown sequence similarity with L-asparaginase from *Rhizobium etli. R. etli* are symbiont bacteria lived with the association of leguminous plants (Borek and Jaskólski 2001; Shi et al. 2017). Peculiar feature of these bacteria was associated with asparagine as they utilized it as a single source of carbon and nitrogen for their living. Asparaginase and aspartase enzymes were employed for this purpose. There are two kinds of asparaginases were reported from *R. etli*; asparaginase I which were constitutive enzyme and thermostable while asparaginase II was thermolabile and expressed only when asparagine is present other than any carbon source. Both the enzyme had not shown any sequence similarity with bacterial asparaginase like *E. coli* and *E. chrysanthemi.* However, type II asparaginase from *R. etli.* was having activity free from glutaminase, which shows a better potential for chemotherapy (Moreno-Enríquez et al. 2012).

5.2 Microbial Sources of Asparaginase

Asparaginase from microbial sources added value to the enzyme as enzymes would inherit the properties of the sources from which they were isolated. Microbial source of asparaginase enzyme is considered as effective as well as an easy way for industrial. A wide range of microbes such as fungi, bacteria, actinomycetes, yeast and algae has been well characterized for the production of L-Asparaginase till date. Different sources of microbial enzyme with their property were listed here in Table 5.1.

Bacillus sp. and *Pseudomonas* sp. are well-known producers of bacterial asparaginase. The prevalence of *Bacillus* sp. in the different environments can be correlated with their typical nature of spore formation, which can be aerially distributed for long distance (Chubukov et al. 2014). The asparaginase production is greatly influenced by media composition as well as the method of production, i.e. solid-state fermentation (SSF) or submerged fermentation (SmF). In bacteria, when the primary carbon source is present along with high concentrations of a variety of

Table 5.1 Different microbial sources of asparaginase with their optimum pH and temperature

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
Bacteria						
1	*Bacillus* sp.	SmF	7	37	–	Moorthy et al. (2010)
2	*B. licheniformis*	SmF	6–10	70	Anticancerous Thermostable	Mahajan et al. (2014)
3	*B. brevis*	SmF	6.7	30	–	Narta et al. (2011)
4	*B. subtilis* BsAIIS40M BsAIID49M	SmF SmF	8 7	65 50	Thermostable	Onishi et al. (2011)
5	*B. subtilis* WB600	SmF	7.5	65	Thermostable GRAS category	Feng et al. (2017)
6	*B. megaterium*	SmF	7	40	Acrylamide reduction	Zhang et al. (2015)
7	*B. subtilis* KDPS1	SSF	5	37	–	Sanghvi et al. (2016)
8	*B. tequilensis* PV9W	SmF	8.5	35	Anticancerous	Shakambari et al. (2016)
9	*B. aryabhattai* ITBHU02	SmF	8.5	40	Antineoplastic activity	Singh et al. (2013)
10	*B. licheniformis* MTCC 429	SmF	8	37	Recombinant Glutaminase-Free l-Asparaginase (ansA3)	Sudhir et al. (2014)
11	*B. subtilis* B11−06	SmF	7.5	40	–	Jia et al. (2013)
12	*Enterobacter cloacae*	SmF	7–8	35–40	Glutaminase-Free l-Asparaginase	Husain et al. (2016a)
13	*E. aerogenes* MTCC111	SmF	8	25	–	Erva et al. (2017)
14	*Pseudomonas otitidis*	SmF	7.5	40	Glutaminase-Free l-Asparaginase	Husain et al. (2016b)
15	*P. aeruginosa* strain SN004	SmF	5.0	50	Asparaginase with low glutaminase activity	Badoei-Dalfard (2015)
16	*P. fluorescens*	SmF	6.3	34	Cloned into *E. coli* BL21 for large scale production	Kishore et al. (2015)
17	*P. aeruginosa* strain EGYII DSM 101801	SmF	8.5	45	Cloned and expressed in *E. coli* BL21(DE3) pLysS	El-Sharkawy et al. (2016)
18	*P. fluorescens*	SmF	7	37	–	Sinha et al. (2015)
19	*P. Aeruginosa* 50071	SSF	9	37	–	El-Bessoumy et al. (2004)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
20	*Erwinia carotovora*	SmF	–	–	Cloned and expressed in *E. coli*	Pourhossein and Korbekandi (2014)
21	*E. aroideae* NRRL B-138	SmF	8.2	45	–	Tiwari and Dua (1996)
22	*E. carotovora*	SmF	7.5	25	Biopharmaceutical properties	Sukhoverkov and Kudryashova (2015)
23	*E. carotovora*	SmF	8.6	35		Kamble et al. (2006)
24	*E. chrysanthemi* 3937	–	–	–		Gilbert et al. (1986)
25	*Escherichia coli* W3110 Free enzyme Immobilized enzyme		7.5 8.5	37 50	Efficiently immobilized in calcium alginate gelatin composites	Youssef and Al-Omair (2008)
26	*E. coli*		7	37	–	Vidya et al. (2014)
27	*E. coli* MTCC 739		7	37	–	Sajitha et al. (2015)
28	*Pectobacterium carotovorum*	SmF	8–10	40	–	Kumar et al. (2011)
29	*Thermococcus kodakaraensis*	SmF	9.5	85	Thermostable enzyme	Chohan and Rashid (2013)
30	*T. gammatolerans* EJ3	SmF	8.5	85	Thermostable enzyme	Zuo et al. (2014)
31	*Pyrococcus furiosus*	SmF	9	85	Thermostable enzyme	Bansal et al. (2010)
32	*Rhizobium etli*	SmF	9	37	Rhizobial-type family of L-Asparaginase	Moreno-Enriquez et al. (2012)
33	*Yersinia pseudotuberculosis* Q66CJ2	SmF	8	60	Cloned and expressed in *E. coli* BL21(DE3)	Pokrovskaya et al. (2012)
34	*Rhodospirillum rubrum*	SmF	9.2	54	–	Pokrovskaia et al. (2013)
35	*Photobacterium* sp. J15	–	7	25	–	Yaacob et al. (2014)
Fungi						
36	*Penicillium* sp.	SmF	7	37	–	Patro and Gupta (2012)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
37	*P. digitatum*	SmF	7	30	–	Shrivastava et al. (2012)
38	*P. brevicompactum* NRC829	SmF	8	37	Anticancerous	Elshafei et al. (2012)
39	*Nocardia levis* MK-VL_113	SmF	7	30	–	Kavitha and Vijayalakshmi (2012)
40	*Aspergillus oryzae* CCT 3940	SmF	8	50	–	Dias et al. (2016)
41	*A.* niger AKV-MKBU	SmF	7	30	–	Vala et al. (2018)
42	*A. aculeatus*	SmF	9	30	–	Dange and Peshwe (2011)
43	*Talaromyces pinophilus*	SmF	8	28	–	Krishnapura and Belur (2016)
44	*Trichoderma viride*	SmF	7	37	–	Lincoln et al. (2015)
45	*Cladosporium* sp.	SSF	6.3	30	–	Kumar and Manonmani (2013)
46	*Rhizomucor miehei*	SmF	7	45	For the food industry and in chemotherapeutics for leukemia	Huang et al. (2013)
47	*Mucor hiemalis*	SmF	7	37	–	Thakur et al. (2013)
Actinomycetes						
48	*Streptomyces* sp.	SmF/SSF	7.5	50	–	Basha et al. (2009)
49	*S. noursei* MTCC 10469	SmF	8	50	Isolated from Marine sponges	Dharmaraj (2011)
50	*S. gulbargensis*	SmF	8.5	40	–	Amena et al. (2010)
51	*S. fradiae* NEAE-82	SmF	8.5	40	Cytotoxicity and anticancer activities	El-Naggar et al. (2016)
52	*S. brollosae* NEAE-115	SSF	–	–	–	El-Naggar et al. (2017)
53	*Nocardiopsis alba* NIOT-VKMA08	SmF	8	37	–	Meena et al. (2015)
54	*Actinobacterial* sp.	SmF	8	35	–	Varma et al. (2016)

(continued)

Table 5.1 (continued)

S. No.	Microorganism	Production method	Enzyme property		Specifications	References
			pH	Temp		
Algae						
55	*Spirulina maxima*	SmF	8.5	37	–	Abd El Baky and Baroty (2016)
56	*Chlamydomonas* species	SmF	–	50	–	Paul (1982)
57	*Chlorella vulgaris*	SmF	–	–	Inducible intracellular l-asparaginase	Ebrahiminezhad et al. (2014)

amino acids under anaerobic conditions, the asparaginase production can be enhanced by 100-fold (Cedar and Schwartz 1968). Since the different metabolites of amino acids like asparagine can contribute directly into the citric acid cycle, and its presence in the medium can trigger the asparaginase gene expression. Although, the model carbon source, glucose may serve as a catabolic repressor for enzyme expression.

The next important and limiting factors for production of asparaginases are their respective microbes. The enzyme catalytic property is importantly influenced by pH, temperature, substrate specificity and other kinetic parameters. It may vary among the same genus and or species. L-asparaginase from most of the *Bacillus* sp. exhibited optimum pH in the range of 6–8 and optimum temperature in the range of 30–40 °C while *B. licheniformis* (Mahajan et al. 2014), *B. subtilis* BsAIIS40M (Onishi et al. 2011), *B. subtilis* WB600 (Feng et al. 2017) and *B. aryabhattai* ITBHU02 (Singh et al. 2013) showed optimum pH around 8.5 and optimum temperature around 60–70 °C. Thermostable asparaginases from bacterial source like *Thermococcus kodakaraensis*, *Thermococcus gammatolerans EJ3*, *Pyrococcus furiosus* etc. are having optimum pH and temperature of 9 and 85°C respectively and can be of interest for industrial applications (Chohan and Rashid 2013; Zuo et al. 2014; Bansal et al. 2010).

When bacterial L-asparaginase used for medicinal purpose, it was found that there is some side effect such as hypersensitivity, pancreatitis, thrombosis, etc., which is mainly due to glutaminase activity (Killander et al. 1976; Ramya et al. 2012). To minimize the side effect, the search of eukaryotic microorganism like yeast and fungi was taken up for production of the enzyme. Several researchers reported that fungi and yeast can produce reasonable production of the enzyme under both solid-state fermentation (SSF) and submerged fermentation (SmF) (Dias et al. 2016; Kumar and Manonmani 2013). The fungal genera such as Aspergillus (Dias et al. 2016; Vala et al. 2018; Dange and Peshwe 2011), Penicillium (Shrivastava et al. 2012; Elshafei et al. 2012; Patro and Gupta 2012) and Trichoderma (Lincoln et al. 2015) are vastly studied for the production of asaparaginases.

Oceans were the cradle of life and they still host an enormous biodiversity which is rather under-explored. Research to explore the marine environment for microorganisms has rapidly increased over the past two decades. Marine microbes majorly include bacteria, archaea and actinomycetes (Izadpanah et al. 2018). Marine actinomycetes were also explored for L-asparaginase production and their application. Genus Streptomyces is the most studied actinomycetes till date (Basha et al. 2009; Dharmaraj 2011; Meena et al. 2015; Varma et al. 2016; Amena et al. 2010; El-Naggar et al. 2016, 2017).

5.3 Application of Asparaginase as Food Enzyme

In recent years, L-asparaginase has been successfully used as food enzyme as it has found an application in reducing acrylamide formation in food system. Asparagine is present in most of the starchy foods, which reacts with their reducing sugar during thermal processing leads to formation of neo formed contaminants named acrylamide which is a bi-product of Maillard reaction (Mottram et al. 2002). It is a potent chemical carcinogen and neurotoxic which forms adduct with the haemoglobin when consumed in high dose and causes reproductive disability (Tyl et al. 2003). Acrylamide toxicity is dose dependent and when ingested in high amount leads to cytotoxicity and necrosis. Acrylamide cytotoxic studies were conducted by Kacar et al. (2018) where acrylamide's cytotoxicity and anti-proliferative properties were analysed on human cell lines A549 and acrylamide (IC_{50}) dose was attained in 24 h was 4.5 mM. Hence, such a small concentration of acrylamide had a deep and regressive effect on human body.

In a view of food system and prevention of human health hazards, acrylamide toxicity and reduction of its content in food systems were studied extensively. Several International Food related agencies like U.S. Food and Drug Administration (FDA), European Food Safety Authority (EFSA) initiated the battle to reduce acrylamide contents in deep fried and baked food products formed during heat processing after its surprise discovery by Swedish scientists in 2002 (Tareke et al. 2002). FDA had issued several magazines and scientific reports claiming the formation of acrylamide in different kinds of food products and formation of other contaminants in heat-processed system (Robin and Clanci 2007). EFSA had done extensive research work on the contents of acrylamide in different food products along with the toxicity levels on human. They had also reported the acrylamide toxicity and its metabolism pathway inside human body where acrylamide is metabolized into glycidamide (GA) which forms an adduct with haemoglobin and leads to genotoxicity and neurotoxicity (EFSA 2015; Bandarra et al. 2013).

Hence, there was a sudden rush to cope up with this major issue. In the wake of this, several studies were conducted and guidelines for cooking were finalized (Robin and Clanci 2007). After that, several parameters were studied like decreasing pH, lowering the temperature for frying and blanching of the potato chips before frying for decreasing the levels of acrylamide in French fries

(Pedreschi et al. 2007). Enzymatic pretreatment of food with asparaginase enzyme leads to reduction of acrylamide at significant levels apart from other treatment procedure (Meghavarnam and Janakiraman 2018). Different food products have been subjected to pretreatment with asparaginase and clearly shown the reduction of acrylamide in variety of food products. Some of the food products with high acrylamide contents and successful reduction of acrylamide using asparaginase enzyme will be listed here (Table 5.2).

Table 5.2 Strategy and use of asparaginase in acrylamide reduction in foods

S. No.	Food products	Enzyme source	Conditions for pretreatment	Acrylamide reduction	References
1	French fries	*Aspergillus oryzae*	Soaking time—1 min, enzyme load—10.5 ASNU/mL	60–85%	Hendriksen et al. (2009)
2	Sliced potato chips	*A. oryzae*	Blanching for 20 min, enzyme load—10.5 ASNU/mL	60%	Hendriksen et al. (2009)
3	Fried dough pastry	Commercial	Different enzyme load—100 U/kg, 500 U/kg and 1000 U/kg	Upto 90%	Kukurová et al. (2009)
4	Potato chips	Commercial	Blanching 85 °C for 3.5 min, enzyme load—10 ASNU/mL	Upto 90%	Pedreschi et al. (2011)
5	Gingerbread	Commercial	Pretreatment time −30 to 60 min, enzyme load—1000 U/kg of dough	97%	Ciesarova et al. (2009)
6	Potato	*Bacillus licheniformis*	30 IU/mL	80%	Mahajan et al. (2012)
7	Potato crisps	*B. subtilis*	Enzyme load—0–40 U	80%	Onishi et al. (2015)
8	French fries	*Thermococcus zilligii*	Enzyme load 0–20 U	80%	Zuo et al. (2015)
9	Fried Kochchi Kesel chips	*A. terreus*	5 U/mL, pretreatment-at 60 °C for 20 min	55%	Ravi and Gurunathan (2018)
10	Potato chips	*Fusarium culmorum*	300 U/L	85%	Meghavarnam and Janakiraman (2018)
11	Sweet bread	*F. culmorum*	300 U/L	78%	Meghavarnam and Janakiraman (2018)

ASNU is defined as the amount of asparaginase that produces 1 μmol of ammonia per min under the conditions of the assay (pH = 7 ± 0.005; 37 ± 0.5 °C) using Acrylaway®

5.4 Asparaginase Sequence Analysis Combined with Mode of Action

Many asparaginase gene sequences have been deposited into GenBank and crystal structure of functional enzymes also available in Protein Database (PDB) which comprises of various microbial sources. Type I asparaginase is from *Lactobacillus reuteri* (PDB ID: A5VMR3), *Escherichia coli* (PDB ID: 2HIM), *Yersinia pestis* (PDB ID: 3NTX), *Vibrio cholera* (PDB ID: 2OCD), *Thermococcus kodakaraensis* (PDB ID: 5OT0), *Pyrococcus furiosus* (PDB ID: 4Q0M) and *P. horikoshii* (PDB ID: 1WLS) while Type II asparaginases are from *E. coli* (PDB ID: 3ECA), *E. chrysanthemi* (PDB ID: 5F52), *Erwinia carotovora* (PDB ID: 2HLN), *Wolinella succinogenes* (PDB ID: 5K3O), *Pseudomonas* (PDB ID: 1DJP), *Helicobacter pylori* (PDB ID: 2WLT), *Campylobacter jejuni* (PDB ID: 3NXK) and *Acinetobacter glutaminasificans* (PDB ID: 1AGX). The phylogenetic relationship among the microbial asparaginase protein sequences retrieved from Protein Database (PDB) revealed high divergence among the Type I and Type II asparaginases (Fig. 5.2). For example, Type I and Type II asparaginase from *E. coli* shares only 24% of protein identity. The protein sequences of both Type I and Type II asparaginases consisted with larger N and smaller C terminal domains, connected with approximately 20 amino acid long linker region (Guo et al. 2017) (Fig. 5.3). The active sites of the asparaginase can be grouped into rigid and flexible regions (Nguyen et al. 2016). The rigid regions consisted of residues between first and third parallel β strands of N-terminal and residues of the C terminal of neighbouring monomer. The flexible region represent the residues involved in

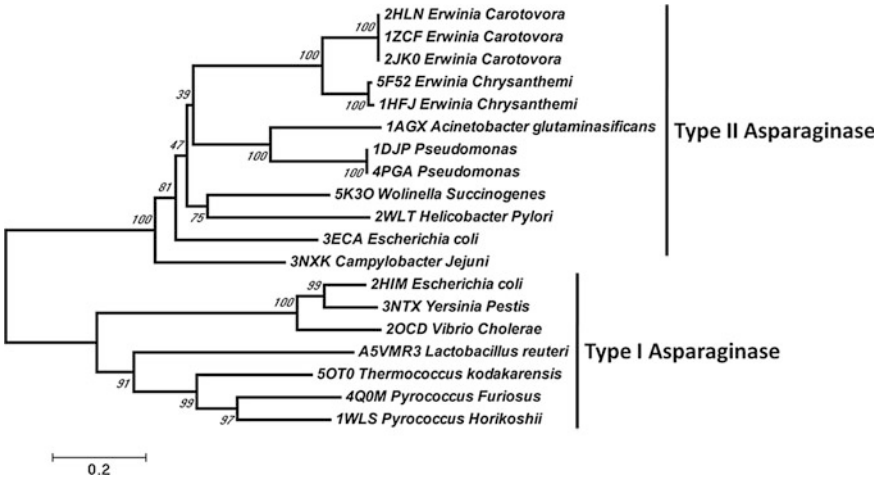

Fig. 5.2 Phylogenetic analysis of Type I and Type II asparaginase protein structures based on the protein sequences retrieved from Protein Database

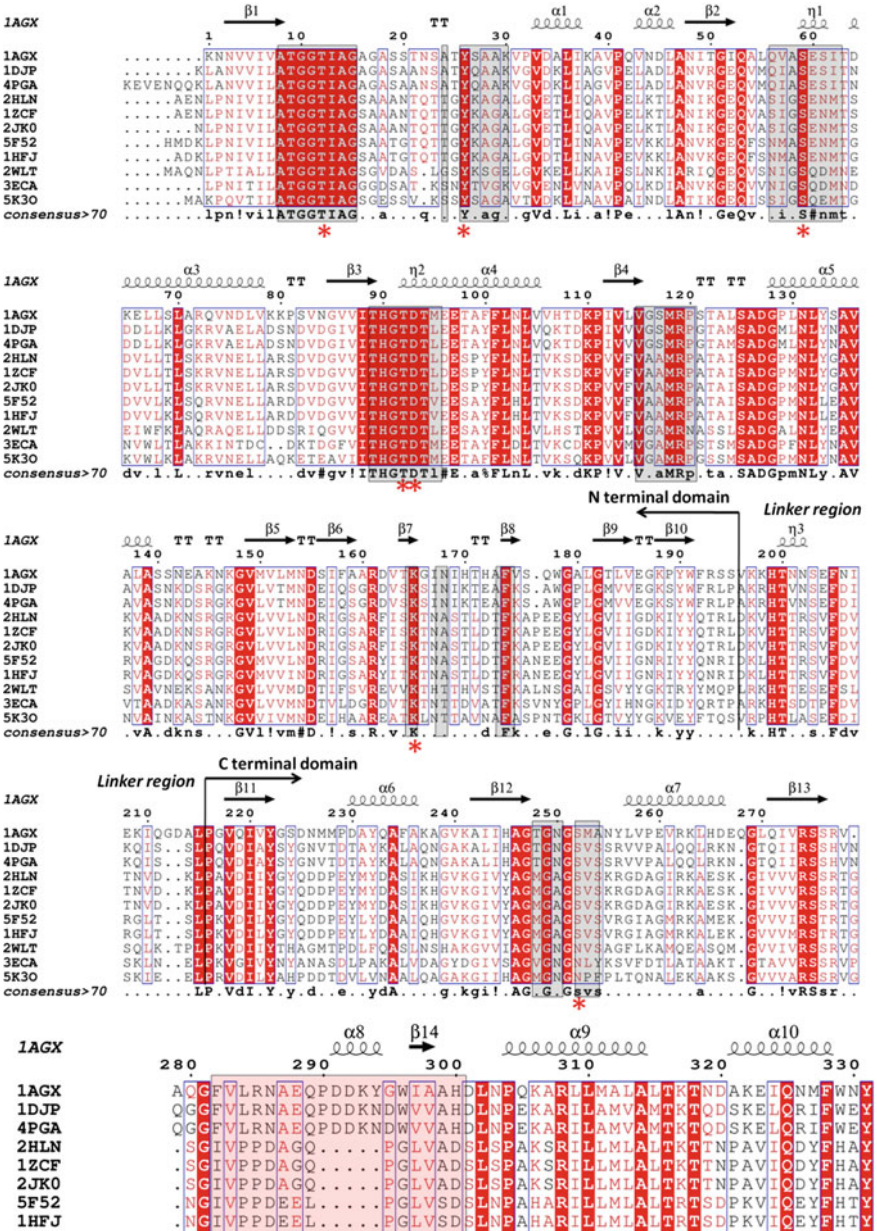

Fig. 5.3 Multiple sequence alignment of the asparaginase protein sequences showing the secondary structure characteristics. All the conserved residues are boxed and the fully conserved residues are coloured white with red background. The less conserved residues are coloured red. The alignment was performed using ESPript 3.0

substrate-enzyme binding pocket and nucleophilic initiation of the reaction, which is situated at the starting of the N-terminal region (Borek and Jaskólski 2001).

The protein sequence of asparaginase may reveal its mechanism of action which shows similarity with serine protease (Tomar et al. 2014). The 'catalytic triad' composed with a nucleophilic residue, a general base and acidic residue which connected by hydrogen bonds define the complete mechanism of asparaginase. As per the reported crystal structures of asparaginases, the active site has two catalytic triad named as Triad I and Triad II. The Triad I is composed with Thr-Tyr-Glu residues are present at flexible region and act as nucleophilic (Thr), a base (Tyr) and an acidic (Glu) residue (Singh et al. 2015). In a similar way, Triad II is represented by Thr (nucleophilic)-Lys (base)-Asp (acid) residues (Bansal et al. 2012). These two triads function intensively for the conversion of substrate into the product in the presence of water as second nucleophile.

The reaction mechanism of asparaginase composed of two-step sequential 'ping-pong' nucleophilic mechanism (Ehrman et al. 1971; Yao et al. 2005). This reaction is initiated by the activation of the enzyme's nucleophile via strong H bonding to the nearby basic residue which attacks the carbon atom of amide bearing substrate (Fig. 5.1). The rearrangement of tetrahedral transition state leads to the formation of acyl-enzyme intermediate product. In transition state, the negative charge on the O atom of amide group is stabilized by the H bond donors known as 'oxyanion hole'. In the second step, activated water is acted as the second nucleophile and attacked on C atom of ester group and again a transition state with oxyanion hole is developed and finally converted into free enzyme and product. These two steps reactions are common in Type I and Type II asparaginase with some structural differences. The 'open and closed' conformations for substrate binding can be defined by a highly flexible β-hairpin at N-terminal domain (Aghaiypour et al. 2001). The enzyme to ligand binding induces the transition of nucleophilic Thr residue which allows the change in the conformation from open to close. This binding of substrate and induced conformational change of Thr residue for the closer of flexible N-terminal loop also revealed the substrate selection of L-asparagine or L-glutamine (Nguyen et al. 2016). The involvement of other residues in enzyme mechanism has also been discussed by various researchers as the sequence variation among various microorganisms leads to change the catalytic proximity of asparaginase.

5.5 Improvement in Asparaginase Characteristics

The biocatalyst gained its properties from the native living source like microorganism, plant, animal, etc. However, it is essential to alter the native properties of the biocatalyst within the purview of its utilization in applied sectors like industrial, agricultural or medical. Microbial production of asparaginase displayed several bottlenecks like less enzyme activity and narrow range of kinetic parameters like substrate specificity, reusability and thermal stability. These incompatibilities of

microbial asparaginase need to be addressed for robust industrial physiochemical conditions. Several attempts have been made to optimize the asparginase enzyme at the molecular level which leads to higher productivity combined with improved properties favourable for industrial or medical applications. For asparaginase enzyme, these selected modifications were carried out by chemical or genetic modifications. The following sections specifically describe the most recent studies, advantages and disadvantages of both modifications.

5.5.1 Chemical Modifications of Asparaginase

The chemical modifications are important to rationalize the immunological responses as well as other characteristics. The immobilization of microbial asparaginase on nanofibers (Ghosh et al. 2012), nanoparticles (Ulu et al. 2018), PEG (Zhang et al. 2004), Sepharose CL-6B (Kotzia and Labrou 2007) agarose-glutaraldehyde (Balcao et al. 2001) and aluminium oxide pellets (Agrawal et al. 2018) which shows advancement of various chemical modifications and its contribution to the asparaginase desired properties. Most of the research has been carried out to possess the asparaginase activity for therapeutic applications (Table 5.3). However, few reports are also available which revealed that the asparaginase immobilization can induce higher reduction of asparagine content that decreases the acrylamide formation in heat-treated food materials. Studies show that immobilized asparaginase-based reduction of asparagine was significantly higher as compared with free asparaginase enzyme. Aiswarya and Baskar (2018) revealed that asparaginase immobilized on magnetic nanoparticle possess 75% mitigation of acrylamide formation in fried potato chips. Similar result was also obtained by Agrawal et al. (2018) and Hendriksen et al. (2016), where asparaginase immobilized on aluminium oxide pellets and silica-based carrier also reduce the asparagine content in fried food materials, respectively. The studies were also suggested that immobilization of asparaginase not only improve the kinetic parameters but thermostability was also shown to be enhanced which is essential of food applications. Different immobilization carrier materials including organic, inorganic and hybrid materials have been used in the studies. However, no study has been reported on the impact of these immobilized asparaginases or other carrier materials on the food quality, texture and flavours. Hence, the research has to be extended in a view of food sample specific carrier materials used for asparaginase immobilization.

5.5.2 Genetic Modifications of Asparaginase

The developments of biotechnological and bioinformatics techniques allowed to attain various approach to improve and assess diverse range of biocatalysts with desired characteristics. These tools can be used to rational or random alteration in

Table 5.3 Chemical and fusion proteins based modifications of microbial asparaginase with improved properties

S. No.	Microbial source	Type of chemical modification	Improved property	References
1	Commercial enzyme	Thiol silane functionalized magnetic Fe_3O_4@MCM-41 core–shell nanoparticles	• 63% higher thermostability • 1.15 fold lower K_m	Ulu et al. (2018)
2	*E. coli*	Poly (2-vinyl-4,4-dimethylazlactone)-functionalized magnetic nanoparticles	• Retain 95.7% activity after 10 repeated uses • Maintain 72.6% activity after 10 weeks storage	Mu et al. (2014)
3	*E. coli*	Covalent immobilization onto agarose-glutaraldehyde	• Prevents enzyme inactivation by subunit dissociation • Improved thermostability	Balcao et al. (2001)
4	*E. coli*	Covalently immobilized on aluminium oxide pellets	• Lowering enzyme level in potato chips • No activity loss after nine cycles	Agrawal et al. (2018)
5	*Bacillus circulans*	Polyaniline nanofibers	2.05-fold lower K_m	Ghosh et al. (2012)
6	*E. coli*	Encapsulated within erythrocytes	• Reduction in the number and severity of allergic reactions • Less coagulation disorders	Domenech et al. (2011)
7	*E. coli*	Variable region antibody fragment (ScFv) conjugated asparaginase	• Resistance to proteolysis • Increased biological half-life	Guo et al. (2000)
8	*Cladosporium* sp.	*N*-Bromosuccinimide-modified asparaginase	Prolonged half-life and proteolytic digestion	Kumar et al. (2013)
9	*E. coli*	Conjugated with modified polyethyleneglycol (mPEG2)	• Improved half-life • Complete abolishment of immunogenicity	Zhang et al. (2004)
10	*Erwinia chrysanthemi*	Immobilized on epoxy-activated Sepharose CL-6B	• Retaining 82% of its activity • Resistance to proteolysis	Kotzia and Labrou (2007)
11	*Aspergillus terreus*	Immobilized on nanomagnetic particle	• No reduction on activity after three cycles • Higher reduction in acrylamide content in fried potato chips	Aiswarya and Baskar (2018)
12	*Pyrococcus furiosus*	Immobilization on silica-based carrier	• Reduced level of asparagines in heat-treated food materials	Hendriksen et al. (2016)

the genetic information embedded in the native organism or recombinant host. Several asparaginase genes from microbial sources like *Bacillus subtilis* (Jia et al. 2013), *Thermococcus kodakaraensis* (Chohan and Rashid 2013), *Pseudomonas fluorescens* (Kishore et al. 2015), *Nocardiopsis alba* (Meena et al. 2016), *Thermococcus litoralis* (Washio and Oikawa 2018), *E. coli* (Vidya et al. 2011), *Streptomyces* (Sudhir et al. 2012), etc. were extracted, cloned and overexpressed by recombinant DNA technology. The molecular analysis of asparaginases has opened up a new avenue for exploring such information for the development of structure–function relationship, substrate binding and selectivity phenomenon, mechanism reestablishment and other identical physicochemical properties. Hence, this information can also be used to rearrange the molecular structure of asparaginase to obtain previously described characteristics. Improvement in thermostability of asparaginase has already been proven and most crucial factor which can broaden its application in therapeutics as well as food for eliminating the toxic content of acrylamide. Therefore, many researchers have attempted to alter the molecular state of native asparaginase proteins by three different and validated strategies: rational design, directed evolution or preparation of synthetic gene construct. Rational design through protein engineering can be defined as hypothesis-driven manipulation of the protein sequence that can change its biophysical properties in a controlled manner. However, the complete crystal structure of validated protein sequence with high resolution and enzyme–ligand binding modeling information is essential for rational protein engineering. The rational hypothesis can be made by algorithmically simulated and optimized designing of available natural sequences for desired protein properties. Few examples are available showing rational designing of asparaginase for the improvement of yield (Verma et al. 2014; Gervais and Foote 2014), substrate specificity (Nguyen et al. 2016) and thermostability (Long et al. 2016; Vidya et al. 2014). Most of the research has been made on the improvement of thermostability of asparaginase. Previous reports concluded that the thermostability of asparaginase can be improved by enhancing the hydrophobicity, H bond and salt bridge, low Ser/Cys content, high Tyr/Arg/Pro content, extra loop deletion, and increased surface polarity and area. Table 5.4 displayed different examples of the rational designing of asparaginase enzyme for the desired properties.

Table 5.4 Rational designing based protein engineering of asparaginase for improved characteristics

S. No.	Enzyme source	Modifications	Conditions and improvement	Reference
1	*E. coli* Asparaginase II	Site-directed mutagenesis	Enhanced stability with activity as compared with wild type	Verma et al. (2014)
2	*E. coli* Asparaginase II	Site-directed mutagenesis	Variants are designed replacement of two positively charged residues (K139 and K207) on the surface loops with neutral and reverse charges; thermally stable	Vidya et al. (2014)
3	*Bacillus subtilis* B11-06	Site-directed mutagenesis	Variant mutants G107D, T109Q, T109S and S166A; G107D displayed superior thermal tolerance and activity	Long et al. (2016)
4	L-asparaginase	Protein-polyelectrolyte complex (PPC) containing L-Asnase and poly-l-lysine (polyK); precipitation-redissolution method	Tolerance towards thermal and shaking stress, protection from oxidation stress and stabilize Asnase from physicochemical stress	Maruyama et al. (2015)
5	*Helicobactor pylori*	Site-directed mutagenesis	HpASNase (T16, T95 and E289); E289A reduced catalytic activity but increase thermo-tolerance	Maggi et al. (2015)
6	*Erwinia chrysanthemi*	Two mutations with single deamination site; (N41D and N281D)	N281D have lowest glutaminase activity; specific activity increase in both mutations	Gervais and Foote (2014)
7	*Bacillus subtilis*	Quadruple mutant NTFS (S180 N/D289T/E260F/ E292S) Site-directed mutagenesis	8.1 fold increased Half-life at 65 °C	Feng et al. (2018)

5.6 Conclusion

The book chapter comprehends to the evaluation of microbial asparaginase in terms of its utilization for food industries for the elimination of toxic acrylamide content. Getting insight into the precise mode of action will find the exploration of new microbial sources for mining novel asparaginase. The integration of Omics approaches needs to be explored to mine new candidates of asparaginase from the environments especially extreme environment. The sequence-based diverse interpretation of structure–function relationship has to be utilized for its future existence in dramatically improving the food products and generation of sequence-based rational protein engineering technology can help to decipher improved functional characteristics to meet the industrial desirability.

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