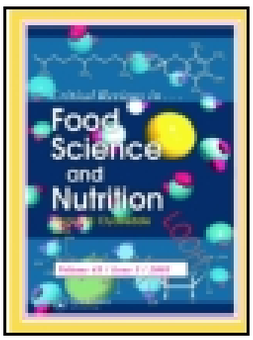

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Extremozymes from metagenome: Potential applications in food processing

Mahejibin Khan[a,c] and T. A. Sathya[b,c]

[a]CSIR-Central Food Technological Research Institute-Resource Centre Lucknow, India; [b]CSIR-Central Food Technological Research Institute, Mysore, India; [c]Academy of Scientific and Innovative Research, New Delhi, India

ABSTRACT

The long-established use of enzymes for food processing and product formulation has resulted in an increased enzyme market compounding to 7.0% annual growth rate. Advancements in molecular biology and recognition that enzymes with specific properties have application for industrial production of infant, baby and functional foods boosted research toward sourcing the genes of microorganisms for enzymes with distinctive properties. In this regard, functional metagenomics for extremozymes has gained attention on the premise that such enzymes can catalyze specific reactions. Hence, metagenomics that can isolate functional genes of unculturable extremophilic microorganisms has expanded attention as a promising tool. Developments in this field of research in relation to food sector are reviewed.

KEYWORDS

Extremophiles; food processing; functional food; metagenomics

Introduction

Food processing involves conversion of raw, perishable, inedible food materials into shelf-stable, palatable foods, and beverages. It also combines raw food ingredients to improve nutrition for specific target groups. Thus, processing involves a chain of events beginning raw material harvesting to the finished product.

The dominating Food and beverage industries with over 35% market shares has led to increased demand for enzymes, specifically from microorganisms, since they are environmentally friendly and safe for industrial applications. From time immemorial, they were used in food sector for processing starch, meat, milk and fruits (Muir, 1996; Fox, 1998; Gurung et al., 2013). The breakthrough in enzyme industry can be dated back to starch processing with amylases for the production of glucose and fructose syrups. The environmentally friendly process technology combined with high conversion efficiency replaced traditional acid hydrolysis (Poulsen and Buchholz, 2003; Vasic-Racki, 2006). Thus industrial enzymatic food processing became an acceptable feature and Figure 1 depicts the classes of enzymes adopted by food and feed industries (Henrissat, 1991; Coutinho and Henrissat, 1999).

Most of the food processing at industrial levels are carried out under unusual physicochemical conditions like, non-aqueous environment and above or below ambient temperature, pH, and pressure. Though enzymes being proteinaceous in nature are prone to denaturation under the rigours of food-processing conditions, characterization of enzymes that perform under extreme conditions has resulted in their acceptance by food industries. Modification of existing enzymes by molecular methods for unique properties followed to meet the requirements of specific enzymes for the evolving food industries. Consequent information on the active site consensus and changes in enzyme properties following amino acid alterations by site directed mutagenesis also suggested that enzymes with distinctive properties can occur in nature,within microorganisms, and can be identified by analyzing unculturable microbes. In this context, metagenomic mining for functional genes of unculturable microbes from varied environment became relevant (Rondon et al., 2000; Schloss and Handelsman, 2003; Khan et al., 2013a).

Metagenomics protocols involve creation of a library of cloned, functionally significant environmental DNA, of diverse groups of microorganisms, after their extraction using defined primers. Further screening of the clones by sequencing and studies on the expressed product, directly identifies unique properties of the cloned genes in terms of industrial applications. Reviews by Handelsman et al. (1998); Daniel (2005); Kakirde et al. 2010 and Angilina and Khan (2014) have described the protocols on this subject.

Extremozymes

The enzymes that function in extreme or unusual physicochemical conditions such as, high and low temperature, pH and pressure, high salinity, low water activity, low oxygen, etc., are generally considered as extremozymes. In industrial processing, extremozymes speed up or maximizes reactions under extreme conditions where non-extreme counterparts get inactivated. Thermophilic, psychrophilic, acidophilic, alkaliphilic, and halophilic microorganisms are generally the sources of these enzymes.

Increaed temperature industrial processing has its inherent advantages like, reducing microbial contamination, low

CONTACT Mahejibin Khan mahejibin@gmail.com CSIR, Central Food Technological Research Institute-Resource Centre, Lucknow 226019, India.
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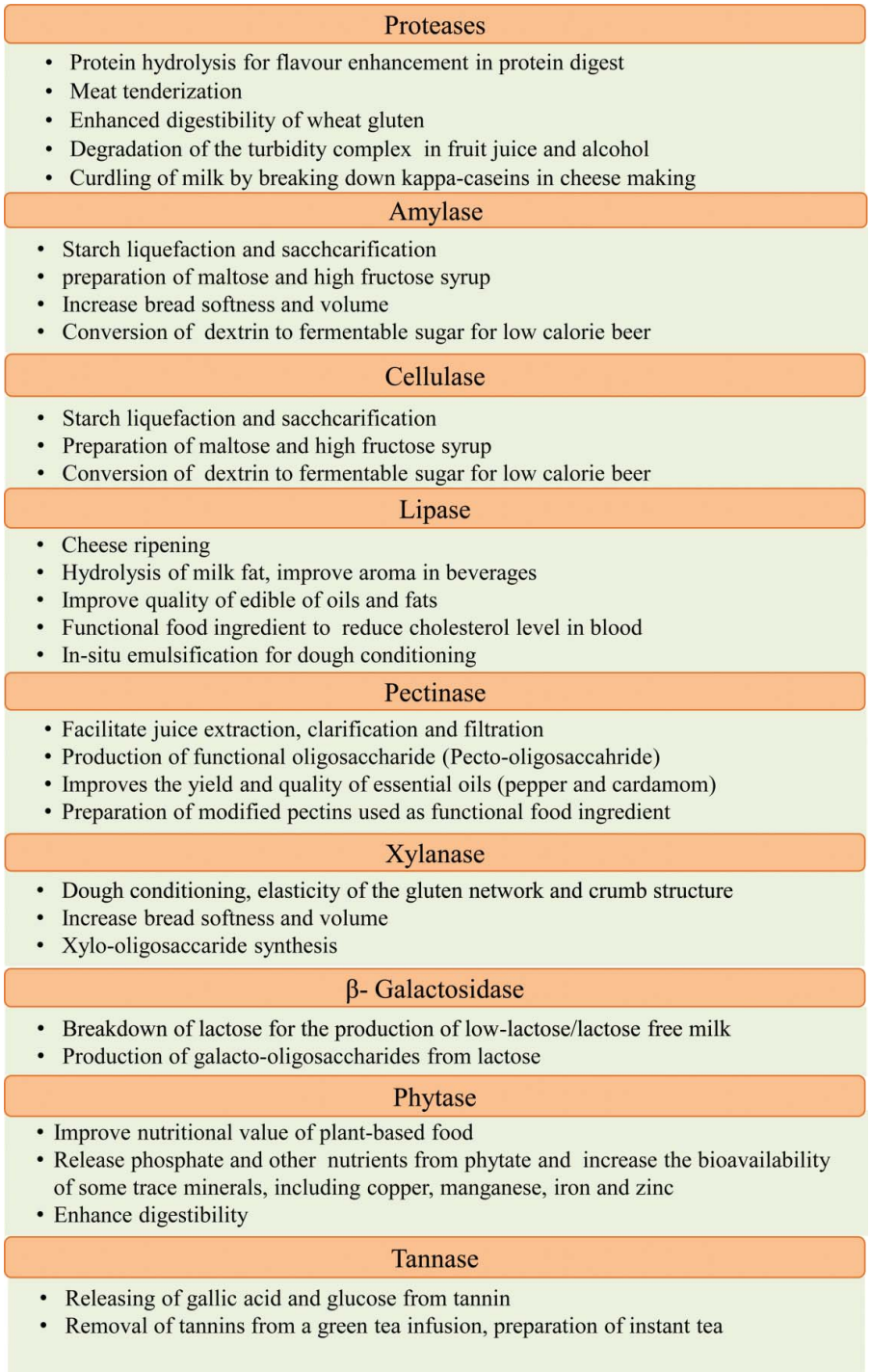

Figure 1. Application of enzymes used in food industry.

substrate viscosity and higher solubility of many polymeric substrates (Gomes and Steiner, 2004). Even though use of proteases, cellulases, amylases, pectinases, lipases, esterases, tannases, and phytases are industrially in vogue, thermophilic enzymes are desirable since they can increase reaction rates during high temperature processing. More than 40 extremophilic enzymes, active at high temperatures, characterized from the hot springs of Yellowstone National Park have found applications in food processing specifically, starch processing, and ethanol production.

Low-temperature active enzymes have various industrial potentials (Demirjian et al., 2001; Burg, 2003; Feller and Gerday, 2003; Georlette et al., 2004). Cold-active β-galactosidase is used in the dairy industry to prepare lactose free milk and cheese. In bakery industry, glycosidases active at low temperatures (amylases, proteases, and xylanases) improve texture and flavor. Fruit processing with low-temperature active pectinases retain flavour and aids clarification. Likewise, proteases tenderizes and enhances flavour of meat during refrigerated storage (He et al., 2004). Other applications include brewing and wine industries, and for the production of animal feed supplements.

A group of enzymes that can catalyze reactions under more than one extreme condition, high or low temperature and pH, nonaqueous medium or in low water concentration is called

polyextremohilic enzymes. Their ability to tolerate more than one unusual environmental condition make them valuable for fundamental and biotechnological research.

Molecular adaptation of extremozymes

Microorganisms rapidly evolve to live and multiply under extreme conditions by changing their gene structure to suit to physiological needs. Studies have revealed that, for faster adapation, flexibility is provided to the enzyme structures to prevent denaturation by nonspecific interactions at high temperatures. Hence their enzymes have shorter loops with less number of prolines (Hoyoux et al., 2004). Increased hydrophilicity with more number of polar groups and few hydrophobic residues (Fields, 2001; Saelensminde et al., 2007), more negatively charged enzyme surface for stabilizing solvent interactions and larger catalytic sites, allowing better accessibility to ligands (Siglioccolo et al., 2010), have also been cited as reasons for their easy adaptation to extremes of environment. According to Liszka et al., 2012, the reaction rates (kcat) of cold active enzymes are independent of temperature due to which they maintain high catalytic efficiency even at low temperatures.

Comparative analysis of three-dimensional structures of several thermophilic enzymes and their mesophilic counterparts (Sterner and Liebl, 2001; Sadeghi et al., 2006; McDonald, 2010) revealed the factors that contribute to thermostability. The increased rigidity of the protein was associated to high number of hydrophobic residues that enabled tighter packing (Fields, 2001). Stacking interactions enhanced thermostability due to more aromatic side chains increasing hydrophobicity of the core (Fedøy et al., 2007; Saelensminde et al., 2009). Modifications in thermophilic proteins also included added ion pairs and hydrogen bonding by increased positively charged residues in the interior and solvent-exposed surfaces (Ladenstein and Ren, 2008; Ma et al., 2010; Kim and Ishikawa, 2013). The finding that disulfide bonds stabilize proteins even above 100°C (Cacciapuoti et al., 1994; Choi et al., 1999; Toth et al., 2000) was reasoned to their presence in variable regions since disulfide bonds were not always found in conserved regions of thermophilic proteins (Rigden et al., 2011).

Halophilic enzymes are known to develop various adaptive strategies to support catalysis in high salt environments. The proteins occur in solution since surfaces contain more acidic amino acids, like glutamic and aspartic acids, for binding significant amounts of salt and water (Frolow et al., 1996, Britton et al., 2006). Comparative structural analysis of fifteen halophilic and nonhalophilic proteins revealed differences in the hydrophobic residues of halophilic and mesophilic proteins (Siglioccolo et al., 2011). Longo et al. (2013) studying prebiotic proteins supported the report of Müller-Santos et al. (2009) that halophilic proteins are adapted for salt-dependent folding and utilize salt to fold for survival and function in high salt environment.

It can be concluded from the data above that, most of the extremophilic enzymes and their nonextremophilic counterparts are highly similar in three-dimensional structures. Structural flexibility of extremozymes through specific modifications in the nature and content of amino acids, number of surface charged residues and enhanced noncovalent interactions appears to have evolved for adaptation to environment (Liszka et al., 2012; Charbonneau and Beauregard, 2013; Pezzullo et al., 2013; Khan and Kumar, 2016).

Extremozymes discovered through functional metagenomics

Important exteremozymes of interest to food industries are described in Table 1. Employing metagenomic techniques, new biocatalysts from the environmental samples have been isolated and employed successfully (Steele et al., 2009).

In the food industry, the raw material starch is processed using amylases for the synthesis of diverse products (Pandey et al., 2000; Maarel et al., 2002). Thermophilic amylases have application in the liquefaction of gelatinized starch and saccharification. Though bacterial thermostable α-amylases are currently used in industrial processes (Declerck et al., 2003), there is still a need for thermostable amylases active at different pH and temperatures. In search of such enzymes, studies have described thermophilic, psychrophilic and salt tolerant amylases of bacteria belonging to the genera, *Desulfurococcus, Pseudoalteromonas, Pyrococcus, Rhodothermus*, and *Thermococcus* (Duffner et al., 2000; Gomes and Steiner 2004).

Biochemical characterization of amylases from a metagenomic library resulted in the identification of enzymes optimal for corn wet milling process (Richardson et al., 2002). In another metagenomic study, Yun et al., (2004) characterized a multifunctional amylase exhibiting α-amylase, maltogenic amylase, and 1,4-α-glucanotransferase activities. An endo acting amylase from a metagenomic library showing no sequence homology to any known amylases or any amylolytic domain was described by Delavat et al. (2012). This report was especially significant since metagenomic screening provided unconventional genes from extreme environments. A cold active amylase, homologous to α-amylase of clostridia, isolated after metagenomic screen was found to retain over 70% activity at 1°C (Vester et al., 2015).

Proteases, that account for more than 60% of the total worldwide commercial enzymes, have been used in food industry from time immemorial. These enzymes have also been used for extracting proteins and carotenoids from marine sources. Thermophilic alkaline proteases are used to digest extra meat attached to the bones (Chaplin, 2015). Cold active proteases enhance flavors and tenderize meat during refrigeration. They aid in hydrolysing protein turbidity complexes in fruit juices during cold storage. Heat resistant proteases are used to improve nutritional quality and functionality of protein hydrolysates (Synowiecki, 2010). Halophilic proteases have several advantages in fermentation processes (Vidyasagar et al., 2009).

Though several thermostable bacterial and fungal proteases have been characterized (Rahman et al., 1994; Jensen et al., 2002; Merheb-Dini et al., 2010; Kranthi et al., 2012), significant challenges in downstream processing and maintenance of their unusual properties during large-scale production has resulted in levying higher costs for their industrial use. Functional metagenomics has conquered these barriers to some extent by expression of genes for unique enzymes from unusual environment.

Table 1. List of some extremozymes used in food processing industry discovered through functional metagenomics.

Enzyme	Properties	Environment	References
α-amylase	Active in low pH, thermostable, optimal for the corn wet milling process	Deep sea and acid soil	Richardson et al. (2002)
α-amylase	Enzyme exhibiting mixed properties of several different amylases, such as α-amylase, maltogenic amylase (or neopullulanase), and 4-α-glucanotransferase.	Soil sample from the junction of the ground and the water of Seoho stream, Korea	Yun et al. (2004)
Two serine proteases	Protease DV1and M30 had an optimum pH of 8 and 11 and showed optimal activity at 55°C and 40°C	Gobi and Death Valley deserts	Neveu et al. (2011)
An alkaline serine protease	Alkali stable	Goat skin	Pushpam et al. (2011)
Serine protease	Alkaline in nature	Forest soil	Biver et al. (2013)
Alkaline protease	Saline serine proteases	Saline habitat	Purohit et al. (2013)
Two protease (metallopeptidase)	Unique modular structure and biochemical analyses showed that the optimum pH and temperature of both proteases were 8.0 and 65°C,	Mining shaft Fortuna (Harz Mountains, Germany	Waschkowitz et al. (2009)
Cellulase	Active in broad pH range, from 5 to 8, retained 11.8% of its maximum activity at 0°C and 28.7% at 10°C, 87% activity after a 20 h pre-incubation in 3M NaCl or 4M KCl	Metagenome on brown alga *Ascophyllum nodosum*	Martin et al. (2014)
Cellulases	Halo- and Thermophillic, ionic liquids tolerant	three different hydrolytic communities (an enrichment culture inoculated with an extract of the shipworm Teredo navalis, a biogas plant sample and elephant faeces	Ilmberger et al. (2012)
Endoglucanse	Organic solvent and salt-tolerant enzyme	Mangrove soil	Mai et al. (2014)
Cellulases	Active in the acidic pH range (4.5–6.5), showed high activity at high temperatures (60–70°C), extreme tolerance to 2 M NaCl	anaerobic beer lees converting consortium enriched at thermophilic conditions	Yang et al. (2016)
Cellulase	Novel thermostable genes	Enriched thermophilic cellulose-degrading sludge	Xia et al. (2013)
Endoglucanse	Stable at 40°C for up to11 d and displayed activity at pH 5.5 and 9.0.	Soil	Voget et al. (2006)
β-galactosidases	optimal temperature of 38°C and 54% residual activity at 20°C	Soil	Wang et al. (2010)
β-galactosidases	pH optimum of 6.8 and a temperature optimum of 78°C, but stable in the temperature range of 40–70°C for 60 min.Showed high tolerance to galactose and glucose	Soil samples of Turpan Basin in China	Zhang et al. (2013)
β-galactosidases	Lactose hydrolytic activity at 5°C	Material from ikaite columns	Vester et al. (2014
Pectinase	Retained more that 80% of activity at pH 5–9 and temperature 20–60°C.	Forest soil	Sathya et al. (2014)
Lipases	SMlipA, highly stable in organic solvents, stable in pH and temperature ranging from 6.0–9.0 and 20–60°C, respectively	Forest soil	Khan and Kottur (2012)
Lipases	SMlipD moderately thermostable optimum temperature 50°C, highly resistant to 50% (v/v) organic solvents	Forest soil	Khan et al. (2013)
Esterase	Thermostable familyVII esterase with high stability in organic solvents	Compost	Kang et al. (2011)
Lipase	highly thermostable esterase EstE1 with thermal stability and activity upto 95°C	metagenomic library of mud and sediment mixtures	Rhee et al. (2005)
Lipase	Highly alkaline lipase	Chinese marine sediment metagenome	Peng et al. (2014)
Lipase	Active over a pH range of 5.0–10.0 and was insensitive to divalent cations, moderately thermostable, active between 50 and 60°C	Forest Soil	Faoro et al. (2012)
Lipase	Two thermostable lipases, belongs to novel lipase families, LipS had an optimum temperature at 70°C and LipT at 75°C.	Sand and humus-rich soil	Chow et al. (2012)
Lipase	Unique lipase, loses its secondary structure completely at 35°C, optimal activity at 50°C	Hot springs in Manikaran	Sharma et al. 2012
Fifteen new lipolytic enzyme	Activity range of 0–60°C Two lipase belongs to new family	South China Sea deep sediments	Fu et al. (2011)
Lipase	Cold active	Oil-contaminated Soil, Northern Germany	Elend et al. (2007)
lipase	Optimal activity at acidic pH	Carnivorous plant's pitcher fluid metagenome	Morohoshi et al. (2011)
Phytase	pH optimum of 2.0	Ground water	Tan et al. (2015)
Phytase	Optimal activity at 55°C (pH 5) and exhibited good stability at 5°C within the acidic pH range	Mehsani buffalo rumen	Mootapally et al. (2016)
Phytase	Optimum activity at 37°C but strong activity at high temperature. Displayed longest half-life time at 100°C for 27 min and at 80°C up to 2.1 h	Metagenomes of fungus gardens	Tan et al. (2016)
Xylanases	Alkali and thermostable	Compost-soil samples	Son-Ng et al. (2009)
Xylanases	Synthesize xylooligosaccharides and ferulic acid from wheat straw	A Holstein cattle rumen metagenomic library	Cheng et al. (2012)
Tannase	Halotolerant and moderately thermostable	Cotton field soil	Yao et al. (2011)

Neveu et al. (2011) identified two alkaline serine proteases, optimally active at 55°C and 40°C, from the metagenome of Gobi and Death Valley deserts. Sequence analysis classified the enzymes as subtilisin (S8A). Another serine protease gene cloned from a saline habitat after metagenome screen was active under saline conditions (Purohit and Singh, 2013). The metagenomic libraries from composting soil and mining shaft Fortuna (Harz Mountains, Germany) resulted in metallopeptidase secreting clones, optimally active at pH 8.0 and 65°C, with unique modular structure (Waschkowitz et al., 2009).

Cellulases are a group of enzymes that hydrolyze β 1–4 glycosidic bonds of cellulose to evolve simple sugars. The enzymes are used for coffee processing, extraction of oil and carotenoids from plants, production of vegetable purees, juices, nectars (Bhat, 2000; Cinar, 2005; de Faveri et al., 2008), and in beer and wine making (Bamforth, 2009). Cellulases also find application in animal feed processing to improve nutritive value of forages and digestibility of cereals (Dhiman et al., 2002).

Xia et al. (2013) through metagenomic approach, mined genes of carbohydrases from cellulose-degrading sludge. Their phylogenetic analysis revealed only 50% similarity to the available genes. Functional metagenome analysis of *Ascophyllum nodosum* microbial consortia (Martin et al., 2014) resulted in the identification of an esterase and β-glucosidase displaying only 42% sequence similaity to the known cellulase genes. The library also detected a cold active halophyllic cellulase with activity over a broad pH range. Screening for functional cellulase genes from mangrove soil metagenome identified a salt tolerant endoglucanase coding sequence containing catalytic domain of GH 44 family stable in organic solvent (Mai et al., 2014). Similar results from metagenomes were also described by Ilmberger et al. (2012) and Yang et al. (2016).

β-Galactosidases (EC 3.2.1.23), that hydrolyze lactose of milk to glucose and galactose, have relevance for the production of lactose free or low-lactose milk and dairy products. In the functional food industry, the transgalactosylation reaction of β-Galactosidases is used to transform lactose to prebiotic galacto-oligosaccharides (Neri et al., 2008). Most of the commercially available β-galactosidases are produced from *Aspergillus* and some yeasts (Husain, 2010). Potential β-Galactosidases for milk industry from metagenomic sources have also been described by Wang et al. (2010, 2012a), Zhang et al. (2013) and Vester et al. (2014).

Pectinases encompassing pectolyases, pectozymes, and polygalactouranase are classified under GH28 glycoside hydrolases family. They are widely used in fruit juice industry and in combination with plant cell wall degrading enzymes, for the production of pepper and cardamom essential oils (Chandran et al., 2012). Recent interest for the production of low methoxy pectins and pectic oligosaccharides of defined chain length for use as functional foods (Khan et al., 2013a) has resulted in search for newer enzymes. Singh et al., (2012) reported a thermostable polygalacturonase from soil metagenome possessing novel biochemical properties. Functional genes of polygalacturonase with similar property were also isolated by Sathya et al., (2014) from a soil metagenomic library.

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are ubiquitous enzymes that hydrolyze triacylglycerols to glycerol and fatty acids. These are produced from Plants, animals, and microorganisms. Prominently used strains of microorganisms for the commercial production of lipase are, *Candida, Pseudomonas, Mucor, Rhizopus, Geotrichum, Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia*, and *Chromobacterium* (White and White, 1997).

Lipases are the most versatile and multifunctional enzymes. They catalyse polymerization, esterification, interesterification, transesterification reactions and also hydrolyze carboxylic ester. Their hydrolytic and/or chiral properties have ben exploited by food industries for cheese ripening, hydrolysis of milk fat, and to improve aroma of beverages (Bárcenas et al., 2003). Lipases are used to enrich polyunsaturated fatty acids in oils and improve DHA contents in tuna oil (Moore and McNeill, 1996; Wang et al., 2012a, b; Chaurasia et al., 2016). Rahmatullah et al. (1994) used selective hydrolysis approach to enrich gamma linolenic acid contents of borage oil. Ability of whole cell lipases of *Rhizopus chinesis* to synthesise short-chain fatty acids (Xu et al., 2002) and enzymatic esterification using lipase for the synthesis of phytosterols and their fatty acid esters have attracted functional food industries (Negishi et al., 2003; Zeng et al., 2015).

Cocoa butter is an important fat for making chocolates. It consists of a specific structure of triaceylglycerol (TAG) to lower the melting temperature to that of the body. Lipase is used to produce a similar fat from vegetable oils as cocoa butter substitute. In infant foods, 1,3-oleoyl-2-palmitoyl glycerol acid is a substitute for human milk fat. It is made by transesterification reaction of 2-palmitoyl TAGs with oleic acid by lipases (Roland et al., 2003; Mohamed 2012). Liapses are also used to transform butterfat into low-trans spreadable fat by interesterification reaction (Jung-Ah et al., 2010).

Lipases act in aqueous and nonaqueous systems. They also catalyze reactions in supercritical fluids. The polymerization and transesterification reactions are generally carried out in harsh conditions at extremes of temperature, pH, and organic solvents with chemo-, regio-, and enantio-selectivity. Enzymatic reactions in organic media increase the solubility of substrate, shifts the equilibrium toward synthesis and makes product recovery easy (Sharma and Kanwar, 2014).

A thermostable esterase that showed activity up to 95°C was characterized from the metagenomic library of mud and sediment mixtures (Rhee et al., 2005). Morohoshi et al. (2011) isolated two lipases with optimal activity at acidic pH, useful for dairy and food industries, from the carnivorous pitcher plant fluid. Expression of South China deep sea metagenes recognized fifteen new lipolytic genes active even at 0°C (Fu et al., 2011). According to the authors, sequence comparison classified two of them to a new family. Metagenomic library search for lipases in the Indian hot springs at Manikaran discovered an enzyme with unusual properties (Sharma et al., 2012). Thermo- and alkali-stable lipases with novel properties and high specificity for buttermilk fat esters were discovered from a metagenomic library constructed from Chinese marine sediment (Chow et al., 2012; Faoro et al., 2012). The enzyme produced palmitic and myristic acids and gave a distinctive flavor when treated with butter. It also maintained the cheesy flavor of the short-chain fatty acids. Functional metagenome analysis of a forest soils resulted in the characterization of lipases with high tolerance to isopropanol, ethandiol, DMSO, methanol, and xylene (Khan and Kottur, 2012; Khan et al., 2013b).

Phytase is an enzyme that has the ability to liberate phosphate and mineral residues from phytic acid. In most of the cereals, grains, wheat and plant based food, phytic acids are formed during the maturation process which chelate di- and tri-valent minerals and decrease their bioavailability. Phytic acids also reduce protein digestibility and availability by forming insoluble complexes resistant to enzymatic hydrolysis (Dersjant et al., 2015). Phytases makes phosphorous and essential minerals available by hydrolysing phytic acid.

Tan et al. (2015) overexpressed a phytase gene from the ground water metagenome in *Escherichia coli*. Even though the cloned enzyme was classified as a histidine acid phosphatase, its optimal activity at pH 2.0 differentiated it from the phosphatases of this family. Mootapally et al. (2016) identified a full-length phytase gene from the Mehsani buffalo rumen metagenome and expressed it in *E. coli*. The enzyme showed optimal activity at 55°C and pH 5.0. Tan et al. (2016) screened out and overexpressed 11 putative HAP phytase genes from metagenomes of fungus garden. One phytase from the metagenome of south pine beetle fungus garden showed optimum activity at 37°C and retained most of the activity at high temperatures. The enzyme exhibited half life time of 27 min at 100°C and 2.1 h up to 80°C.

Tannases catalyse the hydrolysis of gallic acid esters and hydrolysable tannins. In food industries, they are used to remove tannins from green tea infusion and for the preparation of instant tea. Yao et al. (2011, 2014) characterized a tannase from metagenomic library and used it to remove tannins from green tea infusions.

Xylanases used in bakery and feed additive industries have found interest in the functional food industry for the synthesis of xylo oligosaccharides and ferulic acid. Different extremophilic xylaneses with unique properties have been reported from diverse metagenomic libraries (Hu et al., 2008; Son-Ng et al., 2009; Cheng et al., 2012; Ziemer 2013).

Conclusion

Functional metagenomic databases detailing overabundance of enzymes with varied properties in nature has given an impetus to the demands of the evolving food-processing industries. Easy accessibility to natural extremozymes, through metagenomic mining approach, provides food industries avenues for environmentally safe processing methods and enhanced performance. Further advancements in metagenomic methodology, use of more suitable expression vectors, host strains, screening and selection methods with faster sequencing, computational modeling and bioinformatics, will together help to fill gaps between genes of unknown functions and function assignation.

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