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Review

High pressure enhancement of enzymes: A review

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ABSTRACT

While most current applications of high pressure (HP) are for inactivating deleterious enzymes, there is evidence that high pressure can induce stabilization and activation of some enzymes. Various other strategies have been employed to enhance enzyme stability, including; genetic engineering, immobilization, and operating in non-aqueous media. While each of these strategies has provided varying degrees of stability or activity enhancement, the application of high pressure may be a complementary, synergistic, or an additive enzyme enhancement technique. Over 25 enzymes that have exhibited high pressure stabilization and/or activation were compiled. Each enzyme discussed responds differently to high pressure depending on the pressure range, temperature, source, solvent or media, and substrate. Possible mechanisms for pressure-induced stabilization and activation are discussed and compared with current enzyme enhancement techniques. The compiled evidence of high pressure enzyme enhancement in this review indicates that pressure is an effective reaction parameter with potential for greater utilization in enzyme catalysis.

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Contents

1. Introduction 332
   1.1. History 332
   1.2. Overview of current enhancement techniques/technologies 332
   1.3. Mechanisms of pressure-induced stabilization 333
   1.4. Mechanisms of pressure-induced activation 333
2. Enzymes enhanced by high pressure 335
   2.1. Oxidoreductases (EC 1) 335
      2.1.1. Dehydrogenase (EC 1.1.1.1) 335
      2.1.2. Hydrogenase (EC 1.1.1.2) 335
      2.1.3. Peroxidase (1.11.1.1-16) 335
      2.1.4. Lipoxygenase (EC 1.13.11.12) 335
      2.1.5. Polyphenoloxidase (EC 1.14.18.1) 335
   2.2. Transferases (EC 2) 339
      2.2.1. Polymerase (2.7.7.7) 339
   2.3. Hydrolases (EC 3) 339
      2.3.1. Pectin methylesterase (EC 3.1.1.11) 339
      2.3.2. Lipase (EC 3.1.1.3) 339
      2.3.3. Acetylcholinesterase (EC 3.1.1.7) 340
      2.3.4. Polygalacturonase (EC 3.2.1.15) 341
      2.3.5. α-Amylase (EC 3.2.1.1) 341
      2.3.6. β-Amylase (EC 3.2.1.2) 341

Abbreviations: HP, high pressure; SC-CO_2, supercritical carbon dioxide; BPTI, bovine pancreatic trypsin inhibitor; NMR, nuclear magnetic resonance; GAPDHs, glyceraldehydes-3-phosphate dehydrogenases; GDH, glutamate dehydrogenases; YADH, yeast alcohol dehydrogenase; TBADH, *Thermoanaerobium brockii* alcohol dehydrogenase; POD, peroxidases; HPT, high pressure treatment; LOX, lipoxygenase; PPO, polyphenoloxidases; PCR, polymerase chain reaction; PME, pectin methylesterase; SCSF5, sulfur hexafluoride; CALB, *Candida antarctica* lipase B; CALA, *Candida antarctica* lipase A; E_a, activation energy; SEM, scanning electron microscope; PG, polygalacturonase; GRAS, generally recognized as safe; CT, α-chymotrypsin; BLG, β-lactoglobulin.

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2.3.7. β-Glucanase (EC 3.2.1.2) ... 341
2.3.8. Glucoamylase (EC 3.2.1.3) ... 341
2.3.9. Lysozyme (EC 3.2.1.17) ... 341
2.3.10. β-Glucosidase (EC 3.2.1.21) ... 341
2.3.11. β-Galactosidase (EC 3.2.1.23) ... 342
2.3.12. Invertase (EC 3.2.1.26) ... 342
2.3.13. Naringinase (EC 3.2.1.40) ... 342
2.3.14. Myrosinase (EC 3.2.3.1) ... 342
2.3.15. α-Chymotrypsin (EC 3.4.21.1) ... 342
2.3.16. Thermolysin (EC 3.4.24.27) ... 343
2.3.17. Pepsin (EC 3.4.23.1) ... 343
2.3.18. Unidentified protease ... 343
2.3.19. Pyrophosphatase (3.6.1.1) ... 343
2.4. Lyases (EC 4) ... 343
2.4.1. Aspartase (4.3.1.1) ... 343
3. Conclusions ... 343
References ... 344

1. Introduction

1.1. History

In June of 2007, the scientific community celebrated the 30th anniversary of the discovery of volcanic hot vents at the Galapagos Rift at the bottom of the Pacific Ocean. The most notable finding was not the vent itself, but the abundance of entire ecosystems at these extreme conditions (up to 120 MPa, 2–100 °C, absence of sunlight, and scarce supply of organic nutrients) which led to a fundamental change in our understanding of life on Earth. While the presence of barophilic (also termed piezophilic) bacteria was first hypothesized in 1949 [1] and later discovered in various trenches during the Galathea Deep-Sea Expedition of 1950–1952 [2], this was the first discovery of multi-cellular life in the deep sea. These discoveries spawned a global quest for life from extreme conditions. This search has resulted in the discovery of over 550 species thriving at various extreme pressures and temperatures and new species continue to be discovered at a rate of about two per month in environments as extreme as 470 °C [3]. Discoveries of both thermostable and piezostable microorganisms and their enzymes have enabled the field of high pressure enzyme modification to progress dramatically by uncovering several high pressure-induced enzyme applications [4] and modifications.

Since the discovery of microorganisms in deep-sea sediments [5,6] in the 1880s, high pressure (HP) and its application to bioscience was only briefly demonstrated in the early 1900s [7]. While HP was effective at inactivating various deleterious enzymes [8–14], its effect on enzyme stabilization and activation was explored less extensively [4,15–19]. Enzymes from mesophilic and extremeophilic microorganisms were stabilized by HP [20,21]. Extremophiles are defined as organisms that have evolved to exist in extreme environments and fall into at least 15 classes of which the most common are thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles, among others [22]. Within the context of this review, thermophilic organisms will be subcategorized into moderate thermophiles (45–65 °C), thermophiles (65–85 °C), hyper-thermophiles (85–100 °C), and extreme thermophiles (>100 °C).

Extremely heat-tolerant (stable up to 150 °C) enzymes have potential application in organic media [22–24]. However, the boiling point of most organic solvents is well below 100 °C. As pressure is raised, the boiling point of organic solvents increases allowing studies above conventional temperatures at 0.1 MPa [25]. The ability to operate with organic or volatile solvents at temperatures above their conventional boiling point is an important yet often overlooked advantage of HP.

The cost of high pressure processing (HPP) decreased over the last decade and has become more widely implemented particularly in the food industry. In 2007 there were about 120 HPP industrial installations operating worldwide [26] with 80% of their equipment installed since 2000. HPP food became a two billion dollar global market and is expected to comprise 450 million pounds/year in 2008 [27]. As demand for HPP equipment grows, innovation is expected to continue to reduce capital and operating costs [26]. Although HPP of bulk foods is currently more widespread, the much higher profit-margin sector of enzyme-catalyzed synthesis of high added-value products such as pharmacological peptides, antibiotics, carbohydrates, and food flavors has greater potential for adopting HPP.

The effects of HP on proteins and enzyme inactivation were discussed and reviewed over the past 20 years [16,18,19,28–31]. Theories of pressure effects on enzymes and the activation volumes of several enzymes were compiled in the early 1980s [32]. However, the application of HP to enhance enzyme catalysis has not been extensively explored or comprehensively reviewed.

1.2. Overview of current enhancement techniques/technologies

The major drawback to the extensive use of many enzymes compared to chemical catalysts is their relatively low stability in their native state and their often prohibitive cost [33]. There is a great interest in developing competitive biocatalysts for industrial applications by improvement of their activity, stability, and re-usage capacity. Such improvements have been approached by chemical, physical, or genetic modification of the native enzyme. The effects of immobilization on activity, stability, and even selectivity of some enzymes have been well documented [34–36]. For example, immobilization of lipase can improve activity by shifting the equilibrium between open and closed form towards the open, more active form [35], chymotrypsin can be stabilized by a factor of 60,000 by immobilization [37], and the enantiomeric ratio of lipase products may be changed from 1 to almost 100 by using different immobilization preparations [35]. Furthermore, modification of the reaction environment was explored using alternative solvents including organic solvents such as hexane [38–41], solvent-free systems [42–48], dense or supercritical gases such as supercritical carbon dioxide (SC-CO_2) [49–51], and more recently ionic liquids [52–54]. For example, stability and selectivity of lipase in ionic liquid increased 2.7-fold and by 2%, respectively, when compared to in hexane [54]. Likewise, lipase activity dramatically increased in SC-CO_2 with a negative activation volume of 1340 $cm^3\,mol^{-1}$ [55]. Enzymes suitable for use in industrial biocatalysis may require application of a combination of these improvement methods.

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1.3. Mechanisms of pressure-induced stabilization

Although the mechanisms of HP-induced stabilization has yet to be fully elucidated, it appears that intramolecular interactions, hydration of charged groups, disruption of bound water, and stabilization of hydrogen bonds may all play a role. The pressure effects on intra- and intermolecular interactions within proteins were reviewed in depth [56].

According to Mozhaev et al. [57], a possible explanation of pressure-induced stabilization of enzymes against thermal inactivation rests in opposing effects of pressure and temperature on the ability of protein functional groups to interact with water. The promotion of charged species in aqueous medium is favored by a high-pressure environment, because the electrostriction of water around the charges decreases the molar volume of water [58]. This means that an increase in pressure will weaken the electrostatic or coulombic interactions. Hydration of charged groups by water molecules is strengthened by pressure and weakened at high temperature [18,28]. Evidence of this phenomenon was presented by Kitchen et al. [59] who completed a molecular dynamics simulation of bovine pancreatic trypsin inhibitor (BPTI) in solution at HP and found large pressure-induced changes in the structure of the hydration shell, the shell appeared more ordered at HP, and that HP-induced greatest ordering for non-polar surface groups. Enzymatic catalysis in organic media also depends on enzyme hydration state which is greatly influenced by pressure [60]. Mozhaev et al. [57] hypothesized that, at an initial step of thermal inactivation, a protein loses a number of essential water molecules, and this loss may give rise to structural rearrangements in a protein. HP may hamper this process by favoring hydration of both charged and non-polar groups. In other words, application of high hydrostatic pressure fortifies the protein hydration shell thus preventing thermal inactivation.

Hydrogen bonding may also be slightly strengthened by an increase in pressure, because of, according to Kunugi [58], the decrease in the inter-atomic distance leading to a smaller molecular size. This phenomenon was initially based on the negative activation volumes associated with hydrogen bond formation in organic compounds such as formic acid, ε-caprolactam, phenol, and poly-L-lysine [61]. Later, the lengths of existing hydrogen bonds within proteins have been observed to be shorter under HP as first detected by nuclear magnetic resonance (NMR) [62,63] and then by low-frequency Raman spectroscopy [64].

Hydrophobic interactions also greatly affect enzymatic conformation. The relationship between HP and temperature effects on hydrophobic interactions has yet to be fully understood [56]. It was suggested that hydrophobic interactions are strengthened by HP because the hydration of non-polar surface molecules is not favored due to the corresponding volume increases [30,65]. Evidence supporting this hypothesis was provided by Hei and Clark [66] by demonstrating how the stability of several glyceraldehydes-3-phosphate dehydrogenases (GAPDHs) with varying hydrophobicity of their "s-loops" (structural motifs consisting of residues 178–201 which are an important determinant of GAPDH thermal stability) is correlated to pressure-induced stabilization. Further evidence was provided by Mei et al. [67] who studied the role of hydrophobic residues on pressure-induced stabilization. Through site-directed mutagenesis, Mei et al. [67] modified the hydrophobicity of azurin and pressure-induced stabilization increased with increasing hydrophobicity. However, this was in disagreement with Mozhaev et al. [18] who said "it is generally accepted that formation of hydrophobic contacts proceeds with positive $\Delta V^{\neq}$ and is therefore disfavored by pressure" and cited the formation of hydrophobic interactions when transferring methane from a non-polar solvent to water, the micelle formation by surfactants, and a dimerization of carboxylic acid [68,69] which all resulted in a positive $\Delta V^{\neq}$. However the dimerization of formic, acetic, propionic, and n-butyric acid all had negative $\Delta V^{\neq}$ [69].

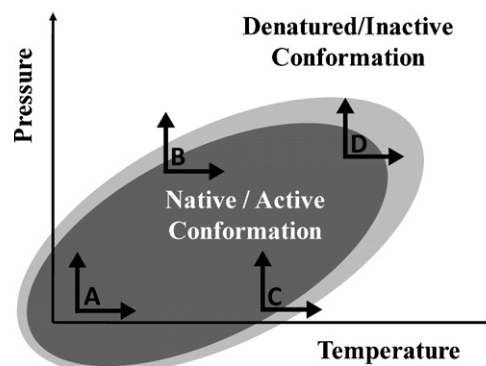

Fig. 1. Theoretical generic elliptical pressure—temperature diagram with native and denatured protein regions inside and outside of the ellipse respectively, separated by a zone of reversible denaturation.

Pressure and heat are generally thought to be antagonistic factors in molecular terms (from the principle of microscopic ordering, an increase in pressure at constant temperature leads to an ordering of molecules or a decrease in the entropy of the system) and more recently with regards to enzyme conformation within defined pressure and temperature regions [17–19,28]. This defined region must be determined experimentally as the complexity of using an enzyme catalyst prevents accurate modeling without pre-determination of enzyme stability and activity at various pressure–temperature combinations. The relationship between pressure and temperature effects on enzyme conformation often results in an elliptical pressure versus temperature diagram as shown in Fig. 1 with native and denatured protein regions inside and outside of the ellipse, respectively, separated by a zone of reversible denaturation with the contour line representing where the equilibrium concentrations of native and denatured forms of the enzyme are equal. A comprehensive review of pressure–temperature phase diagrams of protein unfolding was compiled by Smeller [70]. Increases in temperature and pressure can have both, stabilizing or denaturing effects on enzymes depending on the initial conditions, as represented by points A–D in the elliptical diagram and the magnitude of the increase. For example, moderately increasing temperature and pressure from point A stabilizes the enzyme. However, in point D, increases in both, temperature or pressure destabilizes the enzyme. In the case of point B an increase in pressure denatures the enzyme but an increase in temperature stabilizes it and vice-versa for point C. Such diagrams were first shown by Hawley [71] and later reviewed and discussed by several others [16,17,70,72]. However, they are typically representations of the rate of denaturation which does not always directly correlate to inactivation. These diagrams have been used to describe pressure stability of chymotrypsin [71], polyphenoloxidase [73,74], pectin methylesterase [75–77], myrosinase [78], naringinase [79], β-glucanase [80], α- and β-amylase [81,82], and glucoamylase [83].

1.4. Mechanisms of pressure-induced activation

Explanations for pressure-induced changes in the rate of enzyme-catalyzed reactions can be classified into: (1) direct changes in the structure of an enzyme, (2) changes in the reaction mechanisms; for example, a change in the rate-limiting step, and (3) changes in the substrate or solvent physical properties (e.g. pH, density, viscosity, phase) that affect enzyme structure or the rate-limiting step.

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Early studies suggested that the activities of monomeric enzymes activity were stimulated, while the activities of multimeric enzymes were inhibited by application of high hydrostatic pressure [84]. However, at least 15 dimeric and tetrameric enzymes have been reported to be activated by pressure [32].

It is understood that pressure effects on reaction rates adhere in most cases to Le Châtelier's principle: that elevated pressures favor changes that reduce a systems' overall volume. Thus, by comparing the total volumes of the reactants versus products, of the ground state versus activated state, or of the dissociated versus bound complex, the effect of pressure on reaction rate and equilibrium can be estimated [28] using the Eyring equation (Eq. (1)) [85],

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT} \tag{1}$$

where p is the pressure, T is the absolute temperature, R is the ideal gas constant, $\Delta V^{\neq}$ is the activation volume that represents the dependence of the reaction rate with pressure, and k is the rate constant. After integration and rearrangement, Eq. (1) can be rewritten as Eq. (2),

$$\ln k_0 = -\frac{\Delta V^{\neq}}{RT} \cdot P + \ln k_{P_0} \tag{2}$$

where k_0 is the rate constant at a reference pressure P_0. Thus, any rate with a negative volume change will be shifted towards the more compact state by increasing pressure and any reaction with a positive activation volume will be slowed.

Compared to chemical catalysis, it is more difficult to give a precise physical meaning to $\Delta V^{\neq}$ for enzyme reactions because pressure affects several factors including: enzyme conformation, enzyme solvation (interaction with surrounding medium, other proteins, water, ions, etc.), chemical equilibrium, and changes in immobilization support or immobilization bonding. Depending on the reaction or conformational changes of the enzyme (which are dependent on the temperature–pressure range), activation volume can be positive, negative, or negligible. Typically $\Delta V^{\neq}$ ranges from $-70\,\text{cm}^{-3}\,\text{mol}^{-1}$ to $+60\,\text{cm}^{-3}\,\text{mol}^{-1}$, with most having a magnitude of less than $30\,\text{cm}^{-3}\,\text{mol}^{-1}$ [28].

Separating the effects of HP on the rate constant from those on enzyme conformation and thus on the availability of active enzyme at elevated pressures is difficult because they both occur simultaneously affecting the rate of catalysis. The simple one-substrate Michaelis–Menten mechanism and equation are shown in Eqs. (3) and (4).

$$E_n + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} E_nS \overset{k_2}{\rightarrow} E + P \tag{3}$$

$$v = \frac{v_{\max}[S]}{K_M + [S]} = \frac{k_2[E_T][S]}{K_M + [S]} \tag{4}$$

where E_T is the total enzyme concentration ($[E]+[ES]$), $[S]$ is the substrate concentration, v is the rate of reaction, and k_1, k_{-1}, and k_2 represent rate constants and K_M is Michaelis–Menten constant. Large increases in pressure, temperature, or addition of a denaturing agent causes the native enzyme (E_n) to first become reversibly (E_r) and then irreversibly (E_d) inactivated as described in Eq. (5). However, moderately increasing pressure can produce a more active enzyme (E_{act}) as shown in Eq. (6) which accounts for pressure-induced activation.

$$E_n \leftrightarrow E_r \rightarrow E_d \tag{5}$$

$$E_n \leftrightarrow E_{\text{act}} \leftrightarrow E_r \rightarrow E_d \tag{6}$$

The rate constant is also affected by temperature and generally expressed by the Arrhenius equation (Eq. (7)).

$$\ln k = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln k_{T_0} \tag{7}$$

where E_a is the activation energy and k_{T_0} is the rate constant at a reference temperature T_0. Both temperature and pressure may be complementary to activation at a range of temperatures and pressures; however, it can also hinder the reaction rate by inactivating the enzyme. The combined effect of pressure and temperature is currently impossible to predict without some preliminary data,

In the past 25 years, enzyme catalysis in organic media (mainly n-hexane) was the focus of many studies and reviews [24,39,40,86–93]. The pressure dependence of the solubility parameter and molar volume of organic solvents was recently described at pressures up to 300 MPa at 30 °C [94]. Pressure monotonically increased the solubility parameter (∼20%) and decreased the molar volume (16%) of many of the commonly used organic solvents. Use of non-polar solvents results in better esterification (i.e. higher conversion rates, increased stability, etc.) as they preserved the catalytic activity without disturbing the aqueous mono-layer of the enzyme [95]. The pressure effect on increasing polarity of organic (non-polar) solvents may hinder esterification as product formation is favored in less polar solvents. However, the flexibility of some enzymes is increased with increasing polarity of the organic solvent [96]. Therefore, pressure may increase conformational flexibility, thus improving reaction rates as conformational flexibility is required for activation of enzymes that show interfacial activation in the presence of substrate.

Activity of lipase [41,97], thermolysin [98], esterase (from *Bacillus licheniformis*) [99], chymotrypsin [100,101], xanthine oxidase [102,103], horseradish peroxidase [104], polyphenol oxidase [104], subtilisin [101], protease (from *Bacillus subtilis*) [101], and alcohol dehydrogenase [105] are active in organic or non-aqueous media. Enzyme activity in organic solvents can be correlated with the solvent polarity [40,41,106]. However it has also been reported that activity might be affected by the solvent without correlation to the polarity [107,108]. It was suggested that enzymes that are active in organic solvents retain their native structure upon transfer from water to organic solvents [101]. This is evidenced by the phenomenon of pH memory discussed by Klibanov [104] which suggests the presence of trapped active sites in organic solvents [92,101] and the observation that the addition of strong acids to lipase in hexane does not appreciably inactivate the enzyme [109]. However, it is essential to have small amounts of water to maintain stability and flexibility of lipases in organic solvent. Thus, enzyme-bound water is essential for catalysis and serves as a "lubricant" for the enzyme [92,101]. In contrast, fully dry enzymes are inactive and enzymes in organic solvents with high amounts of water show denaturation [92,101]. Therefore, it is reasonable to hypothesize that pressure-enhanced activity of enzymes in low or non-aqueous solvents may be partially attributed to the pressure-induced hydration of charged groups.

Solvent compression at HP increases substrate molar concentration producing an increase in reaction rate. Solvent compression also results in increased viscosity, which in results in a decrease in reaction rate particularly in heterogeneous systems. Because most enzymes utilized in organic media are immobilized (due to limited solubility), viscosity effects may be significant unless adequate turbulence is provided.

The objective of this paper is to compile and critically present reports of HP enzyme enhancement as an effective reaction parameter with potential for greater utilization in enzyme catalysis.

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2. Enzymes enhanced by high pressure

Discussion of the effects of pressure on enzyme stability and activity is organized by enzyme commission (EC) number. Table 1 provides an abbreviated list of enzymes that are enhanced by elevated pressure and includes source, reaction conditions and, relevant results. Not all enzymes discussed in the text are included in Table 1 for brevity.

2.1. Oxidoreductases (EC 1)

2.1.1. Dehydrogenase (EC 1.1.1.1)

Dehydrogenases and hydrogenases are widely distributed in nature and have been found in many microorganisms, plants, and animals and are the key enzyme involved in the metabolism of H_2. Studies in the 1960s with malic dehydrogenase from *Bacillus stearothermophilus* had no activity at 101 °C from 0.1 to 70 MPa. However, activity was induced above 70 MPa with optimal activity at 130 MPa at 101 °C [110]. This relative activation was attributed to pressure counteracting the increased volume associated with enzyme denaturation. HP effects on the oxidation of benzyl alcohol by yeast alcohol dehydrogenase were measured by substrate capture. Moderate pressure increased the rate of capture of benzyl alcohol 4-fold at 150 MPa and was attributed to activating the hydride transfer step [111].

Pressures up to 75.9 MPa stabilized two glutamate dehydrogenases (GDH) from the hypothermophile *Pyrococcus furiosus* and a recombinant GDH mutant [112]. The stabilizing effects of pressure increased with temperature reaching 36-fold for recombinant GDH at 105 °C and 75.9 MPa. Likewise, GDH from the liver of the Antarctic fish *Chaeonocephalus aceratus* remained active up to 140 MPa and had slightly increased activity from 2 to 100 MPa compared to 0.1 MPa, although reaction rate decreased with pressure above 100 MPa [113]. Fish GDH was rapidly inactivated above 200 MPa while bovine GDH was inactivated slowly in the range of 220–290 MPa [113,114].

Pressure inhibited the tetrameric mesophilic yeast alcohol dehydrogenase (YADH), whereas the thermostable *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) was activated by pressure up to 100 MPa [115]. These results supported the hypothesis that HP can stabilize enzymes from thermophiles and that pressure stabilization may be related to increased hydophobicity of surface groups typically associated with thermophilic enzymes as discussed earlier in Section 1.3. Furthermore, differences in genetic sequences (Ala-180 and His-229) of dehydrogenases have been discovered between two dehydrogenases from the same organism (*Moritella* sp. strain 2D2) found in vastly different pressure and temperature environments [116,117] and provided further evidence that pressure-induced stabilization or activation may be related to a specific genetic sequence or set of sequences as discussed in Section 1.3.

2.1.2. Hydrogenase (EC 1.1.1.2)

The relationship between thermophilicity and pressure stabilization was explored using partially purified hydrogenases from a mesophile, *Methanococcus maripaludis*; a moderate-thermophile, *Methanococcus thermolithotrophicus*; a hyper-thermophile, *Methanococcus igneus*; and the extreme-thermophile deep-sea methanogen, *Methanococcus jannashii*. HP from 1.0 to 50.7 MPa increased the half-life of the hydrogenase from *M. jannashii* and *M. ignius* at 90 °C 4.8- and 4.5-fold, respectively, while pressure decreased the half-life of *M. thermolithotrophicus* and *M. maripaludis* [66]. Hydrogenase activity from *M. jannashii* more than tripled by an increase from 0.75 to 26 MPa at 86 °C with a corresponding $\Delta V^{\neq}$ equal to $-140 \pm 32\ \mathrm{cm^3\ mol^{-1}}$ [118]. These results further supported the hypothesis that barophilicity increases with thermophilicity.

2.1.3. Peroxidase (1.11.1.1-16)

Peroxidases (POD) constitute a group of thermostable heme proteins that oxidize substrates (monophenols, diphenols, etc.) while utilizing H_2O_2 [119]. Although POD often has a deleterious role in food quality, it can also be used as a catalyst in many industrial applications, including; wastewater treatment, fine chemical biosynthesis, and even paper pulp treatment [120]. While POD is widely used, its main limitations are enzymatic stability and low production yield.

High pressure treatment (HPT) increased activity of strawberry POD by 13% and 1% at 400 MPa when applied for 5 and 10 min, respectively [121]. Similarly, crude extract of mate tea leave POD increased activity 25% after exposure to compressed CO_2 at 30 °C and 7 MPa [119] and was attributed to the opposite effects of pressure and temperature on the ability of the protein functional groups to interact with water. Carrot and apple POD were also quite resistant to pressurization below 900 MPa for 1 min, and activation was observed for treatments at 300–500 MPa [122].

2.1.4. Lipoxygenase (EC 1.13.11.12)

Lipoxygenase (LOX) is a non-heme iron-containing dioxygenase that is found throughout the plant kingdom, especially in legumes [123]. It causes chlorophyll destruction and off-flavor development in frozen vegetables and is, therefore, a target for inactivation. Green pea LOX demonstrated resistance to thermal inactivation above 60 °C under 200 MPa [123]. Likewise, LOX from soybean activity increased 120% after treatment at 200 MPa and 55 °C for 15 min in 30% sucrose solution compared to residual activity at 0.1 MPa [124]. Although LOX is generally considered a deleterious enzyme in foods and a target for inactivation, pressure-induced stabilization or activation provide further evidence towards the role of HP in enzyme enhancement.

2.1.5. Polyphenoloxidase (EC 1.14.18.1)

Polyphenoloxidases (PPO) are a group of copper proteins that catalyze the oxidation of phenolics to quinones that produce brown pigments. They are widely distributed in plants, animals, and microorganisms. The oxidation of phenolic substrates by PPO is a major cause of browning in foods during ripening, handling, storage, and processing. It is estimated that over 50% of losses in fruits occur as a result of browning [125]. However, PPO is vital for the desirable color and flavor generation characteristic of many plant based foods.

Mushroom PPO activity increased 140% after treatment at 400 MPa for 10 min compared to untreated sample; and even after 10 min at 800 MPa about 60% residual activity remained [126]. The enhancement in activity was hypothesized to be from pressure-induced changes in the interactions with other constituents in the extract or from the release of membrane-bound enzymes. Similarly, PPO from pears was activated at 400 MPa with a maximum 5-fold increase activity at 600 MPa and 20 °C for 6 h [127]. Unlike the mushroom extract, PPO from pear showed pressure-induced activation which could not be explained by interactions with other constituents as the PPO was extracted and purified to homogeneity. It was suggested that such activation is a result of a limited conformational change in the enzyme, release of the enzyme from an enzyme–inhibitor complex, or activation via limited proteolysis such as zymogen activation [128]. Asaka et al. [128] concluded that PPO of La France pear fruit in its latent form is activated by a limited conformation change; however, such a mechanism is not known.

Victoria grape must PPO experienced a synergistic effect between pressure and temperature for inactivation at 20–60 °C and 200–800 MPa and antagonistic effects at above 60 °C and

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Table 1
Abbreviated list of enzymes that are stabilized or activated by elevated pressures.

Enzyme (EC class)	Source	Solvent	Pressures tested (MPa)	Temperatures tested (°C)	Optimal conditions	Effects	References
Dehydrogenase (EC 1.1.1.1)	*Pyrococcus furiosus*	Aqueous	0.5, 27.5, 50, 75	103–109	75 MPa 105 °C	Pressure stabilized enzyme with increased temperature (36× at 105 °C at 75 MPa)	[112]
	Thermoanaerobium brockii		0–200	40	100 MPa	Activity was enhanced up to 200 MPa with a maximum at 100 MPa	[115]
	Purchased from Sigma (information not provided)		0.1–250	25	150 MPa	Rate of capture of benzyl alcohol increased with pressure up to 150 (4× increase), then decreased beyond that	[111]
	Bacillus stearothermophilus		0–150	28–101	130 MPa 100 °C	Stabilized at 101 °C by application of 130 MPa	[110]
Hydrogenase (EC 1.1.1.2)	*M. jannaschii*, *M. igneus*, *M. thermolithotrophicus*, *M. maripaluaids*	Aqueous	1.0, 50.7	90	50.7 MPa	Hydrogenase has longer half-life at 50.7 MPa versus 1.0 MPa. 4.8× longer from *M. jannashii*, 4.5× longer from *M. igneus*, 0.3× longer from *M. thermolithotrophicus*, 0.07× longer from *M. maripaludis*	[66]
	M. jannaschii		0.65–0.85 25–26.4	86	25–26.4 MPa 86 °C	Hydrogenase activity at 86 °C more than tripled with increasing pressure from 0.75 to 26 MPa with a corresponding $\Delta V^{\neq}$ equal to $-140 \pm 32\ cm^3\ mol^{-1}$	[118]
Peroxidase (EC 1.1.1.x)	Red raspberry, strawberry	Aqueous	400, 600, 800	18 and 22	400 MPa	Activity increased by 13% and 1% after exposure to 400 MPa for 5 and 10 min respectively	[121]
Lipoxygenase (EC 1.13.11.12)	Soybean	Aqueous	0.1–625	−15 to 70	>200 MPa <60 °C	Resistance to thermal inactivation at temperatures above 60 °C by pressures below 200 MPa	[123]
	Green pea	Aqueous (30% sucrose)	0.1–600	35–90	200 MPa 55 °C	Pressure up to approximately 200 MPa at 55 °C increased activity up to 120% of the residual activity compared to at 0.1 MPa	[124]
Polyphenoloxidase (EC 1.14.18.1)	Avocado	Aqueous	0.1–750	25–77.5	<250 MPa >62.5 °C	Pressure increase causes a decrease in inactivation rate	[74]
	Onion		0–700	25	500 MPa	Activity was increased with increasing pressure up to 500 MPa (activity of 142% versus untreated)	[130]
	Mushroom		0–800	20, 40, 60	400 MPa	Extract exhibited increased activity (140% of untreated) after pressurization at 400 MPa for 10 min	[126]
	Tomato		0.1–800	60–105	300–600 MPa 70 °C	Inactivation rate decreased at pressure between 300 and 600 MPa)	[139]
	Apple		10–90	20	30 MPa	Residual activity at 30 MPa was 110% compared to at 0.1 MPa	[122]
	Grape must		10–800	20–70	<200 MPa >60 °C	At high temperature (>60 °C) and low pressure (200 MPa), pressure increase at constant temperature resulted in a decrease in inactivation rate	[73]
	Red raspberry, strawberry		400, 600, 800	18 and 22	400, 800 MPa	PPO activated 15 and 8% at 400 and 800 MPa respectively after 5 min treatment	[121]
DNA polymerase (2.7.7.7)	*Pyrococcus strain ES4*, *Pyrococcus furiosus*, *Thermus aquaticus*	Aqueous	3, 45, 89	110–112.5	89 MPa	Half-lives increased from 5, 6.9, 5.2 min at 3 MPa to 26, 39, 13 min at 89 MPa for each enzyme respectively	[21]
Pectin methylesterase (EC 3.1.1.11)	Recombinant *Aspergillus aculeatus*	Aqueous	0.1, 200, 400, 700	55	<700 MPa 60 °C	No enzyme inactivation up to 700 MPa. At 200 MPa >60 °C was necessary to cause significant loss of activity	[137]
			0.1–500	25–55	200–300 MPa 50–55 °C	Highest rate of PME-catalyzed pectin de-esterification was obtained when combining pressure 200–300 MPa with temperature 50–55 °C	[136]
			0.1–500	20–60	300 MPa 55 °C	Optimal temperature increased as the pressure increased (from 45 °C at 0.1 MPa to 55 °C at 300–500 MPa)	[135]
	Orange		0.1–900	20–65	<600 MPa 20–65 °C	Antagonistic effect of pressure and temperature on inactivation of heat stable fraction	[77]
	Carrot		0.1–600	20, 40, 60	300–500 MPa >50 °C	Optimal activity registered at 50 °C in combination with 300–500 MPa	[134]

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			100–825	10–65	<300 MPa >50 °C	At lower pressures (300 MPa) and higher temperatures (>50 °C) an antagonistic effect of pressure and heat was observed	[76]
	Tomato		100–500	4, 25, 50	300 MPa 50 °C	PME activity enhanced by treatments at 300–500 MPa and various temperatures. Maximal activity was at 300 MPa and 50 °C and 1.7× the control	[138]
Lipase (EC 3.1.1.3)	*Rhizomucor miehei*	SC-CO_2	0–50	50	10 MPa 50 °C	Catalytic efficiency improved up to 10 MPa and decreased above 10 MPa	[168]
		Polyhydric alcohols	0.1–500	25 or 50, 55	50–350 MPa	At 50–350 MPa enzyme maintained 60% and 20% initial activity at 50 °C and 55 °C respectively compared to <5% at 0.1 MPa	[170]
		SC-CO_2 n-butane n-propane mixture of butane/propane	6.5–450	40–80	8 MPa 60 °C	Almost no loss of activity in all gases. Pressure up to 40 MPa increased reaction rate at 40 °C. SC-CO_2 is the better reaction medium due to enzyme deactivation in n-butane	[51]
		Aqueous	0–600	40, 50, 55, 60	100–300 MPa 55 °C	Maximum protective effect of pressure decreases as temperature increases. Increased stability as pressure increases, more dramatic effect at 50 and 55 °C	[169]
	Mucor miehei	SC-CO_2	12.2–18	36–50	12.2 MPa 36 °C	Optimized conditions at 12.2 MPa, 36 °C with butanol (1.2 M) as substrate	[157]
	Candida antarctica		8, 9.5, 12, 15	40, 50, 60	8 MPa/60 °C	84× increased activity in SC-CO_2 versus organic solvents	[158]
	Porcine pancreas	Near-critical propane	30	40	30 MPa 40 °C	4× improved activity after incubation as compared to non-solvent system	[160]
	Rhizopus javanicus		30	40	30 MPa 40 °C	9× improved activity after incubation as compared to non-solvent system	[160]
	Yarrowia lipolytica	SC-CO_2 compressed butane and propane	0.1–28	35–75	N/A	YLL lipase better than lipozyme, but inferior to Novozyme 435. Propane and n-butane are superior to SC-CO_2	[166]
	Pseudomonas cepacea	SC-CO_2 $SCSF_6$ (supercritical sulfur hexafluoride)	4.5–25	50	10 MPa 50 °C	$SCSF_6$ had higher initial rate than SC-CO_2. Activity increased as pressure approached critical pressures (∼10 MPa)	[55]
	Crude hog pancreas	SC-CO_2	6.8	30–60	45 °C at supercritical conditions	The optimum temperature of reaction was higher under supercritical conditions (45 °C) versus solvent free (35–40 °C)	[231]
Acetylcholinesterase (EC 3.1.1.7)	Human	Aqueous buffer	0–14	42	14 MPa	Activity increased at 14 MPa when compared to 0.1 MPa. Linear protection of activity with increasing pressure at denaturing temperature (42 °C)	[178]
Polygalacturonase (EC 3.2.1.15)	Tomato	Aqueous	300–600	5–50		5% enzyme fraction was not affected by prolonged pressurization at all levels	[180]
α-Amylase (EC 3.2.1.1)	Barley malt	Aqueous	0.1–800	30–75	152 MPa 64 °C	Maximum rate is at 152 MPa and 64 °C yielding ∼25% higher conversion as compared to maximum at ambient pressure and 59 °C	[81]
β-Amylase (EC 3.2.1.2)	Barley malt	Aqueous	0–700	20–70	106 MPa 63 °C	Highest depolymerization of starch rate was found at 100 MPa and 62 °C, yielding 15% more compared to optimum conditions at ambient pressure	[82]
β-Glucanase (EC 3.2.1.2)	Barley malt	Aqueous	0.1–900	30–75	400 MPa	Pressure increases thermostability of β-glucanase, highest at 400 MPa. Enzymatic catalysis was found to be delayed by pressure up to 600 MPa	[80]
Glucoamylase (EC 3.2.1.3)	*Aspergillus niger*	Aqueous	0.1–1400	40–90	318 MPa 84 °C	The pressure liable form of glucoamylase was inactivated quickly at pressures above 850 MPa whereas the stable isoform was slow to inactivate up to 1400 MPa at 50 °C	[83]
β-Glucosidase (EC 3.2.1.21)	Red raspberry, strawberry	Aqueous	400, 600, 800	18 and 22	400 MPa	Highest increase of activity (76%) at 400 MPa after exposure for 15 min	[121]
β-Galactosidase (3.2.1.23)	*Aspergillus oryzae*, *Kluyveromyces lactis*, *Escherichia coli*	Aqueous	0–400	55	50–400 MPa	Moderate pressures exerted a protective effect against thermal inactivation. For enzyme from *E. coli*, 80% activity at 100 MPa versus 60% at 0.1 MPa, from *K. lactis* 5× higher activity at 100 MPa versus 0.1 MPa, from *A. oryzae* 100% activity at 250 MPa versus 0% at 0.1 MPa	[200]

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Table 1
(*Continued*)

Enzyme (EC class)	Source	Solvent	Pressures tested (MPa)	Temperatures tested (°C)	Optimal conditions	Effects	References
Invertase (3.2.1.26)	*Saccharomyces cerevisiae*	Aqueous	0–350	60	200 MPa	Half-life increased until pressure reached 200 MPa. Enzyme protected by pressure from 50 to 200 MPa	[169]
Naringinase (3.2.1.40)	*Penicillium decumbens*	Aqueous (acetate buffer)	0.1–200	25–60	160 MPa 35–40 °C	65% higher rate and 70% lower K_M at 160 MPa versus 0.1 MPa	[79,211–213]
Myrosinase (EC 3.2.3.1)	Broccoli	Aqueous	0.1–450	30–60	350 MPa 35 °C	Protective effect of pressure against thermal inactivation	[214]
			100–600	10–60	200 MPa >50 °C	Antagonistic effect of pressure and temperature on inactivation	[78]
α-Chymotrypsin (EC 3.4.21.1)	Bovine pancreas	Aqueous w/wo 40% glycerol	0.1–500	20–60	40% glycerol 180 MPa 65 °C	Increased pressure increased rate 6.5× at 470 MPa at 20 °C. CT is instantly inactivated at 55 °C at 0.1 MPa, but retains activity at 180 MPa. Activity of CT in 40% glycerol is 7× higher than in aqueous buffer	[57]
		Reversed micelles in octane	0.1–200	10, 20, 30	N/A	Observed a $\Delta V^{\neq} = -13 \pm 2\,cm^3\,mol^{-1}$ while maintaining activity up to 200 MPa	[60]
	Not-given commercial	Aqueous	480–760	25	N/A	No pressure-induced activation, but remarkable stability up to 760 MPa after 5 min	[215]
			0.1–500	20–55	200 MPa 45 °C	Increasing pressure increased CT hydrolysis of *p*-nitrophenyl acetate at temperatures ranging from 10 to 65 °C with maximal activity at 45 °C and 200 MPa	[69]
Thermolysin (3.4.24.27)	Protease type × from *Bacillus thermoproteoyticus rokko*	Aqueous (tris buffer)	0.1–350	25	<200 MPa	Rate increase of 22-fold at 200 MPa versus 0.1 MPa	[218]
Pepsin (3.4.23.1)	Porcine stomach mucosa	Aqueous (tris buffer)	0.1–350	25	<200 MPa	270-fold rate increase at 300 MPa versus 0.1 MPa	[218]
Protease (Unidentified)	*Methanococcus jannashii*	Aqueous	1, 25, 50	90–130	50 MPa 116 °C	Enzyme activity and thermostability increased with pressure; increased reaction rate at 125 °C 3.4× and thermostability 2.7×. Reaction rate increased 2× at 50 MPa at 116 °C and by 5× at 130 °C	[20]
Pyrophosphatase (3.6.1.1)	*Bacillus stearothermophilus*	Aqueous	0.1–150	90–150	130 MPa 105 °C	Elevated optimal temperature from below 100 °C to above 105 °C when going from ambient to 130–170 MPa. 25× increased activity at 70 MPa versus 0.1 MPa	[226]
Aspartase (4.3.1.1)	*E. coli*	Aqueous	0.1–100	45–56	40–70 MPa 50–56 °C	Stabilized and activated at 45–56 °C by pressure up to 100 MPa	[230]

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below 200 MPa [73]. Above 600 MPa, a temperature increase from 50 to 80 °C was needed to accelerate PPO inactivation by a factor of 10 [129]. Below 300 MPa, the inactivation rate constant decreased with increasing pressure which was ascribed to the antagonistic effect of pressure and temperature [129]. A similar antagonistic effect was observed for avocado PPO at high temperature ($T \geq 62.5$ °C) and low pressure ($P \leq 250$ MPa) where a pressure increase caused a decrease in inactivation rate [74]. It was also shown that avocado PPO exhibits HP stability (900 MPa for 270 min produced only 1 log-reduction of activity) at room temperature [74]. Red raspberry PPO was activated at 400 and 800 MPa by 15 and 8%, respectively [121]. Likewise, PPO cell-free extracts from carrots and apples showed a dramatic increase in enzyme activity at pH 4.5 and 5.4 after pressurization at 100–300 MPa for 1 min [122]. The activation effects, observed for moderate pressure treatments was attributed to reversible configuration and or conformation changes on enzyme and/or substrate molecules [122].

Onion extract PPO activity increased up to 500 MPa with maximal activity of 142% compared to untreated enzyme [130]. The mechanism of this pressure-induced enhancement was not explored but was suggested to be due to pressure-induced protein conformational changes or separation of a part of the protein molecule and subsequent liberation of a second active site. However, there was no indication of major changes in native and pressure-treated onion PPO when analyzed using SDS-PAGE [130].

2.2. Transferases (EC 2)

2.2.1. Polymerase (2.7.7.7)

With the advent of PCR (polymerase chain reaction), DNA polymerases have become vital in molecular biology [131]. DNA polymerase is the most heat labile enzyme from the *Pyrococcus* species [132]. DNA polymerases derived from three thermophilic microorganisms, *Pyrococcus* strain ES4, *Pyrococcus furiosus*, and *Thermus aquaticus* were stabilized by HP at 111, 107, and 100 °C, respectively [21]. Half-lives of the three DNA polymerases increased from 5.0, 6.9, and 5.2 min, respectively, at 3 MPa to 12, 36, and 13 min, respectively at 45 MPa [21]. Pressure above 45 MPa did not significantly increase the half-life of *P. furiosus* DNA polymerase, whereas pressure increase up to 89 MPa did further increase the half-life of DNA polymerase from *Pyrococcus* strain ES4 and *T. aquaticus* [21]. Pressure-induced stabilization was attributed to pressure effects on thermal unfolding or retardation of unimolecular inactivation of the unfolded state. While application of HP for conducting a PCR assay may not be currently suitable with today's technology, any method for stabilizing an enzyme that is as widely used as polymerase is worthy of further investigation.

2.3. Hydrolases (EC 3)

2.3.1. Pectin methylesterase (EC 3.1.1.11)

Pectin methylesterase (PME) catalyzes the demethylesterification (or demethoxylation) of pectin releasing methanol and protons (creating negatively charged carboxyl groups) [133,134] which reduces viscosity and forms low-methoxyl pectin precipitates resulting in cloud loss of fruit and vegetable juices. This enzyme has been subject to recent reviews which summarize PME genetic sequencing, elucidation of three-dimensional structure, the multiple roles of PME in plants, and the existence of multiple isozymes which differ in their thermal and pressure stability [76].

The rate of recombinant *Aspergillus aculeatus* PME enzymatic de-esterification was optimal at 200–300 MPa combined with 45–55 °C [135]. Similarly, the highest rate of PME-catalyzed pectin de-esterification was at 200–300 MPa at 50–55 °C [136]. This increase in reaction rate of PME was explained using Le Chatelier's principle which may be applicable for de-esterification reactions because charged groups are formed, and solvation of these charged groups is accompanied by reduced volume resulting from electrostriction; i.e. the compact alignment of water dipoles owing to the columbic field of the charged groups [16]. However, Le Chatelier's principle is only applicable as an all-encompassing explanation if pressure does not significantly affect PME stability. In exploration of this caveat to the Le Chatelier's principle explanation, Fourier transform IR spectroscopy was used to reveal the pressure stability of β-helices of *A. aculeatus* PME [137]. No significant changes in spectra could be observed up to 1 GPa. At pressures above 1 GPa, unfolding takes place as indicated by broadening of the deconvoluted amide I′ band from approximately 1638 cm^{-1} towards approximately 1635 cm^{-1} [137]. Pressure stabilization was explained by pressure-induced suppressive effect on the aggregation of the partially unfolded protein due to the counteracting hydrophobic forces to a certain extent and by the substantial amount of protein in the β-helix conformation [137].

Tomato juice PME was activated by pressure above 300 MPa at all temperatures, and maximal at 300 MPa and 50 °C which was almost 1.7 times the control at 0.1 MPa [138]. This activation was attributed to reversible conformational changes or the enzyme and/or substrate molecules. In a related study, PME from tomato was quickly inactivated at 0.1 MPa and 70 °C but the inactivation rate constant dramatically decreased as soon as pressure was raised reaching a minimum between 300 and 600 MPa [139].

Extracted and purified carrot PME showed notable pressure resistance (up to 600 MPa) to inactivation in combination with increased catalytic activity with increasing pressure [134]. The most pronounced activity of carrot PME was observed at 50 °C and 500 MPa. Similarly, PME activity in shredded carrots (*in situ*) was most pronounced at 50 °C and 200–400 MPa and 100–400 MPa at 60 °C in intact carrots [134]. Similar results were found with extracted and purified carrot PME at lower pressures (300 MPa) and higher temperatures (>50 °C) where an antagonistic effect of pressure and heat was observed [76]. At 60 °C, the carrot PME inactivation rate was reduced by greater than 75% at 300 MPa versus 100 MPa [76].

Pressure-treated green peppers showed increased PME activity after treatment between 100 and 200 MPa for 10 or 20 min compared to the native control [140] and up to 500 MPa at room temperature [141].

Extracted and purified PME from bananas exhibited the antagonistic effect of pressure and temperature in the low pressure (≤300 MPa) and high-temperature domain ($T \geq 64$ °C). Increased pressure resulted in a decrease of the observed inactivation rate constant [137]. Inactivation rates of orange PME were also slower at 80 °C and 900 MPa compared to atmospheric pressure and was more pronounced in the presence of Ca^{2+} ions (1 M) but less pronounced for the inactivation in an acid medium (pH 3.7) [77].

While PME is often a target for inactivation in cloudy juices, it is vital for reducing viscosity of high pectin mixtures and the clarification of cloudless juices such as apple juice. It appears that HHP may be utilized to stabilize PME where needed while inactivating various other undesirable enzymes during a combined pressure-heat treatment.

2.3.2. Lipase (EC 3.1.1.3)

Lipases (triacylglycerol acylhydrolases) occur widely in nature where they catalyze the hydrolysis of esters from glycerol and long-chain fatty acids releasing alcohol and acid moieties. Since it became accepted that lipases remain enzymatically active in organic solvents [93] and react in the opposite direction (synthesis of esters), their application grew and have been the subject of many reviews and studies [49,142–151]. Lipases now constitute the most important group of biocatalysts for the synthesis of

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biopolymers and biodiesel, resolution of racemic mixtures, transesterifications, regioselective acylation of glycols and menthols, synthesis of peptides, and the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds [148]. Because of their importance to a variety of industries, lipases have been explored and documented more than any other enzyme at ambient pressure and at HP. Therefore, lipase is discussed in this review to a greater depth than any other enzyme.

Lipase stability and activity was examined at HP in compressed or supercritical fluids/gases including carbon dioxide [49,152–165], propane [51,160,165,166], butane [51,166], sulfur hexafluoride ($SCSF_5$) [51], and mixtures of butane and propane [51]. *Candida antarctica* lipase B (CALB) was effective at synthesizing isoamyl acetate in SC-CO_2 at higher initial reaction rates when compared to n-hexane while maintaining activity from 8 to 30 MPa [156]. These results were explained as being due to improved diffusivity of the reactants in the medium. CALB was also successfully used as a catalyst to synthesize butyl butyrate in SC-CO_2 [158]. All supercritical conditions assayed enhanced activity 84-fold with respect to synthesis in organic solvents while maintaining stability showing a 360 cycle half-life. Best results were achieved at 60 °C and 8 MPa and were explained as improved micro-environments around the enzyme [158].

The apparent kinetics of immobilized CALB synthesis of isoamyl acetate at HHP in hexane has also been examined. Activity of CALB increased by 110% at low pressure versus a 239% increase at 350 MPa when the temperature was increased from 40 to 80 °C. Increasing pressure up to 350 MPa increased lipase activity, while pressures greater than 350 MPa maintained or decreased lipase activity. $\Delta V^{\neq}$ appeared negative between ambient pressure and 200 MPa in contrast to a positive $\Delta V^{\neq}$ between 300 and 600 MPa. Besides increasing activity, HHP also reduced thermal inactivation of CALB by up to 152% after 4 h at 80 °C and 400 MPa when compared to incubations at low pressure [167]. Lipase activity at 63.5 and 80 °C at 400 MPa was greater (from about 20 to 96% increase) than at low pressure [167].

Rhizomucor miehei lipase was used in the esterification of lauryl oleate from oleic acid and 1-dodecanol in a batch stirred reactor using dense CO_2 as a reaction medium where the catalytic efficiency increased up to 10 MPa and was attributed to either substrate–solvent clustering, stabilization effect of SC-CO_2 treatment, or to the stabilization of lipase in the "open" form by hydrophobic interactions [168]. HHP in an aqueous medium has also been shown to improve lipase from *R. miehei.* At denaturing temperatures (50, 55, and 60 °C), application of 50–350 MPa protected the lipase. This protective effect increased with temperature [169]. Similarly, the protective effect of pressure against thermal inactivation (50 and 55 °C) was observed for *R. miehei* lipase at 50–450 MPa [170]. Conformational studies using fluorescence spectroscopy suggested that the conformational changes induced by pressure were different from those induced by temperature [171]. Noel and Combes [171] also concluded that *R. miehei* lipase has a high stability under pressure as it represents a slight change in volume (0.2%) in comparison to the initial volume of the native protein. Conversely, Osorio et al. [172] found for lipase from *Thermomyces lanuginose* increasing pressure improved selectivity while it linearly decreased stability.

Catalytic efficiency of *Pseudomonas cepacea* lipase transesterification of 1-phenylethanol and vinyl acetate in SC-CO_2 increased up to 15 MPa [55] and was more thermostable in supercritical propane than in water (optimum temperature increased from 36.8 °C in water to 49.8 °C in propane) [165]. This was hypothesized to be a consequence of protein structural and conformational rigidity in propane. In the same study lipases from *Pseudomonas fluorescences*, *Rhizpous javanicus*, *Rhizopus niveus*, and *porcine pancreas* were stable in SC-CO_2 and near-critical propane as activity remained constant after 24 h at 30 MPa and 40 °C. Recently, activity of lipase from porcine pancreas, *C. antarctica*, *Aspergillus oryzae*, *Candida cylindracea*, *Penicillium roqueforti*, *Aspergillus niger*, *Rhizopus arrhizums*, *Mucor miehei*, and *Pseudomonas cepacia* was increased after reacting at 40 °C and 15 MPa in SC-CO_2 [173]. The most remarkable increase in activity was from *R. arrhizums* lipase where activity increased 50-fold [173].

Lipase from *C. cylindracea* used for the esterification between n-valeric and citronellol was shown to have a reaction rate dependence on pressure [163] which increased significantly until reaching a maximum at 7.55 MPa near the critical point of SC-CO_2 [163]. Increased activity was explained by interactions between carbon dioxide and enzyme molecules inducing drastic conformational changes of the enzyme, causing active sites to emerge and catalyze the stereoselective synthesis [163].

Crystallographic and molecular-modeling studies of CALB determined that lipase belongs to the α/β hydrolase fold family with a catalytic triad consisting of Ser, His, and Asp/Glu [174]. Early X-ray diffraction data suggests that a lipase's helical part of the α-helix near the active site forms a "lid" that rotates almost 90°, from lying flat on the surface to extending nearly perpendicular to it on rearrangement to the open conformation [175]. This "lid" is shifted aside thus exposing the active site and generating the "active" form of lipase. This mechanism was suggested [168] for lipase (lipozyme RM-IM) activation at pressure close to 10 MPa in CO_2. Knez et al. [168] explained in such a condition the "lid" is moved away (rolls back) and the hydrophobic CO_2 molecules stick out around the active site, following the opening of the lid, unblocking the entrance of the tunnel. Uppenberg et al. [174] suggested that the α-helix (α5) can be stabilized by hydrophobic molecules at the entrance of the active site pocket.

Recent studies conducted in aqueous media have concluded that in contrast to most lipases, CALB may not have a "lid" blocking access to the active site and shows no interfacial activation [96] as compared to *C. antarctica* lipase A (CALA) which does possess the characteristic "lid" structure and shows interfacial activation [176]. Furthermore, flexibility of CALB is reduced in organic solvents, and is limited to: (1) a short α-helix which form the entrance to the active site, (2) three surface loops, (3) the medium binding pocket for secondary alcohols, (4) the acyl binding pocket, and (5) the large binding pocket for secondary alcohols [96]. Flexibility limited to these five elements vital to catalysis may offer possible explanation for relatively HP stability, while still allowing modification of activity. Since lipases are widely used in high value product (fine flavors, pharmaceuticals, etc.) biosynthesis, the use of HP technologies may be uniquely suitable due to cost effectiveness associated with these products.

2.3.3. *Acetylcholinesterase (EC 3.1.1.7)*

Acetylcholinesterase is indispensible in synaptic transmission by catalyzing hydrolysis of acetylcholine and plays a role in stress response, degenerative diseases, and neural development in humans [177]. Acetylcholinesterase 7.4S was more active at pressures up to approximately 14 MPa when compared to 0.1 MPa [178]. Pressure-induced stability appeared to be related to the 7.4S enzyme having a large volume expansion accompanying the activity loss apparently retarded by application of moderate to low pressure. This enzyme is the only pressure-enhanced enzyme native to human physiology neural synaptic system. Acetylcholinesterase is widely used in biosensors for the detection of pesticides, metals, and organic compounds [179]. Therefore it may be a suitable candidate for a pressure stable biosensor used in HP environments such as deep-sea exploration and deep underground oil, natural gas, and water drilling.

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2.3.4. Polygalacturonase (EC 3.2.1.15)

Polygalacturonase (PG) catalyzes the α-1,4-glycosidic bonds of the product of PME-catalyzed pectin de-esterification. This depolymerization leads to a drastic decrease in viscosity of various fruit and vegetable juices. Unlike PME, increases in pressure and temperature can accelerate the inactivation rate of PG [139]. However, a pressure resistant PG fraction from tomato was not affected by prolonged pressurization up to 600 MPa at 50 °C [180]. Furthermore, PG activity increased up to optimal pressure (100–200 MPa) depending on the temperature [181]. Verlent et al. [181] found that PG catalyzed hydrolysis of pectin in the presence of tomato PME remains constant with increasing pressure at 50 °C which is in contrast to earlier work by the same group [182,183] where a shift in optimal temperature to lower values was observed with increasing pressure. The relationship between PG activity with or without PME needs further exploration; however it is clear the HP can be utilized to modulate activities to achieve desired processing conditions such as increasing PG activity to reduce fruit or vegetable juice viscosity.

2.3.5. α-Amylase (EC 3.2.1.1)

α-Amylase catalyzes the hydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units in random order [81] and used mainly to degrade starch to low molecular weight dextrins in the production of sugar syrups [184] as well as for the preparation process of alcoholic beverages such as beer and whisky [81]. Studies from the 1950s described by Laidler [185] identified a "salivary amylase" and a "pancreatic amylase" which both had $\Delta V^{\neq} = -22\,cm^3\,mol^{-1}$ and $-28\,cm^3\,mol^{-1}$ respectively. More recently, α-amylase from barley malt was stabilized against thermal inactivation up to 200 MPa with maximum substrate cleavage at 152 MPa and 64 °C with 25% higher yield as compared to the maximum at ambient pressure and 59 °C [81]. The optimum conditions are explained by the superposition of pressure-induced thermal stabilization of the enzyme and enhanced substrate conversion by temperature. Treatment of α-amylase with SC-CO_2 and 0.1% water at 35 °C and 20 MPa for 1 h resulted in residual activity of 105% compared to prior to treatment [186].

2.3.6. β-Amylase (EC 3.2.1.2)

β-Amylase hydrolyzes the α-1,4-glucosidic linkages of starch from the non-reducing ends. This reaction is utilized in the food and beverage industry for the preparation of fermented foods or maltose production as a precursor for alcoholic beverages. The effects of HP on the flexibility of subgroups within β-amylase have been observed by monitoring intensity changes in the near UV circular dichroism spectrum [187]. β-Amylase activity from barley malt was strongly affected by HP and temperature treatment [82]. Highest depolymerization of starch rate was found at 100 MPa and 62 °C, yielding 15% more compared to optimum conditions at ambient pressure [82]. The shift in temperature optimum was attributed to the stabilization of the enzyme against thermal inactivation. Like α-amylase, HP may be utilized to improve β-amylase catalysis for use in the production of sugar syrups or alcoholic products.

2.3.7. β-Glucanase (EC 3.2.1.2)

β-Glucanase activity in malt is crucial for the brewing process because of its effect on beer filtration. β-Glucanase depolymerizes β-glucans which increase beer viscosity by thus reducing filterability [188,189]. β-Glucanases are synthesized during germination but are inactivated at temperatures above 50 °C during the malting and mashing processes. Application of pressure at 400 MPa significantly stabilized this enzyme against thermal inactivation [80]. However, increasing pressure also decreased activity up to 600 MPa [80]. Counteracting effects of stabilization and reduced activation resulted in maximum conditions at 215 MPa and 55 °C which yielded approximately 2/3 higher degradation of β-glucan after 20 min as compared to the maximum at 0.1 MPa at 45 °C [80]. It is clear that high hydrostatic pressure along with elevated temperature can be utilized to improve β-glucanase activity for depolymerizing β-glucans.

2.3.8. Glucoamylase (EC 3.2.1.3)

Glucoamylase is a multidomain exo-glycosidase that catalyses the hydrolysis of α-1,4- and α-1,6-glucosidic linkages of starch and related polysaccharides to release β-D-glucose from the non-reducing ends and is used in the production of spirits and glucose syrups from starch [83]. Two isoforms of glucoamylase (GA1 and GA2) from *A. niger* were studied for their response to pressure temperature treatments. The pressure liable form of glucoamylase (GA2) was inactivated above 850 MPa whereas the stable (GA1) isoform was slow to inactivate up to 1400 MPa at 50 °C [83]. Pressure stabilized the enzymes against thermal inactivation at 400 MPa for GA2 and at 550 MPa for GA1 [83]. Since elevated pressure contributed to delayed enzyme-substrate interaction up to 600 MPa, the maximum production of glucose by maltose cleavage was observed at approximately 84 °C and 318 MPa after 30 min [83]. β-Glucoamylase from barley malt exhibited a maximum depolymerization rate at 215 MPa and 55 °C which yielded approximately 2/3 higher degradation of β-glucan after 20 min as compared to the maximum at 0.1 MPa and 45 °C [80]. Activity also increased 102 and 105% after treatment with SC-CO_2 and SC-CO_2 + 0.1 wt% water respectively at 35 °C and 20 MPa for 1 h compared to prior to treatment [186]. Like α-amylase, β-amylase, and β-glucanase discussed earlier, glucoamylase is widely used in the brewing industry and shows a clear activity and stability enhancement at elevated pressures. The evidence of pressure enhancement of these four enzymes vital for beer and spirit production are examples of the potential improvement of industrial processes with HP.

2.3.9. Lysozyme (EC 3.2.1.17)

Lysozyme is commonly found in nature and is primarily from egg albumin. Lysozyme hydrolyzes the β-1,4-glycosidic bond between the N-acetylmuranimic acid and the N-acetyl-D-glucosamine of peptidoglycan, which is the major component of gram-positive bacteria cell walls [190]. Lysozyme is relatively low-priced as compared to other antimicrobial agents such as nisin [191], is classified as generally recognized as safe (GRAS), and is effective as a food preservative by inhibition of pathogenic and spoilage bacteria [190,192–195]. Lysozyme activity is relatively stable below 200 MPa while above 200 MPa some enzymatic activity is lost but antimicrobial activity was not reduced [190]. Iuccie et al. [196] also found that lysozyme treated at 100 MPa had increased antimicrobial activity resulting in approximately two logarithmic reductions in microbial death. The increase in antimicrobial activity was attributed to a direct effect of pressure on the integrity of cell walls or outer membranes of the microbes, a consequent increased penetration of enzymes through the damaged walls or membranes, and indirect effects on the processes of the antimicrobial properties of lysozyme [196]. While pressure increased the antimicrobial activity of lysozyme, it may be a synergistic or additive effect and not purely attributed to increased enzymatic activity. Although pressure effects on lysozyme activity above 200 MPa remain unclear, current evidence suggests that pressures below 200 MPa may aid in the action of this widely used, effective, and relatively inexpensive antimicrobial enzyme.

2.3.10. β-Glucosidase (EC 3.2.1.21)

β-Glucosidase catalyzes the hydrolysis of aryl and alkyl β-D-glucosides and are involved in key metabolic events and growth

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related response in plants [197]. This enzyme is involved in the liberation of volatile aglycones from non-volatile glucosides [198] and is important to for subsequent flavor release [121]. In red raspberries, HPT at 400 MPa increased activity 2% while in strawberries HPT of 400 MPa increased activity 76% [121]. β-Glucosidase from *Sulfolobus solfactaricus* was also activated by pressure between 0.1 and 250 MPa with maximal activity at 125 MPa and has a half-life of 91 h at 60 °C and 250 MPa. Conversely almond β-glucosidase was rapidly inactivated by pressure up to 150 MPa [199]. HP activation may be helpful in strawberry or raspberry processing where pressure is used to inactivate some unwanted enzymes while activating others that contribute to flavor development.

2.3.11. β-Galactosidase (EC 3.2.1.23)

β-Galactosidases are widely used in the food industry to hydrolyze lactose into galactose and glucose [200]. Besides their established use for their hydrolytic activity, transgalactosidase activity of β-galactosidases can be useful in synthesis of glyconjugates for food and pharmaceutical applications [201,202]. β-Galactosidases from *A. oryzae*, *Kluyveromyces lactis*, and *Escherichia coli* were investigated for their increased thermostability under HP. After 1 h incubation at 25 °C residual activity did not significantly decrease for β-galactosidase from *A. oryzae* below 450 MPa, below 300 MPa from *E. coli*, and below 200 MPa from *K. lactis* [200]. For the three enzymes tested, moderate pressures protected the enzyme against thermal inactivation. Residual activity of *E. coli* β-galactosidase increased from 60% at 0.1 MPa to 80% at 100 MPa and 55 °C [200]. Residual activity of *K. lactis* β-galactosidase increased 5-fold at 100 MPa versus at 0.1 MPa and 45 °C [200]. Most interestingly, residual activity of *A. oryzae* β-galactosidase increased from complete inactivation at 55 °C and 0.1 MPa to near 100% residual activity at 250 MPa for a 1 h incubation [200]. β-galactosidase from *E. coli* had no decrease in activity below 250 MPa [203] and at 250 MPa the half-life at 55 °C was 32 h versus 1.5 h at 0.1 MPa [204]. While application of HP to β-galactosidase in the food industry may be advantageous, as with many enzymes the application of HP for pharmaceutical synthesis of compounds such as glyconjugates may be more economically viable.

2.3.12. Invertase (EC 3.2.1.26)

Invertase (β-D-fructofuranosidase) is widely used in the food industry for its hydrolytic activity on sucrose for the production of invert sugar (mixture of glucose, fructose, and unhydrolyzed sucrose) [205]. Pressure-induced conformation changes were determined using Raman Spectroscopy [206] and fluorescence spectroscopy [207] where conformational changes induced by pressure and temperature were different. HP caused an increase in low-wave number scatter in solution and to a shift of the amide I bands lower while thermal denaturation showed no such modifications in spectra [206]. Likewise, thermal treatment of invertase caused the fluorescence of tyrosine and tryptophan to decrease slowly while HPT caused these aromatic residues to become more exposed causing increased fluorescence [207]. These differences were hypothesized to be due to HP and temperature exerting opposing effects on weak interactions (hydrogen bonds, ionic and hydrophobic interactions) leading to different denaturation mechanisms [206] and may explain the antagonistic effects of pressure and temperature for invertase pressure-induced stabilization. *Saccharomyces cerevisiae* invertase half-life increased at 60 °C from 50 to 200 MPa [169]. Furthermore, native and immobilized invertase have $\Delta V^{\neq} = -7.3\,\text{cm}^3\,\text{mol}^{-1}$ [208] and $\Delta V^{\neq} = -29\,\text{cm}^3\,\text{mol}^{-1}$ [209] respectively. Conversely, invertase can be readily inactivated by pressure depending on the specific temperature–pressure combination [207,210].

2.3.13. Naringinase (EC 3.2.1.40)

Naringinase is used to reduce bitterness in grapefruit juice by hydrolyzing naringin (a flavonone glycoside and primary bitter component in grapefruit juice) to naringenin, which is tasteless. In a model solution, naringinase activity increased by 72% at 200 MPa and 54 °C versus only a 35% reduction at 0.1 MPa [211]. These results are in agreement with earlier findings where maximum naringinase activity was between 30 and 40 °C and approximately 160 MPa with a $\Delta V^{\neq} = -9$ to $-15.0\,\text{cm}^3\,\text{mol}^{-1}$ [79,212,213] depending on immobilization technique and other reaction conditions. The HP-induced activation of naringinase makes it suitable for grapefruit juice processing as modified conditions can optimize naringinase activity while simultaneously inactivating undesirable pressure-labile microorganisms.

2.3.14. Myrosinase (EC 3.2.3.1)

Glucosinolate hydrolysis products in broccoli catalyzed by myrosinase such as sulforaphane are thought to have beneficial health effects. Therefore, a heat and pressure treatment that results in stabilized myrosinase could be advantageous [78]. Inactivation rate constants decreased up to a maximum around 350 MPa at 35 °C. These results were later substantiated when thermal treatment of 60 °C reduced activity by 97% at 0.1 MPa versus 75% at 100 MPa [78]. The activation energy of myrosinase inactivation was highest at 200 MPa, the pressure at which the antagonistic effect was most pronounced [78]. This antagonistic effect was not observed with a second form of myrosinase for which inactivation rates continually increased with increased pressure [214]. The application of HP may effectively improve the inactivation of deleterious enzymes and microbes while simultaneously increasing or preserving the activity of beneficial enzymes such as myrosinase. Although the relationship between myrosinase activity and pressure is not fully elucidated, it is clear that application of elevated pressure may be a useful tool in the enhancement of health benefits of processed broccoli.

2.3.15. α-Chymotrypsin (EC 3.4.21.1)

α-Chymotrypsin (CT) is a proteolytic enzyme that facilitates the hydrolysis of peptide bonds and is used to aid protein digestion. It is a serine protease with the characteristic three amino acid active site known as the catalytic triad. Stability of α-chymotrypsin at HP was investigated in the 1940s [215]. While no pressure-induced activation was noted, induced stabilization was reported up to 760 MPa. Pressure effects on CT activity and stability were explored again in the 1980s by Taniguchi and Suzuki [69] and in more depth in the mid 1990s by Mozhaev et al. [41,57]. Taniguchi and Suzuki found that increasing pressure increased CT hydrolysis of *p*-nitrophenyl acetate from 10 to 65 °C with maximal activity at 45 °C and 200 MPa [69] and pressure activation was more pronounced as temperature increased. Similarly, Mozhaev et al. found that an increase in pressure at 20 °C resulted in a 6.5-fold increase in activity at 470 MPa versus 0.1 MPa [57]. The acceleration effect became more pronounced at high temperatures as activity at 50 °C and 360 MPa was 30-fold higher than activity at 0.1 MPa and 20 °C [57]. High pressure also increased stability of CT. CT is readily inactivated (less than 5 min half-life) at 0.1 MPa; while at 180 MPa CT remains active for 30 min [57]. Stability and activity of CT was also improved by pressure in organic media when Mozhaev et al. [60] obtained $\Delta V^{\neq} = -13 \pm 2\,\text{cm}^3\,\text{mol}^{-1}$ while maintaining activity up to 200 MPa. This was explained as being due to a transitional conformation between 100 and 150 MPa that renders the enzyme more active. Raman spectroscopy was used to explore pressure-induced structural changes and found only slight changes up to 200 MPa that were attributed to the salt bridge between Asp-194 and Ile-16 [216]. While many enzymes native to muscle tissue are a target for inactivation by HP [217], the pressure-induced stabilization or

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activation of α-chymotrypsin may be useful in modifying the functional and organoleptic properties of a wide variety of processed meat products.

2.3.16. Thermolysin (EC 3.4.24.27)

Thermolysin is used in the production of a precursor for a synthetic sweetener and may be suitable for the production of various peptides through the condensation of amino acids [58]. Thermolysin hydrolysis of β-lactoglobulin (BLG) rate increased by a factor of 22 from 0.1 to 200 MPa before irreversible conformational changes occurred at or above 300 MPa [218]. Similarly, BLG digestion by thermolysin increased from 19.4 to 25.0% at 200 MPa versus at 0.1 MPa [58]. In the same study thermolysin catalysis was accelerated by increasing pressure on nonspecific digestion of proteins from various origins. These proteins included, alcohol dehydrogenase (18.1% versus 45.3%), hemoglobin (13.0% versus 34.6%), bovine serum albumin (0.19% versus 0.23%), and myoglobin (12.0% versus 15.0%) at 0.1 MPa and 200 MPa respectively [58]. Increased reaction rate was attributed to pressure effects on enzymatic activation as well as protein substrate partial unfolding allowing greater catalytic activity on the protein substrate. This combined pressure effect is dually useful when conducting enzymatic catalyzed synthesis or hydrolysis reactions because pressure can act as a denaturation-inducing agent. This pressure effect on substrate denaturation was termed "reagent-less denaturant" by Kunugi [58].

A thermostable neutral thermolysin from *Bacillus thermoproteolyticus* was compared to a non-thermostable neutral thermolysin from *B. subtilis* [219]. The application of 100 MPa resulted in over 13 times increase in reaction rate for the thermostable enzyme versus 3.8 times increase for the non-thermostable enzyme [219]. Also, thermolysin catalyzed hydrolysis of a dipeptide substrate (FA-Gly-Leu-NH_2) had a $\Delta V^{\neq} = -30.2\,cm^3\,mol^{-1}$ [220]. Thermolysin from *B. thermoproteolyticus* was also shown to have $\Delta V^{\neq} = -52 \pm 4\,cm^3\,mol^{-1}$ and up to a 40-fold reaction acceleration at 100 MPa and 40 °C for hydrolysis of the low molecular mass substrate 3(2-furylacrylolyl)-glycyl-L-leucine amide [221]. HP also increased residual activity by 101 and 102% after treatment with SC-CO_2 and SC-CO_2 + 0.1% water at 35 °C and 20 MPa for 1 h resulted compared to before treatment [186]. Similarly to lipase, thermolysin is used in high-value product (synthetic sweetener and peptide) biosynthesis; and therefore, use of HP technologies may be suitable due to the cost effectiveness associated with these products.

2.3.17. Pepsin (EC 3.4.23.1)

Like thermolysin, pepsin is used as a catalyst in the production of a precursor for a synthetic sweetener and may be suitable for the production of various peptides through the condensation of amino acids [58]. The hydrolysis of BLG with pepsin was studied between 0.1 and 350 MPa. Increased pressure showed a significant increase in cleavage rates [218]. Pepsin was unable to hydrolyze BLG at 0.1 MPa versus total hydrolysis of all the BLG substrate in less than 40 min at 300 MPa [218]. The BLG peptic hydrolysis rate increased 270-fold between 0.1 and 300 MPa. Like lipase and thermolysin, pepsin can be used in high-value product biosynthesis. Therefore the use of HP technologies may be suitable due to cost effectiveness associated with these products.

2.3.18. Unidentified protease

Proteases traditionally are applicable for the cleavage or digestion of proteins, which is particularly useful in the food or pharmaceutical industry. The properties of an unidentified and un-named protease from a hyperthermophilic barophilic *M. jannashii*, an extremely thermophilic deep-sea methanogen found at a deep-sea hydrothermal vent, have been explored. The activity and stability of the partially purified enzyme increased with pressure; raising the pressure increased the reaction rate 3.4-fold and the thermostability 2.7-fold at 50 MPa at 125 °C with a corresponding negative activation volume of $-106\,cm^3\,mol^{-1}$ [20] compared to 0.1 MPa. The barophilic behavior of this protease is somewhat expected in view of the unusual barophilicity exhibited by *M. jannashii* [222], as well as other enzymes from this organism such as hydrogenases discussed earlier [66,118].

2.3.19. Pyrophosphatase (3.6.1.1)

Pyrophosphatases are acid anhydride hydrolases that act upon diphosphate bonds and are used in DNA polymerization reactions for *in vitro* RNA synthesis reactions and for pyrophosphate removal which is from biosynthetic reactions that utilize ATP [223,224]. Inorganic pyrophosphatase from yeast was partially stabilized against thermal inactivation by the addition of trehalose and glycerol [225]. Partially purified pyrophosphatase from *B. stearothermophilus* demonstrated increased activity at 90 °C from 10 to 100 MPa with optimal activity at 70 MPa [226]. No activity could be demonstrated at 100 °C at 0.1 MPa while at 105 °C and 103–190 MPa activity increased up to 320% compared to 0.1 MPa [226]. Activation was explained by pressure counteracting the molecular volume increase which results from elevated temperatures. While this explanation is over-simplified, for the time of publication it was considered adequate. Like polymerase, the application of HP for conducting a DNA analysis may not be currently suitable with today's technology, although any method for stabilizing a widely used enzyme is worthy of further investigation.

2.4. Lyases (EC 4)

2.4.1. Aspartase (4.3.1.1)

Aspartase (L-aspartate ammonia-lyase) catalyzes the reversible deamination of the amino acid L-aspartic acid producing fumaric acid and ammonium ion [224,227]. It was used to produce L-aspartic acid on a large scale [228,229]. Aspartase of *E. coli* was shown to be activated by pressure in the early 1960s as activity increased from 0.1 to 100 MPa at 50 °C and from 0.1 to 60 MPa at 53 °C and 56 °C [230]. It was suggested that the application of pressure may increase the incidence of substrate-active site collision as well as prevent enzyme unfolding. Like other early studies, this explanation was adequate for the time of publication but is understood to be an oversimplification.

3. Conclusions

High pressure can be utilized to stabilize or increase activity of many enzymes from various sources and in various solvent systems. Categorized by enzyme class, most reported pressure-enhanced enzymes are hydrolases, followed by transferases, and even fewer oxioreductases, with only one lyase being found. This trend might be explained by the focus of most studies that derived from the food industry being geared towards inactivating deleterious enzymes. For this reason, much of the research has been focused on enzymes important to food, mainly lipases, pectin esterases, amylases, and polyphenoloxidase.

While the effect of pressure on the stability and rates of reaction for many enzymes has been researched, there is still a lack of experimental data for the majority of known enzymes. Also, the effect of HP on enzyme structure is scarce because there are very few laboratories equipped with HP optical cells coupled to the instrumentation necessary for such studies. Currently, most methods require introducing enzyme to substrate then applying incubation conditions for a given time interval. After a given time, the enzyme reaction mixture is removed from the incubator and assayed, and repeating this procedure at time intervals

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allows pseudo-apparent kinetic measurements. While such efforts as precisely controlling temperature and enzyme to substrate ratios among other reaction parameters can effectively slow the reaction to give adequate apparent kinetic measurements, they must compensate for adiabatic heating and cooling and the pressurization and depressurization transient time. For these reasons, future studies are needed to develop *in situ* kinetic measurements at HP with precise control of other reaction parameters such as temperature. Likewise, *in situ* studies need to be conducted to fully elucidate quaternary, tertiary, and even secondary structural changes during pressure changes, at isobaric conditions, and at various combinations of temperature and pressure. These studies will allow greater understanding of enzymatic changes at elevated pressures which may allow for better utilization and optimization of reaction conditions.

Despite its advantages described here, the application of HP for enzymatic reactions may not always be practical due to the high cost of HP systems, lack of appreciable effects, or advancements in alternative enzyme enhancement technologies. However HP has great potential and is only beginning to be explored in the field of enhancing enzyme systems.

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