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Review

Recent Advances in the Application of Enzyme Processing Assisted by Ultrasound in Agri-Foods: A Review

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Abstract: The intensification of processes is essential for the sustainability of the biorefinery concept. Enzyme catalysis assisted by ultrasound (US) may offer interesting opportunities in the agri-food sector because the cavitation effect provided by this technology has been shown to improve the efficiency of the biocatalysts. This review presents the recent advances in this field, focused on three main applications: ultrasound-assisted enzymatic extractions (UAEE), US hydrolysis reactions, and synthesis reactions assisted by US for the manufacturing of agri-food produce and ingredients, enabling the upgrading of agro-industrial waste. Some theoretical and experimental aspects of US that must be considered are also reviewed. Ultrasonic intensity (*UI*) is the main parameter affecting the catalytic activity of enzymes, but a lack of standardization for its quantification makes it unsuitable to properly compare results. Applications of enzyme catalysis assisted by US in agri-foods have been mostly concentrated in UAEE of bioactive compounds. In second place, US hydrolysis reactions have been applied for juice and beverage manufacturing, with some interesting applications for producing bioactive peptides. In last place, a few efforts have been performed regarding synthesis reactions, mainly through trans and esterification to produce structured lipids and sugar esters, while incipient applications for the synthesis of oligosaccharides show promising results. In most cases, US has improved the reaction yield, but much information is lacking on how different sonication conditions affect kinetic parameters. Future research should be performed under a multidisciplinary approach for better comprehension of a very complex phenomenon that occurs in very short time periods.

Keywords: ultrasound; enzyme; food processing; biocatalysts; biorefinery; extractions; hydrolysis; synthesis

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1. Introduction

Enzymes are highly active and specific biological catalysts that are used in highly productive food processes [1]. As catalysts, they increase the rate of reaction by decreasing the activation energy, but different from traditional chemical catalysts, they act under mild conditions and with very high molecular precision [2,3]. These characteristics make enzymes attractive catalysts for use in several food processes, among them: maceration and filtration in brewing, baking, manufacturing of glucose and fructose syrups from starch, winemaking, meat tenderization, juice processing, production of low-calorie sweeteners, and a wide spectrum of applications in the dairy industry [3–7].

At physiological conditions, enzymes develop a unique network of complex reactions conforming to the cell metabolism with stunning performance, responsible for sustaining the life of all organisms. However, their industrial application generally involves extracting them from their cellular confinement, thereby changing the microenvironment that naturally protects them. As a result, enzymes are affected by the operational conditions wherein the reaction is performed, namely temperature, pH, substrate concentration, and the presence of inhibitors or activators, which are quite different from their physiological environment [8]. Hence, a major challenge in biocatalysis is to transform these physiological catalysts into process catalysts that are able to perform under the usually tough reaction conditions of an industrial process [9]. Among the many tools available to perform this transformation, enzyme immobilization is highlighted because of the proven advantages of immobilized enzymes over their soluble counterparts in terms of robustness, operational stability, easy separation from the product stream, and the chance of multiple reuses in a repeated batch operation or prolonged use in a continuous operation [10].

The functions and properties of an immobilized enzyme depend on the enzyme itself, the immobilization system, and the support material [11]. There is a wide range of immobilization strategies that have been thoroughly reviewed in the literature, namely: adsorption [12], covalent attachment and physical entrapment to inert supports [13], and aggregation and crosslinking of the enzyme protein [14,15] or containment within semipermeable membranes in membrane bioreactors [16]. Despite impressive advances in this field, the main drawbacks for the widespread use of immobilized enzymes for food processing are reduced activity per unit of volume, mass transport constraints, time-consuming immobilization procedures, and the additional costs associated with the required support material and reagents [10,17]. Additionally, it is necessary to consider that the immobilization technique should be compatible with the intended use of the enzyme, which may be particularly critical when the products of the enzymatic reaction are intended for human consumption [9]. Several chemical additives are used in most of the procedures for enzyme immobilization. Surprisingly, not much awareness is perceived about the risk of potential contamination of the product by leakage of these chemical compounds into the product and their long-term cumulative effects.

In contrast, ultrasound (US) is an emerging sustainable technology that enhances the rate of several processes in the food processing industry and their efficiency [18]. US involves sound waves at a frequency that exceeds the human hearing threshold (~20 kHz) that are produced by a transducer that converts electrical energy into a vibrational energy [19]. During the propagation of these waves, continuous cycles of compression and rarefaction are produced, thus promoting the development of cavitation bubbles in the liquid medium, which may interact in different ways with a liquid–solid interface [20]. As the pressure fluctuates during the passage of ultrasound waves, it can fall below the vapor pressure of the liquid, forming small, vapor-filled voids that lead to bubbles within the liquid; a phenomenon known as acoustic cavitation [21]. The bubbles' growth and their collapse within the liquid media occur in a very short time domain of approximately 1 μs, leading to a "near" adiabatic heating of the contents of the bubble, reaching localized temperatures ranging from 2000 to 5000 K and pressures of up 1000 atm [22]. As a result, hotspots are produced, thus increasing mass and heat transfer rates, the development of sonochemical reactions, the generation of high turbulence, and liquid jets [23].

Figure 1 summarizes the cavitation phenomenon and its relationship with enzymes. The cavitation phenomenon produced by ultrasound on enzymes during food processing has been investigated from two perspectives. The first is related to those enzymes that have negative effects on food quality, such as polyphenoloxidase (PPO) on vegetables and lipases and lipoxygenases involved in the rancidity in high-fat foods [24]. In such cases, high-intensity ultrasonic cavitation has been shown to deactivate the undesired enzymes, thus increasing the shelf-life and quality of many food products [25] while avoiding the degradation of thermolabile bioactive compounds, as occurs in conventional thermal processing. A second perspective refers to the use of US to improve enzyme bioprocesses.

According to Wang et al. (2018), US has been shown to accelerate enzymatic reactions by acting on different targets, such as by modifying the structure of the enzyme and the substrate or by increasing the substrate mass transfer rate in the reaction system [26].

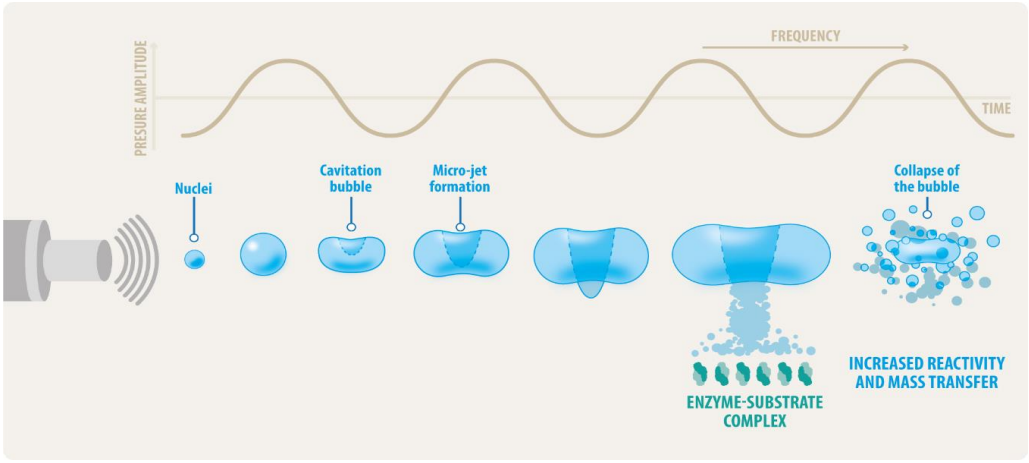

Figure 1. Schematic representation of the ultrasonic cavitation phenomena.

Enzymatic processing in the agri-food sector has experienced constant growth boosted by the development of new food applications, such as the extraction of bioactive compounds, nutrient-rich and quality-improved food production, and the elimination of food safety hazards [4]. With current consumer and industry demands for production in greener systems, the intensification of processes is a must for the development of sustainable processes for the biorefinery concept, and enzyme catalysis assisted by US may offer interesting opportunities for improved biocatalytic performances. In recent years, a few reviews have referred to the lack of information about the ultrasonic processing conditions and their effect on enzyme catalysis, highlighting the necessity to provide complete information on the data to properly compare results [26–28]. To the best of our knowledge, no reviews dealing specifically with the use of US in enzyme agri-food processing have been published. The objective of this work is to review the recent advances in this field, focusing on three main applications: extraction, hydrolysis, and synthesis of bioactive compounds performed with enzyme reactions assisted by US for the manufacturing of agri-food produce and ingredients with improved yields, as well as a strategy for upgrading agro-industrial waste into valuable compounds. A theoretical background with some controversial experimental aspects and the mechanisms by which US processing acts on the enzymes is also included.

2. Ultrasound Technology

2.1. Ultrasound Theoretical Basis

Ultrasound is a hybrid technology characterized by its high processing and economic performance. It allows reducing processing times, with less use of water, solvents, and energy consumption for operations of interest to the food industry, such as extractions of bioactive compounds, emulsification, degassing, membrane filtration, and convective air drying, among others [22,23,29,30]. Two main classifications can be assigned to the application of US in food processing: the low intensity ($\leq$1 W/cm^2) and high frequency (>100 kHz) ultrasound (LI-HF), used mainly for non-invasive and non-destructive food quality monitoring, and the high intensity (>>10 W/cm^2) and low frequency (20–100 kHz) ultrasound (HI-LF), which induces considerable effects on the physical, biochemical, and mechanical properties of foods [18].

The generation of ultrasound is caused by transducers that convert electrical pulses into acoustic energy. There are two types of transducers: those based on the magnetostriction effect and those based on the piezoelectric principle. The former consists of the alteration in length per unit length caused by the application of the magnetic field on a

material with ferromagnetism properties, which causes magnetization and the consequent generation of vibrations [18]. However, piezoelectric transducers are the most used in ultrasonic food processing. They consist of two ceramic elements that change their size in response to an electric field. Thus, if an alternating field is applied, the ceramic elements move up and down in a highly reproducible manner [31].

Frequency is a very important aspect of ultrasound processing because it is directly related to the resonance bubble size (R_r). This refers to the critical size reached by the bubble during cavitation before collapse [32]. R_r can be expressed as follows (Equation (1)):

$$R_r = \sqrt{\frac{3 \cdot \gamma \cdot p_\infty}{\rho \cdot \omega^2}} \quad (1)$$

where γ is the specific heat ratio of the gas inside the bubble, p_∞ is the ambient liquid pressure, ρ is the liquid density, and ω is the angular frequency of the ultrasound [32]. Usually, larger bubbles (~100 µm) can be formed in the range of 20 to 40 kHz, and their collapse is the main cause of the effects of ultrasound processing in foods [31]. On the contrary, at higher frequencies, the rarefaction and compression cycles proceed too quickly, inhibiting bubble growth up to a size sufficient to cause its violent collapse [33].

Ultrasonic intensity (*UI*) is a processing variable of paramount importance for the cavitation phenomenon. According to Pierce (1997), particles of the medium that are set in vibratory motion (v) with ultrasound waves possess kinetic energy [34], which can be expressed as energy density per unit of volume (E):

$$E = \frac{1}{2} \cdot \rho \cdot v^2 \quad (2)$$

The sound energy flowing (E_f) in time t, passing through a cross-sectional area (A) with velocity c is expressed as follows [35]:

$$E_f = E \cdot A \cdot c \cdot t \quad (3)$$

Ultrasonic intensity (*UI*) is defined as the amount of energy flowing per time per area of ultrasound probe (A); that is, $UI = E_f/(A \cdot t)$. Thus, by applying this definition and merging Equations (2) and (3), *UI* corresponds to:

$$UI = \frac{1}{2} \cdot \rho \cdot c \cdot v^2 \quad (4)$$

Usually, *UI* is expressed in units of W/cm^2. Colussi et al. (1998) established that for a plane progressive wave, the particle velocity is related to the acoustic pressure, P_a [36]:

$$\frac{P_a}{v} = \rho \cdot c \quad (5)$$

Replacing Equation (5) with Equation (4) leads to an expression relating *UI* with P_a:

$$UI = \frac{P_a^2}{2 \cdot \rho \cdot c} \quad (6)$$

The variation in the position of the molecules when the sound wave travels through the liquid produces variations that can be related to the maximum amplitude of the wave, P_A, and the frequency, f [37]:

$$P_a = P_A \cdot \sin(2 \cdot \pi \cdot f \cdot t) \quad (7)$$

Then, *UI* can be expressed as:

$$UI = \frac{(P_A \cdot \sin(2 \cdot \pi \cdot f \cdot t))^2}{2 \cdot \rho \cdot c} \quad (8)$$

This last expression shows that *UI* is a parameter strongly correlated to the frequency, to the transducer amplitude, and, consequently, to the amplitude of the sound wave pressure. Thus, increases in pressure amplitude will generate more violent bubble collapses [23]. This is true only to some extent because if the sound intensity is too high, the cavitation bubbles will be too large or will aggregate to form a barrier on the surface of the sound source, avoiding the propagation of sound waves [38].

2.2. US Experimental Considerations

A critical aspect in US processing is the determination of the *UI* value. Some researchers report the percentage of wave amplitude as a way to express the *UI*. Even though this is true, the *UI* value obtained by operating a certain percentage of wave amplitude will vary from device to device and according to the sonication conditions. In such cases, an experimental determination of the *UI* is recommended. There are different approaches to estimate the *UI*, the calorimetric method being one of the most used. The simplest way to measure it is by Equation (9):

$$UI = \frac{m \cdot c_p \cdot \frac{dT}{dt}}{A} \quad (9)$$

where m is the mass of solvent, c_p its specific heat capacity, dT/dt is the rate of temperature change, and A is the area of the transducer. As *UI* is expressed in W/cm^2, the complete term of the numerator is the ultrasonic power, P (W). A plot of T against t has an intercept of T_o (the initial temperature of the solvent) that enables determining the acoustical energy [39]. A consideration to keep in mind about this method is that, unless the solution container is perfectly isolated, it is necessary to consider the heat loss to the environment. Although this can be solved using an energy balance, in practice, most of the works that use Equation (8) for estimating *UI* consider the term dT/dt as the slope of the temperature rise with time. In this regard, it is important to ensure that the estimation is made within a linear behavior range. For instance, Figure 2a shows how the linearity of this relationship is lost at longer sonication times due to the heat losses of the vessel, causing distortions in the *UI* estimation, especially when higher levels of wave amplitude are used. Nevertheless, calorimetric estimation of *UI* results in lower values of the actual cavitation energy measured by hydrophones because only a fraction of this energy is inverted in bubble formation and explosion, leading to a temperature rise [39]. This problem has been solved in most of the updated devices that have systems for measuring the energy delivered during sonication. Another way to express these results is in terms of power density (Pd), which is the ratio of ultrasonic power (P) to the processing volume (W/mL). If the latter is the option chosen to compare results, it is worth mentioning that what occurs in small volumes (<50 mL) is not linearly scalable to larger volumes [40]. Furthermore, from Equation (7) it can be noticed that liquid density has an opposite effect on the *UI*. More concentrated and viscous solutions tend to attenuate the propagation of the wave, a phenomenon known as acoustic impedance [41]. However, in practice, this effect tends to be marginal because of the high reduction in density and viscosity that usually occurs due to the increase in temperature during sonication.

US devices can operate continuously or discontinuously in duty cycles. A duty cycle refers to the actual time that the US application lasts, and it affects either the *UI*, the energy consumption, or the cavitation phenomenon. Some authors have referred to the duty cycle mode as "pulse", which is incorrect because it would imply that the energy system works like a laser, that is, energy stored and accumulated for a certain time and then delivered to the sample, which is not the case with US. If US processing works under a duty cycle, it is important to provide information regarding the number of time units of application per total time units of the cycle (e.g., 5 s/9 s), as a fraction of the unit of time that the US application lasts (e.g., 0.5 s/s) [27], or as alternating ratio of application time with respect to idle time (e.g., 5 s on/5 s off). Figure 2b shows an example of the effects of the operating conditions already mentioned on *UI*. Either in duty cycle or in continuous operation, increasing the amplitude of the wave significantly increases the *UI*

value, regardless of the solution concentration. Likewise, for the same wave amplitude level, the continuous application of US significantly increases the *UI* received by the sample compared to the duty cycle. All these factors must be weighed because the *UI* is the energy that the enzyme "feels" during sonication.

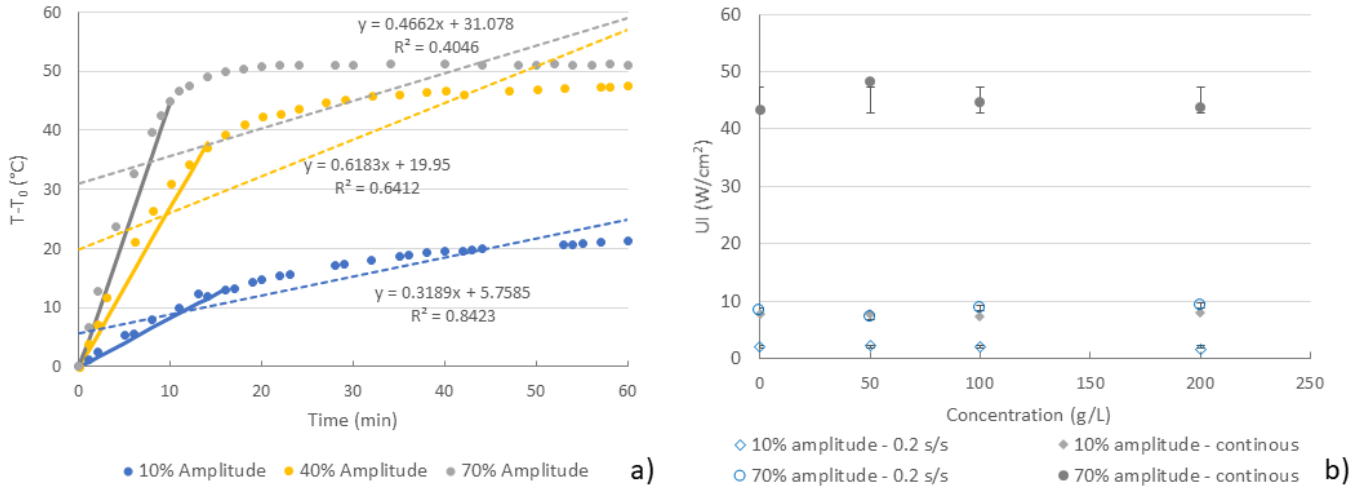

Figure 2. (**a**) Temperature increase with sonication time in distilled water at different wave amplitudes. Continuous lines show experimental data fitted to linear behavior ($R^2 \geq 0.98$); dotted lines show the linear fit assumed if the entire sonication time is considered. Poor fitting can be observed in those cases as shown by their respective equations, seriously increasing the bias of the determined slope value and, by default, the estimated *UI*. (**b**) Effect of wave amplitude and sonication mode (duty cycle and continuous) on UI (W/cm^2) at different substrate concentrations. Determinations were performed in triplicate with lactose solutions (100 mL) of different concentrations in a probe device (20 kHz, 550 W output) within 6 min and using Equation (8) ($R^2 \geq 0.99$).

It is clear that much attention and care should be taken when comparing results reported in the literature regarding the sonication conditions applied to enzyme processes. A review from Delgado-Povedano and Luque de Castro (2015) provides examples of works wherein the input power is mistaken with the *UI*, or when this processing variable is calculated as the ratio between the input power per area of the transducer [27]. Moreover, the authors present cases wherein the frequency or the type of setting are not specified. All these conceptual errors should be avoided because they are critical to understanding the effects of US processing variables on enzyme catalysis.

2.3. Ultrasound Devices

Ultrasonic baths are the cheapest configuration and are mostly used for the extractions of bioactives, but also for some enzyme applications (Figure 3a). US baths come in a range of ultrasonic frequencies, from 20 kHz to 1 MHz [42], and with different sizes, from 500 mL to 200 L. Most operate at one fixed value of frequency, but there are a few commercial devices with the possibility to use two or more frequency levels. Usually, the container with the reaction medium is inserted into the bath with an overhead stirrer that allows the constant mixing of the solution. Although this configuration is useful for preliminary studies, its use entails a series of limitations to bear in mind. US baths are known for the low reproducibility of results [29] because the position, thickness, shape, and distance of the vessel to the transducers have a high incidence on the acoustic field delivered to the reaction medium [40]. Furthermore, the delivered intensity is highly attenuated by the water contained in the bath and the glassware used [23]. In fact, as the water volume of the bath is several times higher than the reaction volume, this kind of configuration is not scalable. On the other hand, commercial ultrasound probes (Figure 3b) have the advantage of directly delivering the ultrasonic field to the reaction with minimal energy loss. Usually,

these devices provide only one frequency to operate within values from 20 to 100 kHz; however, some of them offer different frequency values by exchanging the tip. In fact, because the probe tip also has a high impact on the amount of ultrasonic intensity that is delivered to the solution, most commercial probes have options for changing the shape and size of the tip depending on the reaction volume. For instance, microtips produce highly concentrated intensities in small volumes (<20 mL). However, it is common that the maximum amplitude used in these applications is restricted by the device manufacturer for safety reasons. In contrast, the maximum power levels of the equipment can be used with larger diameter tips. By default, most of the probes come with tips whose diameters rank from 1 to 2 cm. Nevertheless, there is a maximum volume (generally a few liters) in which the cavitation delivered by these probes is effective because their ultrasonic intensities rapidly decrease both radially and axially. Hence, the results obtained with these devices are not directly scalable [22]. Besides, considering the effect of the thermal increase caused by ultrasonic cavitation, the reaction vessel must be jacketed and connected to a heat exchanger to keep the temperature constant, thus avoiding the fast thermal inactivation of the enzyme. The latter is one of the main issues for enzyme reactions assisted by US that are mostly performed in batch. Nevertheless, thanks to the existence of US reactor chambers (flow cells), continuous operations in the "flow-through" mode allow the processing of larger volumes with improved ultrasonic exposure uniformity, simpler temperature control, and results practicable for scaling up purposes [43].

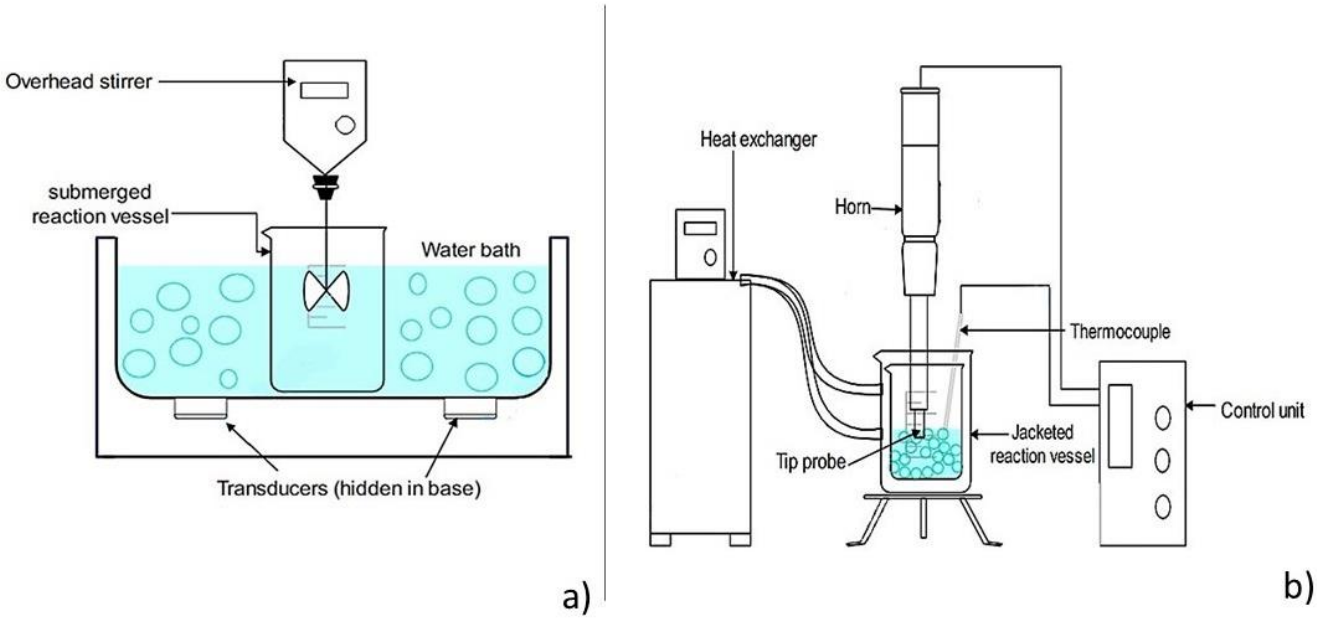

Figure 3. (**a**) Set up of US bath reactor device, (**b**) and US probe reactor device.

Both pilot-scale (up to 50 L) and large-scale applications of ultrasound reactors (500 to 1000 L) have been possible thanks to the development of novel ultrasonic transducers that have been tailored for specific requirements. If larger processing volumes are required, these devices are assembled to work simultaneously in parallel arrangements [23,30,42,44], as well by operating with continuous flow recirculation [43]. Fortunately, industrial applications have been shown to require much less power than reported in laboratory devices [40]. Hence, it could be thought that US laboratory-scale studies are a very good starting point because they show the possible effects occurring in a food matrix under a poorer energy requirement scenario compared to industrial applications. The reader may consult the previous reviews of Chemat et al. (2011) and Chemat et al. (2017), wherein interesting examples of ultrasound extraction of essential oils and bioactive compounds for food and non-food uses have been implemented at an industrial scale [29,45].

3. Effects of Ultrasound on Enzyme Structure

Enzymes are polypeptide chains arranged in a particular configuration on which catalytic activity depends. Catalytic activity resides in the active site, which is a small portion of the molecule made up of a few amino acid residues, which are directly involved in the mechanism of substrate transformation [46]. Although the information about the specific mechanism by which the enzyme is excited by US waves has not been studied in detail, it is well known that enzyme activity is governed by the configurations of the active site and, in this sense, a small variation in the medium conditions and/or composition may cause significant alterations in the three-dimensional structure of the enzyme that is essential for performing catalysis [47,48]. Most of these alterations in the protein structure reflected on enzyme activity have been observed between 20 and 100 kHz [26,49].

As enzyme activity changes as a consequence of structural alterations, it is very important to understand how the enzyme molecules undergo changes under US waves. The crystalline structure of a protein significantly differs from its structure in solution; therefore, techniques such as X-ray diffraction (XRD) or scanning electron microscopy (SEM) are not helpful for unraveling the changes that enzymes undergo in reaction media. One suitable technique is fluorescence spectroscopy, which studies the conformational changes in the local environment of tryptophan units [50]. The fluorescence of tryptophan is affected by its environment, allowing researchers to obtain information about the protein structure and the reaction mechanisms of enzymes [51]. The variation of the intrinsic fluorescence of an enzyme molecule has been used as an indicator of the alteration in the secondary structure of the enzyme by the exposure of tryptophan residues. This results in having a positive effect on enzyme activity by the exposure of the active site, allowing the substrate to interact more easily with the enzyme, and therefore increasing the reaction rate [47,52,53]. It has been proposed that when the enzyme is under the appropriate US condition, it undergoes unfolding that exposes the active site so that the substrate can reach it faster and the reaction rate increases [47,54,55]. Moreover, it has been proposed that, altogether with the enzyme unfolding, US waves improve transport phenomena, which increases the enzyme–substrate interaction and the subsequent product release rate [41].

Circular dichroism (CD) is a helpful, non-destructive technique to explore the structure of proteins in solution. This technique allows evaluating the conformational changes in optically active molecules. Among their characteristics, CD is an easy, consistent, and rapid tool for monitoring conformational changes in protein structures [55]. In enzyme molecules, UV-CD spectra allow identifying the changes in the secondary structure of enzymes. This tool identifies variation in the proportion of α-helix, β-sheet, β-turns, and random coil structures when the enzyme is under any kind of disturbance in solution, as is the case with US [27,56]. Studies from Ma et al. (2011) [57] and Bashari et al. (2013) [58] have evidenced that an enzyme under US treatment experiments changes within its UV spectrum at wavelengths below 200 nm, which indicates variations in the proportion of α-helix and β-sheet structures.

Another widely used technique for studying conformation changes in enzymes is Fourier Transform Infrared (FTIR) spectroscopy. This technique is very sensitive to the chemical composition and distribution of molecules, making it a reliable tool for the study of protein 3D structures and molecular mechanisms of reaction, unfolding and misfolding [46,59]. It has been reported that IR absorption bands between 2551 and 724 cm^{-1} provide valuable information about the amino acid side chains in proteins. The changes in enzyme structure reported by FTIR include an increase in or depletion of peak intensity in the amino or carboxyl regions of the spectrum [46].

The three above-mentioned techniques can be helpful in the study of the effects of US on enzyme structure. Nonetheless, this information is not enough to completely elucidate whether the ultrasonic waves affect only the structure of the enzyme and the substrate or whether the transport phenomena occur in the reaction media, which may cause improved kinetic parameters and/or increased enzyme activity. Despite this lack of knowledge, it is generally well accepted that *UI* is the main parameter affecting the catalytic

behavior of enzymes. Changes in the 3D conformation of the enzyme increase the enzyme activity by the production of stable cavitation bubbles, for which a low-intensity and low-frequency US irradiation must be used. When *UI* intensity increases and exceeds the optimal value, a transition phase occurs, leading to a decrease in enzymatic activity because of the denaturation of the polypeptide chain [60]. Some of these changes may result from mechanical stress, but others may be produced by the sonochemical reactions occurring in the medium. According to Mawson et al. (2011), three zones for sonochemistry have been identified [61]: within the cavitation bubble, at the cavitation bubble surface, and within the bulk liquid surrounding the bubble. The concentration of hydroxyl radicals is highest at the interface between the bubble and the surrounding liquid, therefore surface-active materials that accumulate at this interface are most prone to sonochemical attack. Enzymes in free solution have the potential to act as surface-active agents and associate with the cavitation bubble surface. In this circumstance, enzymes are prone to sonochemically induced radical attack, which may cause the breakdown of hydrogen bonding and van der Waals interactions in the polypeptide chains, leading to the modification of the secondary and tertiary structures of the protein [61].

Another important parameter is the irradiation duty cycle because this controls the time in which the enzyme is exposed to sonication. When a low duty cycle is used, the enzyme reaches its maximum activity over a long process time; however, if continuous and prolonged irradiations are used, more heat is generated, which may also cause partial dissociation of the enzyme structure, with consequent inactivation. It then follows that the increase/decrease in enzymatic activity will not only depend on the ultrasonic processing and the reaction medium, but also on the natural properties of the enzyme [28]. Therefore, the great biodiversity of enzymes does not allow proposing a general mechanism, thus opening a research field to be explored on a case-by-case basis wherein kinetic modeling may offer an important complement for a better understanding of the causes by which a biocatalyst is affected (positively or negatively) under US.

4. Enzyme Bioprocessing Assisted by Ultrasound in Agri-Foods

A biorefinery is a new concept developed to meet the challenges of the 21st century that permits the valorization of the majority of secondary and primary metabolites from plant matrices where the use of green technologies, such as US, deserves special attention [45,62]. In addition, robust enzyme-based biocatalytic systems represent a valuable approach for augmenting the economic and environmental sustainability of food production [4]. Merging these two principles may result in interesting opportunities for the creation of added value in the agri-food sector.

US applications for enzyme food processing can be performed before and during the reaction, usually to improve the accessibility of substrates to the enzyme active site by increasing the reactivity, or when treating with complex macromolecules or in systems that present mass transfer limitations, such as in immobilized enzymes. Figure 4 presents examples of how US may affect enzyme catalysis. In the following section, the application of this hybrid technology refers to three different situations related to the agri-food sector: enzymatic extractions assisted by US, enzymatic hydrolysis assisted by US, and US in enzymatic reactions of synthesis.

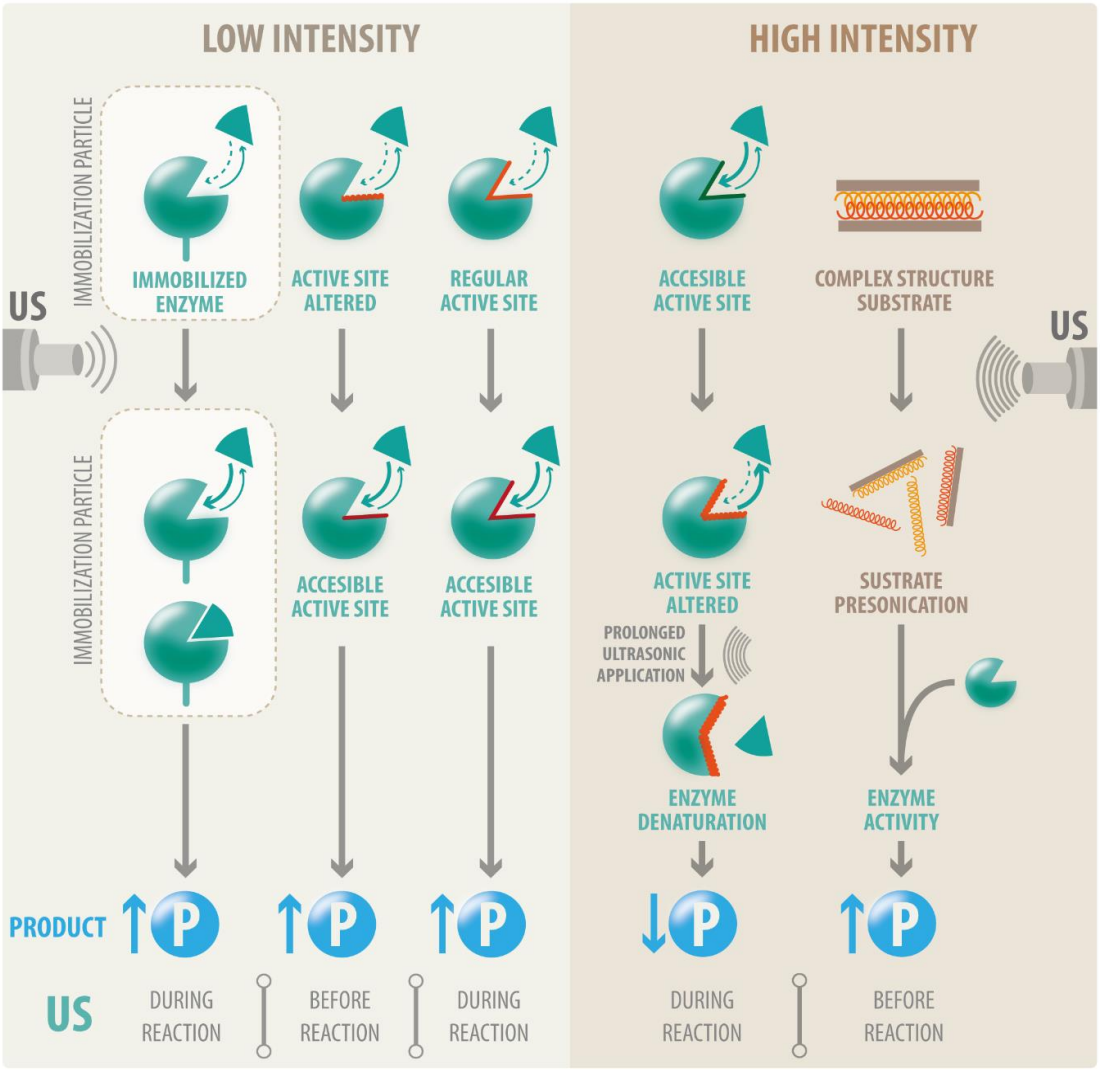

Figure 4. Different strategies of enzymatic processing assisted by US applied in foods.

4.1. Ultrasound-Assisted Enzymatic Extractions

One of the novel methods that has been developed for the extraction of bioactive molecules corresponds to extraction assisted by enzymes combined with ultrasound (UAEE) [63]. The cavitation process causes physical damage to cell walls or cell membranes [64], increasing the cell permeability with the consequent leaching of biomolecules [65]. As US can also increase the activity of the enzyme, a synergistic effect occurs that improves the extraction of compounds from the cell [28]. Table 1 shows examples of UAEE employed for the extraction of bioactive compounds from food and food waste, as well as for improving fruit juice processing.

Ke (2015) [66] investigated ultrasonic extraction based on an enzymatic treatment to extract polysaccharides from *Lentinus edodes* (one of the most widely consumed edible mushrooms). The results showed that the optimal conditions of ultrasonic extraction were an ultrasonic power (P) of 340 W, a material–water ratio 1:30 (g:mL), and a sonication time of 14 min. Under these conditions, better performance in the extraction of polysaccharides was obtained when using US than when using hot water, obtaining a higher yield and shorter processing time; extraction could be carried out at a lower temperature. In another study, Liao et al. (2015) used UAEE to prepare a polysaccharide extract from the Asian clam *Corbicula fluminea* using a commercial papain (1800 UI/mg) followed by the application of US [67]. Comparing the results with those obtained from the enzyme-assisted extraction (EAE), the yield obtained by UAEE was 36.8% higher, reducing the extraction time from 4 h to 32 min. Optimization was carried out by response surface methodology

(RSM), which determined that this improvement was achieved at P of 300 W, 62 °C, and material–water ratio 1:35 (g:mL) using a probe ultrasonic device. However, information regarding the diameter of the transducer and the sonication mode (continuous or duty cycle) was not reported. From a functional perspective, the results also revealed that UAEE produced extracts with lower molecular size and higher superoxide radical scavenging activity compared to those obtained from EAE. These works are examples of extractions performed with highly diluted solutions, which in turn means a high volume of solvent to be removed in the following downstream stages. If UAEE is meant to intensify the process, the results should be evaluated not only in terms of performance but also considering energy consumption and environmental metrics, such as the environment factor (E) and atomic efficiency proposed by Sheldon (2018) [68]. E is a fast metric for the evaluation of the environmental impact that accounts for the total mass of all the waste generated in a technological process per unit mass of the product obtained [69]. In this way, it is possible to establish whether this type of technological innovation can be effectively translated into more sustainable processes.

UAEE has been successful in more concentrated colloidal dispersions. Haji and Taghian (2017) evaluated the suitability of US pretreatment to extract oil from peanut seed powder in n-hexane, material–solvent ratio of 1:4 (g:mL), using a commercial cellulose [70]. At a constant pH of 4.75, the extraction performance gradually increased with time and cellulase concentration, reaching a maximum value at 42.4 min while at a cellulase concentration of 1.2%. Wang et al. (2014) used UAEE to extract arabinoxylan from wheat bran using endo-1,4-β-xylanase (EC 3.2.1.8) from Bacillus subtilis in a US bath reactor (transducer area ~ 27 cm^2) [71]. The processing variables studied were material concentration (20–60 g/L), enzyme dose (2.0–6.0 g/L), extraction temperature (40–60 °C), extraction time (20–100 min), and P (50–300 W). As they provided information with respect to the transducer diameter (5.87 cm), P could be expressed as *UI*, which ranged from 1.85 W/cm^2 to 11.09 W/cm^2. The results showed an increase in the extraction performance with *UI* up to 7.49 W/cm^2, corresponding to a peak of the enzyme activity at this value, which decreased once was exceeded. The optimal processing conditions were: raw material concentration, 50 g/L; enzyme dose, 4.5 g/L; extraction temperature, 50 °C; extraction time, 70 min; and *UI*, 6.65 W/cm^2. The use of US increased the extraction of arabinoxylan by approximately sevenfold. The authors explained this improvement based on two facts: (i) the cavitation effect produces a more homogeneous mixture, which enhances the movement of the reagents towards the active site of the enzyme, and (ii) the oscillation of cavitation can induce a radiation force and microstreaming, which can cause a change in the active stereo-configuration of the enzyme, thus generating a change in the availability of the active site, resulting in improved activity [72].

UAEE has also been evaluated for its potential to improve the quality, yield, and health properties of fruit juices. Tchabo et al. (2015) applied US and enzyme simultaneously to improve the quality of mulberry must [73]. The processing variables were frequency (22 to 42 kHz), enzyme concentration (0.01 to 0.02%), and maceration time (10 to 30 min), keeping a constant temperature (20 °C), P (60 W), and duty cycle mode (10 s/15 s). The authors concluded that UAEE could be employed to enhance the color by increasing the concentration of phenolic compounds and flavonoids in the must and reducing maceration time. Under the optimal processing conditions (33.8 kHz, 11.5 min, and 0.01% enzyme concentration), no damage to the enzyme structure seemed to occur. Later, Bora et al. (2017) used US pretreatment for the extraction of juice from banana pulp followed by enzyme extraction using cellulase and pectinase [74]. The studied variables were ultrasonication time (0 to 30 min), cellulase concentration (0 to 0.2%), and pectinase concentration (0 to 0.2%). US pretreatment alone did not significantly increase the yield of juice extraction, however, when combined with both enzymes, a much higher yield was obtained than in the control (89.4% and 47.3%, respectively). The recovery of valuable compounds from agro-industrial waste has also been studied using UAEE in oil extracted from perilla seeds [64], lycopene extracted from industrial residues of tomato processing [75], phenolic compounds extracted

from *Trapa quadrispinosa* residues [76], and oil extracted from pomegranate seeds [77]. In all cases, significant performance improvements were obtained with respect to conventional extraction methods.

An important fact to highlight is that, in most of the cases mentioned above, UAEE had been optimized through response surface methodology. RSM combines mathematical and statistical tools to analyze the effects of multiple factors and their interactions with minimum experimental data, determining the most relevant factors and their influence ranges [78]. Although this approach may be useful, attention must be paid to the interpretation of the information. For instance, when enzyme concentration, the solute–solvent ratio, or both are selected as variables, the different combinations that the experimental design generates result in reactions that are carried out at different enzyme–substrate ratios, which in turn changes the reaction rate. Therefore, it is crucial to determine if an eventual improvement in the UAEE is due to the effect of the US cavitation itself or to a different kinetic scenario. Furthermore, it is well known that enzymes are more prone to inactivation when they are used at low concentrations, a situation that also occurs when they are subjected to US [61], and therefore this should be weighted in an experimental design.

Table 1. Ultrasound-assisted enzymatic extractions.

Source	Biomolecule/Product	Enzyme	Extraction Conditions	Results	Reference
Lentinus edodes (Edible mushroom)	Polysaccharides	Cellulase	Water/mass of *L. edodes* was 30:1. Treatment was sequential: enzymatic treatment followed by ultrasound treatment.	The optimal conditions of ultrasonic extraction were: ultrasound power 340 W, and ultrasound time 14 min. Under these conditions, the yield of polysaccharides was 14.3% (*w/w*), (weight of polysaccharides/dry weight of *L. edodes*).	[66]
Corbicula lumine (Asian clam)	Polysaccharides	Papain	The volume of extraction was 50 mL, and the experiments were carried out applying ultrasound as pretreatment of the enzyme reaction.	The optimal extraction conditions in ultrasound power of 300 W were: temperature 62 °C and ultrasound time 32 min. The yield of polysaccharides was 36.8% (*w/w*), (weight of polysaccharides/weight of raw material).	[67]
Wheat bran	Polysaccharides	Xylanase	Working volume was 100 mL. Ultrasound was applied simultaneously with the enzyme. The process was carried out in an ultrasonic bath.	The optimum extraction conditions were: temperature 50 °C, 70 min, and ultrasonic power 180 W. Under these conditions, the experimental yield was 142.6 mg/g.	[71]
Curcubita moschata (Pumpkin)	Polysaccharides	Cellulase	Enzymatic extraction and ultrasound were simultaneous. Ultrasonic processing was carried out in a thermostatic ultrasonic processor.	The optimal conditions were: temperature 51.5 °C, ultrasonic power 440 W, and time 20 min. Under these conditions, the maximum yield was 4.33%.	[79]
Blackcurrant	Polysaccharides	Pectinase and papain	Blackcurrant fruits were processed simultaneously by ultrasound and enzymes. The fruits and enzymes were put into a 500 mL beaker, then aqueous solutions were added at different liquid to solid ratios (10:1–50:1 mL/g). The extraction process was carried out at 40 °C in an ultrasonic cell disintegrator.	The optimal conditions were: enzyme concentration 1.575%, temperature 40 °C, and time 25.6 min. Under these conditions, the yield of polysaccharides was 14.3% (*w/w*), (weight of polysaccharides/dry weight of sample).	[80]

Table 1. *Cont.*

Source	Biomolecule/Product	Enzyme	Extraction Conditions	Results	Reference
Perilla frutescens seeds (Medicinal and edible plant of Asian origin)	Oil	Cellulase, Viscozyme L®, Alcalase 2.4L®, Protex 6L®, and Protex 7L®	Perilla seed kernel powder (50 g) was mixed with water at a ratio of 6:1 liquid/solid and treated by ultrasonic, thus totaling approximately 300 mL of extraction volume. Ultrasonic pretreatment was carried out on an ultrasonic homogenizer.	The optimum ultrasonic parameters were: 250 W of ultrasonic power, 30 min, and 50 °C. The highest oil yield was 81.74% and was achieved with cellulase.	[64]
Pomegranate seeds	Oil	Cellulase and Peclyve V	The extraction of pomegranate seed oil by enzymatic treatment was carried out simultaneously with ultrasound treatment. The sonication process was carried out using a probe-type ultrasonic, and the water/seeds ratios were varied between 2:1 and 6:1 mL/g.	Ultrasonic irradiation was applied at 130 W. The combined use of enzymes and ultrasound had a maximum oil recovery of 95.8% at extraction time of 10 min, using Peclyve V at 55 °C.	[77]
Peanut seeds	Oil	Cellulase	The ultrasound was used as pretreatment, the peanut seed powder (40 g) was mixed with n-hexane (160 mL) at a ratio of 1:4 solid/liquid. The sonication was carried out at the ultrasonic bath.	The ultrasound extraction process was applied at frequency of 250 Hz and at 45 °C. The optimum condition was ultrasonic pretreatment for 33.23 min and cellulase concentration of 1.47%.	[70]
Banana	Juice	Cellulase and pectinase	100 g of the banana slices were mixed with distilled water to make pulp. The pulp was subjected to a pretreatment with ultrasonication in an ultrasound bath at 40 kHz.	Ultrasonic irradiation was applied at 50 W for 30 min. Ultrasound combined with both enzymes produced a maximum yield of 89.4% compared to 47.3% in the control.	[74]
Tomato residues	Lycopene	Endozym®-Pectofruit	The ultrasonic pretreatment was performed using a probe-type ultrasound. The extractions were performed in a double-walled cylindrical glass chamber (200 mL).	Combined sonication and enzymatic pretreatments improved the efficiency up to 39%, which was obtained in ultrasound treatment at 50 W for 30 s.	[75]
Morus nigra (Mulberry)	Flavonoids	Pectinex UF	The process was carried out with ultrasonic probe equipment. Mulberry must (300 g) was placed into an Erlenmeyer flask (500 mL) with the enzyme to be simultaneously sonicated.	The ultrasonic treatment was performed at 60 W, duty cycle (10 s on and 5 s off), at 20 °C. The UAEE treatment was employed to enhance the quality of the must and reduce the time during the maceration process of juice.	[73]
Trapa quadrispinosa residues (Water caltrop)	Phenolics	Cellulase	The stem powders (1 g) were placed into 100 mL Erlenmeyer flask. Extractions were carried out in an ultrasonic bath at 40 kHz.	The optimal UAEE conditions were 1.74% cellulase concentration, ultrasonic extraction time of 25.5 min, and temperature of 49 °C. The yield was 53.6 mg gallic acid equivalent/g dry weight.	[76]

4.2. Enzymatic Hydrolysis Assisted by Ultrasound

US may produce enzyme structure modifications with a higher exposure of the catalytic sites, which become more accessible to the substrate, facilitating enzyme–substrate interaction, decreasing the activation energy, and increasing the rate of hydrolysis [81–83]. A list of published works regarding the enzymatic hydrolysis assisted by ultrasound in food processing is provided in Table 2. In a pioneering work by Sener et al. (2006), they reported the effect of P (20–100 W) and duty cycle mode (0.1 s/s–0.9 s/s) at 20 kHz and 37 °C on the hydrolysis of milk lactose with commercial enzyme Maxilact LX 5000 from

Kluyveromyces marxianus var lactis [60]. The best US conditions were P (20 W) and duty cycle (0.1 s/s), obtaining 90% of lactose hydrolysis and residual enzyme activity of 75% after 30 min. More intensified US processing resulted in a fast enzyme activity decline and lower lactose conversion, however, strange enough, these results were not compared with a control experiment. Later on, this research group studied the same reaction and sonication conditions to hydrolyze lactose recovered from milk whey [84], obtaining similar results to before, but including in this instance a comparison with non-sonicated enzyme hydrolysis. It was reported that such US conditions produced an increase in lactose hydrolysis from 81% to 92% and in residual enzyme activity from 68% to 77%. Huang et al. (2020) investigated the hydrolysis of lard catalyzed by a 1,3-specific lipase from *Rhizomucor miehei* combined with a nonspecific mono and diacylglycerol lipase from *Penicillium cyclopiumand* assisted with US pretreatment [85]. The results showed that with a frequency of 53 kHz and P of 250 W for 5 min, the hydrolysis rate of lard was increased from 78.1% to 97%, resulting in a potentially attractive alternative to produce free fatty acids.

Table 2. Enzymatic hydrolysis assisted by ultrasound in food processing.

Enzyme	Hydrolysis Conditions	Results	Reference
Lipases	Hydrolysis of lard catalyzed by 1,3-specific lipases from *Rhizomucor miehei* combined with a nonspecific mono and diacylglycerol lipase from *Penicillium cyclopiumand* assisted with ultrasound pretreatment for 5 min, frequency at 53 kHz and ultrasound power of 250 W.	When using combi-lipases, the hydrolysis degree was 78.1%. When combi-lipases were assisted with 5 min ultrasound pretreatment before the reaction, the hydrolysis degree reached 97%.	[66]
Pectinase, xylanase, and cellulase	The equipment used was an ultrasonic bath of 9.5 L of maximum capacity at ultrasonic frequency of 40 kHz and total ultrasonic power 220 W.	The results show that ultrasound treatment increased enzyme activities by 5% for pectinase, 30% for xylanase, and 25% for cellulase compared with mechanical stirring. The substrates were presonicated.	[67]
α-L-rhamnosidases, β-glucosidases, and limoninases	The sonication treatment was carried out at 40 kHz, 80 W/L, and 90 min. Working volume was ~300 mL.	The process of sonication significantly enhanced activities of α-L-rhamnosidases, β-glucosidases, and limoninases; also, the sonication reduced the hydrolysis time by 33% (30 min).	[71]
Glucoamylase	The ultrasound probe was inserted into a starch solution and glucoamylase solution. The sonication was carried out at different ultrasonic powers (45–360 W), temperatures (35–75 °C), and treatment times (10–50 min). Reaction volume was fixed at 25 mL.	Ultrasound produced a significant intensification of starch enzymatic hydrolysis catalyzed by glucoamylase; furthermore, the ultrasound promoted the enzymatic hydrolysis of amylopectin, significantly enhancing starch hydrolysis.	[79]
Glucoamylase	The glucoamylase solutions were subjected to different ultrasonic conditions of power (0, 420, 540 W) for 10 min at 60 °C. Ultrasonic reactor worked with 2 L.	The glucoamylase activity was increased by 21.07% over the control with ultrasound. However, at the application of high ultrasonic power (540 W), the rate of reaction decreased, probably due to decreased enzyme activity.	[80]

Table 2. *Cont.*

Enzyme	Hydrolysis Conditions	Results	Reference
Alcalase	The ultrasound was used as pretreatment, using a probe at 200 W and five different frequencies (20, 28, 35, 40, 50 kHz). After pretreatments, the solutions were adjusted to temperature of 50 °C. Reaction vessel used was 600 mL.	The results showed that ultrasound pretreatment increased the degree of hydrolysis compared to that of the control for up to 75 min, even so, different substrate concentrations were used (5–25 g/L).	[64]
Alcalase	Ultrasound pretreatment for the enzyme hydrolysis of defatted corn germ protein with single frequency (20 KHz) and with a multi-frequency application (20, 28, 35, and 40 KHz) at constant Pd (100 W/L). Processing volume was ~1 L.	Ultrasound increased the reaction rate constant values in an average of 51%, while under the multi-frequency ultrasound scheme, it was increased by 56%.	[77]
Alcalase	The ultrasound substrate pretreatment with sweeping frequencies in cycles (40 +/− 2 kHz) and 200 was tested using a working solution of 300 mL.	Multi-frequency power ultrasound pretreatment was able to improve the enzymatic hydrolysis; kinetic studies showed that SFPU pretreatment decreased the apparent constant K_M by 32.8%.	[70]
Alcalase	The experiments of multi-frequency power ultrasound pretreatments were conducted under different ultrasound frequency modes: mono-frequency (20, 40, and 60 kHz), dual-frequency (20/40, 20/60, and 40/60 kHz), and tri-frequency (20/40/60 kHz). The system had a volume capacity of 3 L.	Results showed that multi-frequency power ultrasound pretreatments in tri-frequency mode significantly improved the degree of hydrolysis value of casein in 12%.	[74]
β-galactosidase	Enzyme was sonicated at 20 kHz and acoustic power from 20 to 100 W, using milk as substrate in a reaction volume of 250 mL.	Ultrasonic treatment resulted in lactose hydrolysis degree of 90% and residual enzyme activity of 75% at the optimum operational conditions (acoustic power of 20 W, duty cycle rate of 10%, and enzyme concentration of 1 mL/L), resulting in a significant improvement compared to the control reaction without ultrasound.	[74]

Enzyme hydrolysis assisted by US may result in being useful for the beverage industry. An interesting work by Dalagnol et al. (2017) reported the effect of US on a commercial enzyme preparation (Zimopec PX5®) used for juice clarification containing pectinase (PE), xylanase (XLN), and cellulase (CE) activities [86]. The authors assessed the effect of US as a substrate presonication step, for presonication on the enzymes, and also during the hydrolysis reaction using a US bath at 40 kHz and 220 W of P. When xylan and cellulose were subjected to sonication before the enzymatic reaction, the activity of XLN and CE increased by 25% and 17%, respectively, suggesting a modification of the substrates' structure that facilitates their access to the corresponding enzymes, but no modifications to the activity of PE were detected. A similar trend occurred when the enzymes were presonicated. However, when PE was subjected to US during the reaction, the catalytic efficiency (V_{max}/K_M) increased by 24.5%, which was mainly due to a significant decrease in K_M, indicating an enhanced affinity between the PE and pectin. For XLN and CE, the application of US during the reaction increased the catalytic efficiency of both enzymes by approximately 17%, but those changes were attributed to a significant increase in the V_{max}, indicating an increase in the reactivity of the enzyme–substrate complex, accelerating the hydrolysis process. Gao et al. (2021) studied the impact of sonication on the degrees

of enzymatic hydrolysis of the bitter compounds limonin and naringin in Ougan juice (*Citrus suavisima Hort.ex Tanaka*) [82]. The results showed that, when using an *Aspergillus niger* koji extract containing α-L-rhamnosidase, β-glucosidase, and limoninase under US (40 kHz and 80 W/L), the degrees of enzymatic hydrolysis of naringin and limonin were increased by 89.9% and 36.2%, respectively. Furthermore, sonication reduced the hydrolysis time by 33% (30 min). These improvements were mainly due to the increased activity of all enzymes with respect to the control samples. Wang et al. (2020) studied the effect of US for the enzymatic saccharification of starch at 22 kHz at temperature ranging from 35 to 75 °C and P ranging from 90 to 360 W [87]. Using a glucoamylase from *Aspergillus niger,* they tested the US as enzyme pretreatment, as starch pretreatment, and during the saccharification reaction. Enzyme presonication did not cause a significant effect on the degree of hydrolysis, while its application to the substrate produced a slight improvement with respect to the reaction performed without US. Meanwhile, sonicating the reaction medium produced a fivefold increase in the degree of starch hydrolysis compared to the control. Enzyme activity remained unaltered within 50 min if P and temperature were kept below 270 W and 65 °C, respectively. Above these values, the enzyme was drastically inactivated, probably because of the combined effect of temperature and the sonochemical and mechanical effects caused by US [61]. On the contrary, below these critical values, the enzyme hydrolysis assisted by US produced an increase in the reaction rate by helping the dispersion of the aggregates of glucoamylase and starch, reducing the mass transfer barrier and facilitating the removal of the hydrolysis products after the reaction [87]. Similar results were also reported by Meng et al. (2018) with a commercial glucoamylase of unspecified origin having a higher resistance to US cavitation. In such a case, the catalytic constant (k_{cat}) of the enzyme increased by 56 at 420 W and 60 °C, but at 540 W, the rate of reaction decreased below the values obtained in the control sample [88]. CD spectra revealed that a proper US power caused an increase in the content of α-helix structure and random coil and a decrease in the content of β-sheet of glucoamylase, which positively affected its active site.

The production of bioactive peptides is of interest to the food industry because they provide health benefits such as the mitigation of cardiovascular diseases, hypertension, and diabetes, and cancer prevention, while it allows the reuse of proteins of low commercial value. Peptides are encrypted within protein molecules and their release involves the application of controlled enzyme hydrolysis, which usually requires long reaction times with low conversions and low degrees of hydrolysis [89]. In this regard, substrate US pretreatment is an alternative that has been studied for this purpose and that can be performed under mono-, dual-, or multi-frequency systems. Wali et al. (2018) evaluated the effect of sequential dual-frequency ultrasound pretreatments (which implies the alternation of the frequency applied during sonication) on rapeseed protein hydrolysis using Alcalase 2.4 L from *Bacillus licheniformis* as a model enzyme and five different frequencies (20, 28, 35, 40, and 50 kHz) [90]. They determined the Michaelis–Menten constant (K_M) and the average value of apparent breakdown rate constant corresponding to the binding frequency between enzyme and substrate, represented by K_A. Compared to the control, the K_M value decreased by 17.61% while a 10.47% increase in K_A was observed. Musa et al. (2019) tested the US pretreatment for the enzyme hydrolysis of defatted corn germ protein with a single-frequency (20 KHz) and with a multi-frequency application (20, 28, 35, and 40 KHz) at constant Pd (100 W/L) [91]. The results showed that, compared to traditional enzyme hydrolysis, samples subjected to single-frequency US increased the reaction rate constant values by an average of 51%, while under the multi-frequency US scheme, this was increased by 56%. Jin et al. (2015) performed a similar study at dual frequency (20–40 kHz), resulting in a significant improvement in the degree of hydrolysis [89]. It was concluded that US pretreatment modified the conformation of the protein by inducing its molecular unfolding, which caused more hydrophobic groups and regions within the enzyme molecules to be exposed to the medium and be available to the substrate. More recently, Xu et al. (2020) assessed the effect of dual- and multi-frequency US pretreatment

of casein for further hydrolysis with Alcalase [92]. For such purpose, pretreatments were conducted under different US frequency modes, which were mono-frequency (20, 40, and 60 kHz), dual-frequency (20/40, 20/60, and 40/60 kHz), and tri-frequency (20/40/60 kHz) at constant Pd (150 W/L) over 40 min under duty cycle (10 s on/5 s off). Surprisingly, no significant variations with respect to the degree of hydrolysis were found for the samples subjected to mono- and dual-frequency as compared to the control (without US), but under tri-frequency, an improvement of ~12% was obtained. Based on these and other previously reported results, the authors concluded that the discrepancies may be attributed to the fact that US has a significant effect on the protein structure by generating resonance with their own frequencies of proteins. The resonance can cause more enzyme reactive sites to be exposed, thus the efficiency of hydrolysis is improved. As the enzymes have different resonances depending on their molecular structure, which may provoke different behaviors, this interesting finding suggests several research opportunities for evaluating the use of enzyme processing assisted by US with dual- and multi-frequencies during the reaction. Later on, an industrial-scale continuous enzyme bioreactor (500 L) assisted by ultrasound was developed by the same Chinese research group, as stated in a recent review [44]. The mixture can be treated by dual-frequency ultrasound in the sequential or simultaneous working mode under the frequencies of 20 and 40 kHz and the power level of 2 kW. According to the time of enzyme addition, the reaction can be divided into ultrasonic modification on substrate materials or ultrasonic treatment during enzymolysis. The resulting mixture is pumped to a membrane bioreactor for separating the biocatalyst from the reaction products. Subsequent downstream processing by membrane filtration is performed according to the desired particle size of the liquid and then subjected to spray drying to obtain the final products [44]. To the best of our knowledge, this is the largest enzyme bioreactor assisted by ultrasound developed, and according to the authors, it has been successfully applied to produce peptides from rapeseed protein, wheat gluten, defatted wheat germ protein, zein protein, and rice proteins.

4.3. Ultrasound in Biocatalysis

Literature reveals that lipases are the most studied enzymes in synthesis reactions assisted by US. Lipases are enzymes with an outstanding ability to produce a wide variety of biobased products because their mode of action can be reversed in organic media, therefore they can catalyze esterification and transesterification reactions with high enantiomeric selectivity. Despite this, there are cases where poor solubilization of the substrates in a non-toxic reaction solvent would lead to long reaction times and poor regioselectivity, which reduce the benefit of using these biocatalysts [93]. However, there are quite interesting recent works on the use of US-assisted synthesis reactions with lipases. Most of them have been focused in intensifying the production of biobased materials, such as surfactants, perfumes, cosmetic raw materials, and plasticizers [94–97], but applications in the agri-food sector have been seldom considered. One of these applications refers to the enzymatic synthesis of structured triglycerides (ST). ST are triglycerides that are chemically or enzymatically modified to change their structure by altering the fatty acid composition and/or their positional distribution in the glycerol backbone with the purpose of enhancing some nutritional and/or physicochemical property [98]. Liu et al. (2015) studied the effect of US during the first minutes of reaction in the production of 1,3-dioleoyl-2-palmitoylglycerol (OPO), one of the most important triglycerides present in human breast milk, which may result as important to incorporate in infant formulas [99]. Synthesis of this ST was performed from tripalmitin (PPP) and oleic acid (OA) using different commercial lipases. The best results were obtained with Lipozyme RM with sonication at 20 kHz, over 6 min, 50% of wave amplitude (ultrasonic power not declared), 3 s on/9 s off duty cycle, 1:8 PPP/OA molar ratio, 12% enzyme dosage, and at 50 °C. This short US pretreatment was enough for producing 35.9% OPO within 1 h, while under conventional stirring, 4 h was required to produce a similar OPO content. Using the same biocatalyst and immobilized lipase from *Candida antarctica* (Novozyme N-435), More et al. (2019) evaluated the intensified

synthesis of an ST with fish oil, flaxseed oil, and rice bran oil as fatty acids sources to deliver a product with health properties such as LDL-cholesterol reduction, improved cognitive functions and blood circulation, and protection against cardiac and diabetic diseases [100]. The intensification was carried out using supercritical CO_2 and US. With respect to US, the best yield of ST was similar to that obtained with supercritical CO_2 (84%), and was obtained with 6 s on/4 s off duty cycles in 9.6 h while using Novozyme N-435, with a significant increase in yield with respect to conventional enzymatic synthesis (77%), which normally requires more than 24 h. This result can be explained because cavitational effects improved the mixing between two phases. Additionally, Novozyme N-435 could be reused for up to 15 cycles, with a marginal lowering of yield, which was not affected by sonication.

Flavonoids are phenolic compounds that promote health benefits and are usually present in higher concentrations in non-edible fractions of fruits and vegetables. There is increased interest in their consumption, but they are poorly soluble in lipophilic systems, which limits their application as additives in functional foods. Facing this challenge, Zheng et al. (2013) studied the enzymatic esterification of rutin and naringin with different free unsaturated fatty acids (oleic acid, linoleic acid, and α-linolenic acid) to produce the corresponding flavonoid esters with US at 25 kHz, P at 200 W for rutin and at 150 W for naringin, as a pretreatment over 1 h using lipase Novozym 435 [101]. US reduced the reaction time in 24 h with respect to the traditional stirring without a significant reduction in enzyme activity, which was explained because increased emulsification was caused by sonication. More recently, Nieto et al. (2013) performed a US-assisted enzymatic synthesis of xylitol fatty acid esters using Novozyme N-435 in a solvent-free system [102]. Fatty sugar esters impair high emulsifying and stabilizing properties, with tasteless, odorless, and non-toxic effects, making them highly appreciated for industrial applications in foods. Under conventional stirring, the authors detected that the mutual immiscibility between the different fatty acids and xylitol substrates and the semisolid character of the initial reaction mixture precluded product formation. However, substrate presonication for 15 min (20 kHz; 0.75 s/s duty cycle; 40–70% wave amplitude), followed by sonication under the same conditions during the reaction (90 min), resulted in melting the lauric acid, favoring its transfer to the active site of the enzyme to form the acyl intermediate. Meanwhile, water was released as a by-product from the enzyme microenvironment and retained by the undissolved hygroscopic xylitol, avoiding the reversible hydrolytic pathway. The reaction proceeded faster at 70% of wave amplitude, resulting in a product yield of ~89%. These results are quite promising because they allow the production of natural biobased products without the large number of solvents typically used in this kind of system, which may facilitate its scaling up, leading to a more sustainable process.

Galacto-oligosaccharides (GOS) are non-digestible carbohydrates derived from lactose that are properly considered as prebiotics. They can replicate the bifidogenic effect of human milk oligosaccharides by stimulating a healthy intestinal microbiota; this can improve the intestinal motility and the immune system and reduce the levels of blood cholesterol and the risk of colon cancer development, making them attractive to incorporate as functional ingredients in several food matrices, and this represents an attractive technology for whey upgrading [103,104]. However, the synthesis of GOS is challenging because it is a kinetically controlled reaction with low product yield, resulting in high concentrations of unreacted lactose and undesired monosaccharides that reduce the purity of the product, limiting their application. Therefore, downstream operations are mandatory for delivering a commercial product and represent a most significant part of the operation cost [105]. A novel proposal to solve this drawback was reported by Rico-Rodríguez et al. [54] where the impact of US on the production of GOS and gluconic acid (GA) in a multienzyme system was evaluated. GA is an interesting compound due to its technological properties (refreshing sour taste, sequestering power, chelator at alkaline pH, high resistance to oxidation); its production in GOS mixtures could expand the GOS market targets, obtaining a product with reduced calories [106]. The multi-enzyme system included the application of β-galactosidase from *Kluyveromyces lactis* and glucose oxidase from *Aspergillus oryzae* subjected

to US treatments (20 kHz, duty cycle 3 s on/7 s off, and *UI* at 423 W/cm^2) using pure lactose as substrate, and a temperature of 40 °C. Despite this, US did not produce a significant increase in GOS yield compared to the control, glucose consumption and GA production were significantly higher, obtaining the best product composition (49% GOS and 28% GA) after 2 h of reaction. Fluorescence spectrometry analysis revealed that β-galactosidase did not show conformational changes in its surface structure, while significant changes were observed in glucose oxidase causing a positive effect on enzyme activity. In fact, after 60 min of sonication, the activity of β-galactosidase increased by 121% while the activity of glucose oxidase increased by 798%. The explanation for this remarkable difference is that the collapse of acoustic bubbles can dissolve oxygen, thus increasing its available concentration in the reaction medium to produce oxidized products. More recently, the same reaction system assisted by US was evaluated while changing the lactose source (using pure lactose, whey permeate, or cheese whey) and the *UI* (203.4 and 423.1 W/cm^2) [107]. Similar results were found, and the highest yield of GOS was obtained using a lactose solution. The conversion of glucose into gluconic acid increased for all the substrates at 423.1 W/cm^2, but an increase in GOS yield from 35% to 40% was achieved when cheese whey was used as a lactose source. As cheese whey is a substrate with a complex composition (protein, fat, lactose, and mineral), it is probable that the action of US disperses solutes such as protein and fat, favoring the interaction of the enzyme with lactose and glucose. Jambrak et al. [108] report that the use of US in whey proteins (20 and 40 kHz) causes a decrease in particle size and distribution, reflected in a decrease in molecular weight of whey proteins. In addition, it allows increasing the specific free surface in all analyzed samples. These results are not only promising because they improve the performance of the synthesis of GOS directly from by-products of the cheese industry, but they also allow the obtained products to be easily incorporated into different food matrices. Finally, another advantage of the use of US in the synthesis of GOS is the increase in the activity and thermal stability of the enzymes used during the reaction, which is essential for labile enzymes with low half-life time, such as β-galactosidase from *Kluyveromyces lactis* [109]. A summary of recent works on this field is presented in Table 3.

Table 3. Summary of ultrasound applications in biocatalysis.

Synthesized Product	US Reaction Conditions	Results	Reference
Synthesis by lipase of lipid structure with high content of 1,3-dioleoyl-2-palmitoylglycerol.	Lipozyme immobilized with an ultrasound pretreatment of 6 min, 50% power, 20 kHz, and 3 s on/9 s off duty cycle was applied.	With ultrasound, the OPO content increased to 35.9% in 1 h compared to 4 h without ultrasound.	[99]
Lipase synthesis of flavonoid esters with unsaturated fatty acids.	Novozym 435 was used, applying ultrasound pretreatment with a frequency of 25 kHz, power of 200 W for routine and 150 W for naringin for 1 h.	A conversion of 83.2% was obtained, reducing the reaction in 24 h as compared to the mechanical stirring.	[101]
Xylitol fatty acid esters.	Immobilized lipase B from *Candida antarctica* was used, applying a direct sonication pretreatment for 15 min (45 s/min pulses), different amplitudes from 10 to 100%, and 40 °C.	Up to 95% yield was achieved after 90 min at 40 °C.	[102]
Synthesis of structured triacylglycerols from fish, flaxseed, and rice bran oil.	Novozyme N-435 and Lipozyme RM (LRM) lipase were used, applying ultrasound probe at 22 kHz, 240 W, and testing different duty cycles.	84.5% of product yield with an optimal cycle of 6 s/4 s (on/off) in 9.6 h.	[100]

Table 3. *Cont.*

Synthesized Product	US Reaction Conditions	Results	Reference
Production of GOS and GA in a multi-enzyme system.	Commercial β-galactosidase from *K. lactis* and commercial glucose oxidase (Gox) from *Aspergillus oryzae* were used, applying sonifier at 20 kHz, 400 W, 30% amplitude, pulses of 3 s on/7 s off, and temperature between 40 °C and 45 °C.	The best GOS product composition of 49% and 28% GA was obtained after 2 h of reaction.	[54]
Production of GOS and GA in a multi-enzyme system with different sources of lactose.	Commercial β-galactosidase from *K. lactis*, commercial glucose oxidase (Gox) from *Aspergillus oryzae* were used, applying US at 20 kHz, 400 W, submerged at 2 cm depth from the surface of the liquid, amplitude of 0, 15, and 30%, duty cycle of 3 s on/7 s off, and the temperature was kept between 40 °C and 45 °C.	Maximum yield in the production of GOS 44.9% was obtained after 60 min of reaction and the production of GA depended on the intensity of ultrasound, achieving the highest amount of GA when the intensity was 30%.	[107]

5. Future Trends and Challenges

Research on US-assisted enzymatic food processing has increased in recent years given the potential of this technology to intensify these bioprocesses under specific UI conditions. However, it is urgent to have an international research association that establishes standard criteria for the measurement of this parameter and others related to US that have a high impact on enzymatic reactions. Precisely, the lack of information within the many works that report their US processing conditions makes it difficult to compare the results obtained with different enzymes and reaction conditions. Therefore, the classification ranges proposed by some authors to refer to what is understood as high or low ultrasonic intensity may be somewhat discretionary.

Only a few studies have determined how different US processing conditions affect the kinetic parameters of the enzymes used as catalysts. There are some interesting reports, but they mostly referred to hydrolysis reactions; much less information exists on synthesis reactions under this approach. Enzymes are being increasingly used as industrial catalysts for organic synthesis, therefore there is much to be explored regarding combined systems of US/biocatalysis, especially in those systems that use immobilized enzymes and phase mixtures that can be addressed by heterogeneous phase kinetic modeling. Thus, obtaining these models would not only be interesting in terms of creating new knowledge, but also for the design and scaling up of these hybrid reaction systems.

Much future research in this area should be performed under a multidisciplinary approach by merging knowledge from food science, enzyme catalysis, computational chemistry, and multiphysics simulations for better understanding a complex phenomenon like this that occurs in a very short time domain. Last but not least, it is crucial to incorporate energy consumption measurements and environmental metrics in order to evaluate whether this hybrid technology effectively contributes to the sustainability of agri-food bioprocesses beyond the technologies presently used.

6. Conclusions

Recent advances in enzyme food processing assisted by US have been reviewed. This hybrid technology may act by modifying the structure of the enzyme and the substrate, or improving the mass transfer when both are combined in the reaction system. However, much attention and care should be taken when comparing results reported in the literature because of the lack of critical information in some experiments. Furthermore, the need for standardization in the quantification of *UI* is of paramount importance because it is generally accepted to be the main variable affecting the catalytic activity of enzymes.

In terms of number of publications, US-assisted enzyme food processing has been mostly studied for the extraction of bioactive compounds, followed by hydrolysis reactions for juice and beverage manufacturing, as well as in the production of bioactive peptides. Much less research has been conducted with respect to enzyme catalyzed reactions of organic synthesis while it is worthwhile mentioning the results reported in transesterification and esterification reactions to produce structured lipids and sugar esters. In a more incipient stage of research, US application for the synthesis of prebiotic oligosaccharides offers promising results. Most of the works in this field have been explored using the Response Surface Methodology, however, despite the usefulness of this approach, there is still a lack of information regarding specific mechanisms that positively or negatively affect the enzymes when subjected to US. However, the wide variety of enzymes and reaction conditions makes it necessary to conduct studies on a case-by-case basis. Techniques such as CD, FTIR, or SEM have revealed how certain enzymatic structures and molecularly complex substrates are affected by US; however, this information is not enough to completely elucidate whether the ultrasonic waves are affecting the enzyme structure, the substrate structure, or the transport phenomena occurring in the reaction media. In this sense, investigations using a multidisciplinary approach through food science, enzyme kinetic modeling, computational chemistry, and dynamic bubble modeling will be extremely helpful for a better understanding of the complex phenomenon that occurs in fractions of a second.

Finally, energy consumption and environment indicators are mandatory to assess if this type of technological innovation can be effectively translated into more intensified and sustainable food processing, with scaling-up potential to be developed for the biorefinery concept.

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