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USE OF NOVEL TECHNOLOGIES TO INTENSIFY ENZIMATIC REACTIONS. POWER ULTRASOUND AND SUPERCRITICAL FLUIDS

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USE OF NOVEL TECHNOLOGIES TO INTENSIFY ENZIMATIC REACTIONS. POWER ULTRASOUND AND SUPERCRITICAL FLUIDS.

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ABSTRACT

Enzymes are present in food production because food reactions take place under milder conditions of temperature, pressure, and pH than chemical reactions. These mild conditions are aimed to preserve nutritional value, flavor, and texture, which are important elements in the development of functional products. Desired traits in industrial enzymes are the ability to act over a wide pH range, higher reaction speed, thermostability, specificity, and reusability not found under extreme processing conditions. It is important to mention that consumers are looking for products with high nutritional value and quality. The aim of this work is to carry out a literature review about the use of power ultrasounds and supercritical fluids to enhance enzymatic reactions. The use of ultrasound as a pretreatment on the substrate or enzyme, or during the reaction, is described and discussed. The parameters of the reactions catalyzed by enzymes in supercritical fluids in products of interest to the food industry are also described.

KEY WORDS: enzymes, ultrasounds, supercritical fluid, biocatalysis, reaction, food

RESUMEN

Las enzimas se encuentran presentes en la producción de alimentos debido a que las reacciones se realizan en condiciones más suaves de temperatura, presión y pH, en comparación a las reacciones químicas. Estas condiciones suaves tienen por objeto conservar en los alimentos el valor nutricional, el sabor y la textura, elementos importantes en el desarrollo de productos funcionales. Los rasgos deseados en las enzimas industriales son la termo-estabilidad, la capacidad de actuar en un amplio rango de pH, mayor velocidad de reacción, especificidad y capacidad de reutilización, que no se encuentran en condiciones extremas de procesamiento. Es importante mencionar que los consumidores buscan productos con alto valor nutricional y calidad. El objetivo del presente trabajo es realizar una revisión bibliográfica sobre el uso de ultrasonidos de potencia y de fluidos supercríticos para intensificar las reacciones enzimáticas. Se describe y discute acerca del uso de los ultrasonidos como pretratamiento sobre el sustrato o la enzima, o durante la reacción. También se discute sobre los parámetros de las reacciones catalizadas por enzimas en fluidos supercríticos, en productos de interés para la industria alimentaria.

PALABRAS CLAVE: enzimas, ultrasonido, fluido supercrítico, biocatálisis, reacción, alimentos

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1. INTRODUCTION

Enzymes are highly effective biocatalyst proteins, which start or speed up the velocity of a biochemical reaction. They guarantee the specificity of the substrate concerning stereoisomers and enantiomers. These organic molecules have a wide range of structures and functions which have generated interest on an industrial scale (Dubey & Verma, 2018). Since ancient times, enzymes have been present in the production of different foods, such as alcoholic beverages, chocolate, dairy productse, meat products, or bakery products (Kuddus, 2018). Set against chemical products, enzymatic catalysis in food processing generates less waste, reduces energy consumption, and improves the biodegradability of products, so it can be considered as an environmentally friendly process. Furthermore, reactions that catalyze enzymes are carried out under milder conditions of temperature, pressure, and pH, thus functional components can be preserved after processing. For that reason, nutritional value, flavor, and texture are preserved (Singh & Kumar, 2018).

The main food industrial enzymes used on a wide scale are proteases, carbohydrases and lipases. New enzymes such as tanases, phytases and L-asparaginases have been incorporated into the industry in the development of functional foods (Speranza et al., 2018). The desired traits in industrial enzymes are thermostability, the ability to act in a wide range of pH and pressure, higher reaction speed, specificity and reuse capacity (Rastogi & Bhatia, 2018). However, these traits are not present in the enzymes when they are under extreme processing conditions. So, emerging technologies are being investigated, such as: high pressure homogenization, ultrasound, supercritical fluids, pulsed electric fields, etc. (Ma et al., 2016). These technologies are being proposed as alternatives to improve the performance of the enzyme and / or the reaction, in order to developed safe and higher quality foods (Leonelli & Mason, 2010).

Ultrasounds are a technology based on the spreading of sound waves with a frequency that cannot be detected by human ear. The frequency between 20 to 100 kHz is known as power ultrasound, in this range, when the waves are transmitted in liquid media, bubbles are generated (Kentish, 2017). Most of these bubbles are stable, others keep on expanding until collapse, where they implode and build small hot spots, generating energy that causes remarkable chemical and mechanical effects, which have an influence on the molecule chemistry that is exposed. This phenomenon is, known as cavitation (Wang et al., 2018). Ultrasounds as an emerging technology, helps to the processing and preservation of food. One of its applications in the food processing is the modification of the enzyme performance, because they can cause activation (Ma et al., 2016) or inactivation of enzymes (Marques Silva & Sulaiman, 2017).

The ultrasound action mechanisms are observed singly or in combination, on the biocatalyst or in enzymatic catalysis. In the first case, when ultrasounds are used as a pre-treatment reaction on the reagents, they lead to modifications in the structure and an increase in the exposure of the catalytic surface, therefore greater effective contact between enzyme and substrate are achieved (Bansode & Rathod, 2019). In the second processing route, ultrasounds are used throughout the enzymatic reaction, it is thought that the energy generated during cavitation hastens the reaction speed, although the mechanism is not entirely clear (Lerin et al., 2014). Cavitation changes temperature and microenvironment pressure around the bubble, so, it can improve the dissolution of the substrate,

and improves mass transfer. It also induces conformational changes in the enzymes, disturb weak interactions generating free radicals. In other words, they improve considerably enzymatic reactions (Wang et al., 2018). Therefore, the enzymatic reactions assisted by ultrasound, minimize the reaction time, increase the stability and the long-term catalytic activity (Lerin et al., 2014).

Another emerging technology that is currently being explored is the use of supercritical and subcritical fluids as a reaction medium in catalytic processes of enzymes. A fluid is in a supercritical state when its temperature and pressure are over critical values, some fluid properties are similar to properties of a liquid or a gas, for example, the density is similar to that of liquids, providing a high solvation power (Dias et al., 2018). The viscosity resembles that of gases, and diffusivity is somewhere between that of gases and liquids. Generally, supercritical fluids are depressurized to allows the separation of the products in a simple way leaving no solvent residues. All these factors contribute to supercritical fluids replacing conventional organic solvents (Escandell et al., 2015). Due to their high diffusivity and low viscosity, they increase the transport of substrates to the active site of enzymes, thus they increase the conversion to products (Dias et al., 2018). The most important factor in supercritical fluids is that the behavior of the phases can be controlled by varying the pressure and / or the temperature, therefore, the substrates and the products can be present in one or more phases. This fact allows the reaction phase to be homogeneous, thus faster and more efficient (Knez, 2018), without the need of an extra separation stage (Escandell et al., 2015).

This literature review focuses on the current state of research on ultrasound-assisted enzymatic reactions and on reactions catalyzed by enzymes in supercritical fluids, in products of interest for food industry.

2. MATERIALS AND METHODS

The compilation of information was carried out using the Science Direct scientific database with keywords such as: biocatalysis, enzyme, reaction, food, ultrasound, supercritical fluids. Table 1 shows keywords and choosing criteria used in literature data base search.

TABLE 1. Keywords and choosing criteria used in literature data base search

Keywords (connected by AND)			Date	Results	Publications used in this review	Choosing criteria
Ultrasound	Enzymatic pre-treatment	Food	All years	15	5	Lypase, carbohydrases, proteases
Ultrasound	Substrate pre-treatment	Enzyme	All years	18	8	Lypase, carbohydrases, proteases
Ultrasound - assisted	Enzymatic reactions	Lypase	All years	8	6	Lypase
Ultrasound - assisted	Enzymatic reactions	Carbohydrases	All years	10	7	Carbohydrases
Ultrasound - assisted	Enzymatic reactions	Proteases	All years	4	4	Proteases
Supercritical fluid	Enzymatic reactions	Food	All years	6	6	Lypase, carbohydrases, proteases

With the information collected, a research study about the use of ultrasound and supercritical fluids to enhance enzymatic reactions was established, including advantages and optimal process conditions.

3. RESULTS AND DISCUSSION

3.1. Ultrasound-Assisted Enzymatic Reactions

Ultrasounds are used in industrial processes to intensify catalytic reactions and to create a synergistic effect with enzymes (Wang et al., 2020). It is a low-cost and energy-efficient technology (Siewe et al., 2021). Mechanisms to promote ultrasound-assisted enzymatic reactions focus on three aspects: substrate and/or enzyme pretreatment and use of ultrasound throughout the reaction (Wang et al., 2018).

3.1.1. ENZYMATIC PRE-TREATMENT WITH ULTRASOUND

Globally, there has been an industrial increase in food processes catalyzed by enzymes. There are several studies that show the influence of ultrasound application on the activity of enzymes. In one hand, ultrasound application produces inactivation of enzymes. On the other hand, low intensity and short duration treatments redraw the enzyme structure and this leads to an increase in its activity (Wang et al., 2018). Ultrasound also reduces mass transfer resistances in the process by generating turbulence and shear forces during bubble collapse. (Subhedar & Gogate, 2014). Table 2 shows some studies about the influence of the use of ultrasound on the enzyme activity with industrial application.

TABLE 2. Activity of the enzymes found in buffer solution after ultrasound treatment

Enzyme	Optimal parameters						Activity (%)	Reference
	Type US	f (kHz)	Power (W)	Other parameters	T (°C)	t (h)		
Pectinase Xylanase Cellulase	Bath	40	220	pH: 3-7	37 50 50	- - -	- 10,0 + 30,0 - 10,0	(Dalagnol et al., 2017)
Invertase (β-fructofuranosidase)	Bath	25	450	22 (W/L)	23	1	- 13,2	(Souza et al., 2019)
Lypase (Novozymes)	Bath	25	-	22 (W/L)	25, 40	0,75 1	+ 12,0	(Souza et al., 2020)
Cellulase (Endo-1,4-β-D - glucanase)	Probe	20	-	17,33 (W/cm^2)	50	0,5	+ 23,4	(Subhedar & Gogate, 2014)
Glucoamylase (1,4-α-glucosidase)	Probe	22	180	10 mm	35	0,17	-	(Wang et al., 2020)

In Dalagnol et al. (2017), pectinase, xylanase and cellulase were treated with a bath ultrasound at different pH values, to analyze the ultrasound effect on the enzymatic activity. After treatment, it was observed that pectinase and cellulase decreased their activity by 10 %, whilst xylanase increased its catalytic power by 30 %. pH affects the enzyme efficiency and extreme values can change the structure causing a partial or irreversible denaturation, and thereby the loss of the enzymatic activity. In this study it was observed that ultrasounds helped the

enzyme activity to be greater than that obtained without pre-sonication. In the same study, at optimal pH and temperature conditions, the activities of pectinase (37 °C y pH: 4,8) and xylanase (50 °C y pH: 5) were not affected by the previous treatment by ultrasounds. With cellulose (50 °C y pH 4,8) a 50 % of enzymatic activity improvement was obtained with ultrasound pretreatment (Dalagnol et al., 2017).

Another study with cellulase shows that ultrasound treatment at the optimum enzyme pH (4,8) and temperature (50 °C) increased the activity by 23,4% in a treatment time of 30 min compared to the treatment without applying ultrasound. When the time period reached 50 min, the cellulase activity was lower than the control, in other words, it caused harmful effects that led to the degradation of amino acid residues that contribute to the enzyme-substrate binding (Subhedar & Gogate, 2014)

Souza et al. (2019), showed that the enzymatic activity after ultrasound processing reduced or did not modify the enzymatic activity evaluated at different reaction temperatures (40, 55 and 65 °C). At 55 °C and pH 5, which are considered optimal process parameters, the enzyme activity decreased by 13,2% after 1 h of processing, under non-optimal conditions (> 55 °C) the enzyme still lost faster its activity in shorter processing times. Similar results were shown in the glucoamylase activity, the pretreatment with ultrasound did not increase the enzymatic activity, from 270 W of power the activity decreased and to powers greater than 360 W and 40 min of treatment the enzyme was inactivated (Wang et al., 2020).

Souza et al. (2020), evaluated the effect of ultrasounds on lipase at optimal temperature and pH (55 °C, pH: 6) of the enzyme reaction. Ultrasound application did not improve lipase activity. However, the lipase activity showed an increase of 12 % after processing with ultrasound at 25 °C and 40 min; and at 45 °C and 60 min, compared to the enzymatic reaction without ultrasound pretreatment under the same conditions. In this study, it is important to emphasize that the use of ultrasound as a pretreatment on the enzyme enhanced enzymatic catalysis in non-optimal working conditions (temperature below 55 °C or pH other than 6) where it is more difficult to act. This fact is of great interest for the process since production costs can be reduced (Souza et al., 2020).

Each enzyme has optimal processing parameters, where temperature conditions, steam pressure, surface tension and the viscosity of the liquid medium benefit the propagation of ultrasonic waves and the cavitation phenomenon. If the temperature increases, the number of bubbles is increased, and the collapse gets dampened because the vapor pressure increases. On the other side, the higher the temperature, the lower the viscosity, thereby the cavitation is more violent. Therefore, it is important to find an optimal temperature where violent cavitation bubbles are formed and, in enough quantity, to improve enzymatic activity (Patist & Bates, 2008).

3.1.2. SUBSTRATE PRE-TREATMENT

Ultrasound pretreatment of substrates is used in food industry to degrade and convert them into functional and bioactive products that provide essential nutrients. It is also used to improve its physicochemical characteristics such as solubility (Cheng et al., 2017), to get short chain compounds to be used as natural flavorings and flavors (Souza et al., 2020), or to increase the efficiency of

enzymatic degradation (Wang et al., 2020). The ultrasound pretreatment on the substrate modifies its molecular structure and affects the reduction of activation energy, the Gibbs free energy, the Michaelis-Menten constant and increases the initial reaction rate (Cheng et al., 2017). Table 3 shows the parameters used in the ultrasonic pretreatment of some substrates with the enzyme used for its degradation.

TABLE 3. Ultrasonic pretreatment of some food substrates.

Substrate	Enzyme	Ultrasonic parameters						Activity (%)	Reference
		Type US	f (kHz)	Power (W)	Other parameters	T (°C)	t (h)		
Soy sauce residue	Proteases	Probe	20	126,4	1,27 cm; 33 %	50	0,17	+ 47,6	(Chen et al., 2017)
Potato protein	Alcalase	Probe	20/40	250	2 probe 77 %	25	0,17	+ 23,5	(Cheng et al., 2017)
Pectin Xylan Filter paper	Pectinase Xylanase Cellulase	Bath	40	220	-	37 50 50	- 0,08 0,17	- + 25,0 + 17,0	(Dalagnol et al., 2017)
Sucrose	Invertase	Bath	25	450	22 (W/L) pH: 5	30-55	9	-	(Souza et al., 2019)
Goat cream	Lipase	Bath	25	-	22 (W/L) pH:6	25-55	5	-	(Souza et al., 2020)
Corn gluten meal	Glucoa-mylase	Probe	22	90-360	10 mm	35-75	0,17-0,83	+ 21,5	(Wang et al., 2020)
Whey protein	Alcalase	Probe	20	0 – 500	1,5 cm 50 %	25	0 - 0,25	> 20,0	(Wu et al., 2018)
Wheat Gluten	Alcalase	Probe	20	464	2 cm 57 %	30	0,33	-	(Zhang et al., 2015)

In soy sauce, they applied ultrasound to the substrate for 10 min as a pretreatment followed with enzymatic hydrolysis for 20 min, the increase was 47,6% at 50 °C and increased by 24,8 % more during 4 h of reaction, compared to treatment without ultrasound. Also, it helped protein recovery by 8,2 %. In the Scanning Electron Microscope (SEM) study, they observed that the substrate pretreated with ultrasound reduced the size of the particle, which explains the increase in the catalytic surface and improved the mass transfer of the system (Chen et al., 2017). The pretreatment with ultrasound on the substrate stimulated the efficiency of the enzymatic reaction of potato protein with alcalase, the protein hydrolysate increased, and the process time was shortened. Efficiency was related to the decrease of kinetic parameters of the reaction such as energy, enthalpy, activation entropy, etc. That caused the enzyme-substrate binding to be easier and faster, potato protein hydrolysate increased by 23,5 %; 6,3 % and 5,5 % compared to the treatment without ultrasound at 30, 40 and 50 °C, respectively, after 60 min of reaction. After that time, the pretreatment of the substrate had a negative effect, therefore, the percentage of potato protein hydrolysate was lower than the control, that means that the effect of ultrasound on the structure of the protein limits the binding with the enzyme in prolonged reaction times (Cheng et al., 2017). Similar results were observed in the presonication of xylan for 5 min. They obtained a 25 % increase in the activity of the enzyme. Xylan is a complex polysaccharide that requires a set of enzymes for its degradation, the ultrasound process was able to alter the xylose chains and facilitate the access of the enzyme. Pre-sonication on cellulose improved enzyme activity by 17 % (Dalagnol et al., 2017). It can be stated that at optimal

physical and chemical conditions, ultrasound treatment can induce cellulose to have more hydrophobic groups and exposed internal regions, giving a molecule with greater uniformity and flexibility, improving accessibility to the substrate (Dalagnol et al., 2017).

Another study with ultrasonic pretreatment in whey protein found an influence on protein hydrolysis. The optimal value was 300 W and 15 min, beyond those values, hydrolysis decreased, compared to untreated whey protein. The pretreatment may have altered the structure of the protein, so the binding sites of enzymes were exposed. In other words, it induced the breakdown of whey protein by shear forces, which induced an increase in the content of free sulfhydryl and hydrophobic molecules on the surface, the secondary structure also changed (Wu et al., 2018).

Wang et al. (2020) demonstrated that ultrasonic pretreatment promoted starch hydrolysis with glucoamylase. The pretreatment accelerated the liquefaction and saccharification of starch because it could have destroyed starch chains and decreased molecular weight, increased the free fragment of the structure and exhibited more active sites, increasing the reaction rate. However, presonication in other substrates such as pectin (Dalagnol et al., 2017) , sucrose (Souza et al., 2019), goat's milk cream (Souza et al., 2020) did not have an influence on enzyme activity. It is important to determine whether the use of ultrasound on the substrate will have a positive effect on the enzymatic activity during the reaction, as it depends on the structure of the substrate and the treatment conditions.

3.1.3. ULTRASOUND-ASSISTED ENZYMATIC REACTIONS OF LIPASES

Lipases are enzymes that catalyze organic reactions of esterification, transesterification, regio-selective acylation, glycerolysis and hydrolysis. They are frequently used because its selectivity and specificity minimize unwanted reactions and generate less waste and by-products. Enzymatic reaction processes occur under mild physicochemical conditions, lipases are versatile on a wide range of substrates and have high stability at extreme temperatures and pH (Lerin et al., 2014). They belong to the group of hydrolases that do not need cofactors and hydrolyze ester bond substrates with low solubility in water. They are used in industry to produce chiral drugs, prepare cocoa butter substituents, biofuels, fat modification, cosmetics, enhancers flavor and degrade waste (Angajala et al., 2016). Due to all these advantages that are obtained when using lipases in the processes, it is important to achieve greater efficiency in reactions. Table 4 shows some examples of enzymatic activity after treatment in an ultrasound-assisted system.

Souza et al. (2020), determined in their study greater hydrolysis compared to treatment without ultrasound, the increase was 12 %, 23 % and 28 % at 55, 40 and 25 °C, respectively. Additionally, they demonstrated that the assisted reaction at 40 °C presented greater hydrolysis than the conventional process at 55 °C. The formation of cavitation bubbles is affected by temperature, which explains that at 25 °C they managed to form bubbles from violent cavitation and in enough quantity to improve enzymatic activity. Esterification reaction to get tricaprylin under normal conditions takes 24 h or more. More et al. (2017), used sonication with ultrasound in the initial phase of the reaction, which allowed them to reduce the process to 8 h and got a maximum yield of 94 % compared to the 81 % achieved in the conventional way. The results suggested a maximum of 30

min of sonication. A longer time had no significant ($p > 0,05$) effect on increasing the reaction yield. Optimizing the ultrasound time also minimizes any adverse effect on the immobilized enzyme. The study also demonstrated that enzyme activity was not affected during reuse for at least ten times and that the use of ultrasound is a viable mechanism for the synthesis of tricalprylin with immobilized lipase (More et al., 2017).

TABLE 4. Enzyme activity of lipases after ultrasound-assisted treatment

Substrate	Enzyme application	Product	Type ultrasonic	T (°C)	Reaction time (h)	Conversion (%)	Reference
Free enzymes							
Goat cream	Lipase hydrolysis	Fatty acids	Ultrasonic bath (25 kHz, 22 W/L)	25	5	100	(Souza et al., 2020)
Solvent free - immobilized enzymes							
Caprylic acid and glycerol	Lipase esterification	Tricaprylin	Ultrasonic horn (20 kHz, 240 W, 70 %)	50	0,5	94	(More et al., 2017)
Glycerol and olive oil	Lipase hydrolysis and esterification	Mono and diacylgly-cerols	Ultrasonic bath (37 kHz, 132 W)	60-70	2	-	(Fiametti et al., 2012)
Propyl amine and butyl acetate	Lipase amidation	Propyl acetamide	Ultrasonic bath (25 kHz, 100 W, 70 %)	60	2	98	(Bansode & Rathod, 2019)
Solvent - immobilized enzymes							
Aspirin methyl ester	Lipase hydrolysis	Methyl salicylate	Ultrasonic bath (53 kHz, 100 W 50 %)	35	1	65,3	(Chiplunkar et al., 2018)
Methanol and caffeic acid	Lipase esterification	Methyl caffeate	Ultrasonic bath (25 kHz, 150 W)	75	9	99	(Wang et al., 2015)

Similar results with immobilized lipase were obtained by Fiametti et al. (2012). The enzymatic activity did not reveal changes during reuse, indicating that the sonication did not produce conformational modifications in its structure. They also concluded that the ultrasound-assisted enzymatic catalysis achieved high product contents versus the conventional enzymatic treatment that did not exceed 4 % of product during the reaction. In the study presented by Bansode & Rathod (2019), they determined that immobilized lipase after reusing it for 5 cycles lost its activity by 50 %. However, during catalysis, they obtained a conversion of 94 % after 2 h of reaction with a temperature of 60 °C. Besides, at optimal conditions of temperature and pH, they modified the molar ratio of the substrates to 1:1,5 (propylamine to butyl acetate) and increased the percentage of biocatalyst from 2 % to 3 %, obtaining a yield of 98 %. It is important to determine the appropriate percentages of substrates and biocatalysts, thereby the study determined that particle crowding limits internal diffusion and mass transfer, the dissipation energy of ultrasound is also inhibited in reaction mechanisms with immobilized enzymes (Bansode & Rathod, 2019).

To increase the mass transfer and therefore the conversion rate with immobilized enzymes under the influence of ultrasound, several studies used organic solvents as reaction medium. The solvent enhances the cavitation effect. In lipases, organic solvents with low polarity (Chiplunkar et al., 2018) generate better results (Wang et al., 2015).

Chiplunkar et al. (2018), in reactions assisted by ultrasound in the presence of solvent triolein and water, presented a conversion rate of 65,3 % versus 58,9% without ultrasound. Water acts as a nucleophile, while solvent acted as an enzymatic protector and improved ultrasound yield due to its high viscosity and surface tension. They also reported that the enzyme lost 37 % of its activity after the first use. Better results with immobilized enzymes and use of solvent (ionic liquid) were presented by Wang et al. (2015), the reaction time was reduced 0,75 times, the conversion rate was approximately 99 %, and the lipase could be reused 11 times without significant loss of activity.

Some of the advantages of ultrasound-assisted reactions in lipases can be summarized as: minimization of reaction time, decrease in energy consumption, reduction in reagent amounts, increase in conversion rate and reduction of polluting by-products (Lerin et al., 2014).

3.1.4 ULTRASOUND-ASSISTED ENZYMATIC REACTIONS OF CARBOHYDRASES

Carbohydrases are responsible for polysaccharide degradation and structural polymers of plant cell walls, to improve polysaccharide properties and produce functional oligosaccharides. They are used mainly in food industry in juice, wine, and feed industries (Dalagnol et al., 2017). There are other industrial applications to get modified pectin (Ma et al., 2016), microporous starch (Majzoobi et al., 2015), invert sugar (Souza et al., 2019), xyloligosaccharides (Sun et al., 2015). Table 5 presents the activity of polysaccharide enzymes assisted by ultrasound.

Oleuropein aglycone is present in olive leaves, the compound is used as a nutraceutical in functional foods. Delgado-Povedano et al. (2017), synthesized the compound with ultrasound-assisted β-glucosidase. They were able to reduce the time required for complete hydrolysis from 2 h to 18,75 min. The compound yield was 95,7 % with a purity of 94,9 %.

Ma et al. (2016), hydrolyzed pectin to get modified pectin that is used in functional foods. They determined that the optimal conditions of the ultrasound-assisted reaction were: temperature 20 ° C, power 4,5 W/mL, sonication time 10 min, where the enzymatic activity was 32,6 greater than the reaction at the same conditions without ultrasound. They also observed that when the reaction temperature was at 50 ° C (optimal temperature of the enzyme) there were no significant differences between the treatments, the difference was only 7 %. Majzoobi et al. (2015), modified the structure of wheat starch with ultrasound-assisted enzymatic reactions. Microporous starch is used in the industry as a vehicle for colorants, spices, flavorings, vitamins, or bioactive compounds. The synergy between α-amylase enzyme and ultrasound increased the amount and size of the micropores, so, the modified starch showed the best percentage of oil adsorption, hydration capacity and methyl violet adsorption when the treatment was at 50 °C, 40 min of sonication and 0,4 % enzyme. However, sonication for 60 min of the enzyme-treated samples (0,6 %) destroyed part of the starch granules. The sonication and enzymatic catalysis treatments under the same conditions, that were carried out individually on the starch, showed less quantity and size of cavities (Majzoobi et al., 2015). Treatment of sucrose hydrolysis with ultrasound-assisted invertase presented a 33,0 % higher reaction rate versus treatment without ultrasound. Optimal parameters of enzymatic catalysis with ultrasound were: pH 5,0 and at 40 °C (Souza et al., 2019). For obtaining xyloligosaccharides from corn cobs, the yield rate was 55,0 % more at 50 °C, 10

min of sonication, 200 W of power versus the enzymatic reaction without ultrasound (Sun et al., 2015).

TABLE 5. Activity of polysaccharide enzymes assisted by ultrasound

Substrate	Enzyme application	Product	Type ultrasonic	T (°C)	Reaction time (h)	Increase in reaction rate (%)	Reference
Olive leaves	β-glucosidase	Oleuropein aglycon	Probe (200 W, 20 kHz, 3 mm, 60 %)	37	0,31	-	(Delgado-Povedano et al., 2017)
Citrus peel	Pectinase hydrolysis	Polygalac-turonic acid	Probe (4,5 W/mL, 22 kHZ, 900 W, 10 mm)	20	0,17	32,6	(Ma et al., 2016)
Wheat starch	α-amylase hydrolysis	Microporous starch	Bath (240 W, 35 kHz, 100 %)	40	0,67	-	(Majzoobi et al., 2015)
Sucrose	Invertase hydrolysis	Glucose and fructose	Bath (22 W/L, 25 kHz, 400 W)	40	7,0	33,0	(Souza et al., 2019)
Corncobs	Xylanase hydrolysis	Xylooligo-saccharides	Bath (200 W)	50	0,17	55,0	(Sun et al., 2015)
Potato starch	Gluco-amylase hydrolysis	Glucose	Probe (22 kHz, 900 W, 10 mm, 7,20 W/mL)	55	0,67	25,9	(Wang et al., 2017)
Potato starch	Gluco-amylase	Reducing sugar	Probe (22 kHz, 10 mm, 270 W)	55	-	-	(Wang et al., 2020)

Potato starch shows limitations such as low solubility, high viscosity, thermal decomposition and retrogradation. Wang et al. (2017), used ultrasound-assisted enzymatic reactions to catalyze potato starch. The optimal treatment parameters were intensity 7,2 W/L, temperature 55 °C and sonication time 40 min. The reaction speed increased by 25,87 % compared to the reaction without ultrasound, due to the increase in the enzyme-substrate binding. The activation energy was 13 % lower than the enzymatic reaction without ultrasound, showing that the energy barrier necessary for the reaction decreased due to ultrasonic action. Wang et al. (2020), determined that ultrasound-assisted enzymatic reactions are more efficient than ultrasound pretreatments of the enzyme (glucoamylase) or the substrate (potato starch).They concluded that ultrasound dispersed the aggregate formed by glucoamylase and starch modified glucoamylase and degraded starch, reduced the mass transfer barrier and promoted starch to diffuse to the active sites of the enzyme and facilitated the product separation of the enzyme after the reaction. They also determined that amylopectin is the compound that was most hydrolyzed during the reaction (Wang et al., 2020).

The increase in activity is due to the fact that ultrasound generates shear forces because of cavitation that prevents the enzymes from aggregating, alter the configuration and promote the exposure of active sites on the enzyme or catalytic sites on the substrates. It depolymerizes the surface structure of the substrate for the enzyme to penetrate and improves mass transfer during the reaction. However, if the intensity and treatment time is greater than optimal, ultrasound generates many free radicals and shear forces that destroy

carbohydrases structure and therefore it decreases enzyme activity. At temperatures higher than optimum, the enzymes are denatured (Wang et al., 2018).

3.1.5. ULTRASOUND-ASSISTED ENZYMATIC REACTIONS OF PROTEASES

Ultrasound-assisted enzymatic reactions have been used to optimize protein catalysis in order to degrade or change their structures, so it improves characteristics such as protein solubility, and it delivers functional peptides or bioactive peptides with antioxidant capacity (Wang et al., 2018). Ultrasound has a significant effect on accelerating the rate of protein hydrolysis. Table 6 shows some investigations of ultrasound-assisted enzymatic protease reactions.

TABLE 6. Activity of protease enzymes assisted by ultrasound

Substrate	Enzyme application	Product	Type ultrasonic	T (°C)	Reaction time (h)	Increase in reaction rate (%)	Reference
Soy sauce residue	Proteases	Bioactive Polypeptide	Probe (20 kHz, 126,4 W; 1,27 cm; 33,3 %)	50	0,33	33,0	(Chen et al., 2017)
Sesame bran	Alcalase and Viscozyme L	Sesame protein	Bath (35 kHz, 836 W)	43	1,63	10,8	(Görgüç et al., 2019)
Bovine tendón	Pepsin	Collagen	Bath (40 kHz, 120 W, 50 %)	20	16	24,0	(Li et al., 2009)
Rapeseed protein	Alcalase	Hidrolized protein	Bath (600 W, 57 W/L, 77 %, 68/33 kHz)	60	1,27	64,6	(Wang et al., 2016)

Chen et al. (2017), worked about the application of ultrasound in the catalysis of soy sauce residue to get protein hydrolysates. They obtained an increase of 33,0 % versus the reaction catalyzed by enzymes without ultrasound. This happened because of the increase in the area of catalysis of the substrate. In a previous study, they determined that ultrasound reduced the catalytic power or inactivated enzymes, but the positive effect of ultrasound was greater.

Sesame is a seed that has between 18 to 25 % protein. Sesame bran is a by-product obtained from oil production and contains around 15 % protein. Görgüç et al. (2019), determined that the optimal pH, temperature, and time for extraction was 9,2; 43 °C and 1,63 h. They concluded that the ultrasound-assisted enzyme treatment provided the highest yield (87,9 %), in comparison to the enzyme extraction (79,3 %). Through Scanning Electron Microscope (SEM) analysis, they observed that the sesame bran had a rigid cellular structure. After the treatments, the structures exhibited disordered irregular fragments with huge perforations on the external surface. As a result of the research, the authors concluded that sesame bran is a protein source with high values of phenolic compounds and antioxidant capacity (Görgüç et al., 2019).

Collagen is an especially important biomaterial in the food and pharmaceutical industry. It is extracted from the skin and tendons of animals by treatment with neutral salt, acid, alkali and proteases. Li et al. (2009), used ultrasound in combination with a 0,5 M pepsin and acetic acid solution to extract collagen from bovine tendon. The process was carried out at 20 °C, 40 kHz and 120 W. They showed that the yield of collagen increased by 124 % in comparison to the conventional pepsin process. Also, the time was reduced from 2 days to 16 hours

and the enzymatic activity increased up to 106 %. The ultrasound-assisted enzymatic hydrolysis helped to the diffusion of enzyme molecules towards the substrate, it eased collagen fibrils to separate from the bovine tendon, so they keep their molecular structure intact (Li et al., 2009).

Rapeseed protein meal is a by-product in obtaining oil with a protein content of up to 40%, which is used to get functional peptides. Wang et al. (2016) worked about how to extract proteins using alcalase enzymes in combination with ultrasound. They determined that the maximum hydrolysis occurred at 57 W / L, with values greater than the optimal value mentioned, hydrolysis decreased, because the enzyme's center of activity could be destroyed. The optimal time was 76 min, after that, the hydrolysis was reduced due to the denaturation of the enzyme and the accumulation of the product. They also concluded that the frequency combination (68/33 kHz) led to the exposure of more active sites on the enzymes and increased the efficiency of enzymolysis. Through Scanning Electron Microscope (SEM) analysis, they observed that cavitation generated strong shock waves and shear forces modified the enzyme surface that increased the affinity between the enzyme and the substrate (Wang et al., 2016).

In all studies, ultrasound improved hydrolysis because it modified the substrate structure, presenting more active sites for enzyme-substrate binding.

3.2 Supercritical Fluid Assisted Enzyme Reactions (SCF)

Enzyme-catalyzed reactions are considered environmentally friendly because they are highly specific since they have active sites that are adapted to a specific substrate and limit side reactions (Dos Santos et al., 2017). It is important to highlight that the immobilized enzymes on a support are more effective and efficient in biocatalysis processes because this improves their stability and allows their reuse (Escandell et al., 2015). Depending on the industrial process, the use of organic solvents, that are generally toxic, is a limitation in food industry. Supercritical fluids are a viable alternative that is being investigated, due to the ease of controlling their properties as a function of pressure and temperature, which allows the use of a single reactor for the reaction and separation of the final product from the solvent (Dias et al., 2020). For each enzyme / substrate / SCF system, the appropiate reaction parameters that have an influence on the stability and maximum yield of the enzyme must be determined (Hojnik Podrepšek et al., 2019). Supercritical carbon dioxide (SC-CO_2) is the most common to carry out enzymatic reactions, due to its properties: low toxicity, non-mutagenic, non-flammable, inert, respectful with the environment, easy to get and inexpensive. It needs low temperatures (Dos Santos et al., 2017), and pressures (Escandell et al., 2015). Some physical properties of SC-CO_2, such as its higher diffusivity and lower surface tension and viscosity, can overcome the limitations of transport between phases and, so, improve conversion. Other parameters must be also considered, such as solubility of the solvent in the matrix that supports the enzyme and the amount of water that it contains, since an optimal water level (called essential water) is required to keep the active conformation of the enzyme molecules, through hydrogen bond, inside and outside the catalyst (Dias et al., 2020). Table 7 shows several studies where SC-CO_2 is used as the medium for enzymatic reactions.

As mentioned above, it is important to determine the optimal parameters of enzyme / substrate / SCF system. Bourkaib et al. (2018), established different experiments where parameters such as flow, pressure, temperature, and residence time of SC-CO_2 were modified. They obtained a maximum conversion of around 98 %, that is, 1,1 g of geranyl acetate per gram of immobilized enzyme per hour at 65 °C, 15 MPa, residence time of 36,5 s in a short 1 mL reactor. Under these production conditions, geranyl acetate is considered a solvent-free green product, because enzymatic esterification with SC-CO_2 does not generate any secondary or toxic effluents compared to chemical processes and it is more efficient.

TABLE 7. SC-CO_2 assisted enzymatic reactions

Products	Enzyme	Optimal Conditions		Maximum conversion	Type of reactor	Reference
		P (MPa)	T (°C)			
Geranyl acetate	Lipase	15	65	98,0 %	Packed bed	(Bourkaib et al., 2018)
Isoamyl acetate	Glycerol ester hydrolases	15	60	95,8 %	-	(Dias et al., 2020)
Isoamyl Acetate	Lipase	15	40	100 %	Packed bed	(Dos Santos et al., 2017)
Butyl acetate	Lipase	11	60	78,0 %	Packed bed	(Escandell et al., 2015)
Glucose	Cellulase	10	50	143 %	CLEA	(Hojnik Podrepšek et al., 2019)
Polysaccharides	α-amylase	24	41	-	Free enzyme	(Senyay-Oncel & Yesil-Celiktas, 2011)

Dias et al. (2020), produced isoamyl acetate with SC-CO_2 as the reaction medium. The operating conditions were: pressure 15 MPa, temperature 60 °C with a molar ratio of substrates 4:1 (acetic anhydride: fuel oil), with 10 % of enzyme obtained a conversion of 95,8% in 5 min and it was kept constant. Similar results were obtained under the same conditions, varying the molar ratio, which was 2:1. They also indicated that the variation in the percentage of enzyme was not significant, they found similar values in the range of 2,5 to 10 %. SC-CO_2 produced higher conversion and productivity 1,2 and 1,3 for solvent-free systems and for systems with n-hexane, in a molar ratio of substrate 2:1 with 2,5 % of enzyme. In reactions with SCF, the optimal conditions of the process are those where a greater conversion of the substrate into product is achieved and these are obtained when there is a greater volumetric expansion of the substrates and therefore better mass transfer (Dias et al., 2020).

Better results in obtaining isoamyl acetate were presented by Dos Santos et al. (2017), with a 100 % conversion. They first determined the conversion rate between solvent n-hexane and SC-CO_2, obtaining results of 51,1 % and 79,0 % respectively. Then, they were varying reaction parameters in a batch without supercritical fluid, to determine the best results. The molar ratio parameters between the substrates, the amount of enzyme and the agitation were statistically significant in the conversion of isoamyl alcohol; the values obtained in the conversion were from 52,2 % to 100 %. However, temperature and pressure had a small influence on the reaction, instead the diameter of the particles was not

important in the diffusion and mass transfer. Once the optimal parameters were established in the batch reactor, they compared the results with those obtained in the continuous reactor, for which they established the molar ratio of 1:1, 600 rpm, enzyme concentration of 3 % and 3 h of reaction, the conversion rate was 79,0 % for the batch reactor and 100 % for the packed bed reactor under the same conditions, except for the reaction time which was 1 h with a flow rate of 0,13 g/s of SC-CO_2. As a conclusion of the work, the packed bed reactor presented higher productivity of isoamyl acetate than the batch reactor and they showed that the best option to synthesize isoamyl acetate is in SC-CO_2 medium (Dos Santos et al., 2017).

In other studies, such as the one presented by Escandell et al. (2015), they obtained 4,2 times more butyl acetate when the reaction was carried out in SC-CO_2 versus that obtained with n-hexane. SC-CO_2 may improve mass transfer compared to hexane; it is also probable that high working pressures allow a better substrate distribution in the porous granules of the system. Therefore, it improves the enzyme / substrate contact and increases the productivity. The optimal parameters in the reaction with SC-CO_2 were: substrates ratio 1:3 (n-butanol: vinyl acetate), flow rate of substrates 0,5 mL / min, flow rate of CO_2 20 g/min, temperature 60 °C and pressure 11 MPa. The research also concluded that at temperatures above 60 °C there is a significant loss of enzyme activity, as well as an increase in n-butanol concentration, which decreases the reaction yield (Escandell et al., 2015)

Hojnik Podrepšek et al. (2019), experimented with immobilized cellulose in a CLEA system which consists of enzymatic aggregates cross-linked with solvents and glutaraldehyde. They determined the effects of SC-CO_2 on free and immobilized enzyme and a control treatment without using SC-CO_2, in different combinations of pressure (atmospheric, 10 and 20 MPa), temperature (40 and 50 ° C) and different reaction times. (1,3, 4 and 24 h). The maximum residual activity was achieved when the temperature was 50 ° C and 10 MPa, which was 143% at 3 h of treatment. All the reactions in SC-CO_2 presented higher activity than the untreated ones. This can be attributed to the increase in the enzymatic activity and stability because of the modifications of the conformational structure of the immobilized enzyme due to the speed of the dissolved CO_2 in the system. Also, enzyme stability can be attributed to the cross-linked enzyme aggregate (CLEA) methodology. During crosslinking, the amino groups of lysine residues react with the solvents that add them, so they are not available to react with CO_2.

Senyay-Oncel & Yesil-Celiktas (2011), evaluated the effect of pressure (5 - 30 MPa), temperature (28-80 °C), CO_2 flow (2-10 g/min) and processing time (60-180 min) in order to determine the optimal parameters where the free enzyme, which was pretreated with SCF, had its maximum efficiency and stability. They kept all the parameters fixed except for pressure and determined that there was no significant increase in the enzyme activity, but they did observe that the activity was 29,4 % higher compared to the enzyme not treated with CO_2. It is believed that under the pressure of an SCF the enzymatic structure becomes more compact and rigid, minimizing the thermal effects caused by high temperature. If the pressure is increased, the solubility of the SCF improves, that is, thanks to the solvation power and the conformational fluctuations, there is greater exchange of water between the interior of the enzyme and the solvent. These hypotheses explain the improvement in enzyme activity on the treated enzyme. After the first trial, Senyay-Oncel & Yesil-Celiktas (2011) set the pressure at 18

MPa and varied the temperature. The activity with respect to the untreated enzyme was increased up to 46,8 %, that is, when the temperature increased there was no adverse effect on activity. Later, they set the temperature at 54 °C and working time at 120 min, varying only the CO_2 flow. Increasing the fluid flow decreased the enzyme activity, at 10 g/min. The decrease was 28,9 % compared to the untreated enzyme. The process time in the study did not show any significant effect on the enzyme and its catalytic power. Finally, they set new study conditions and the optimal conditions were determined as 24 MPa, 41 °C, 4 g/min-CO_2 flow and 150 min duration of the process, producing 67,7% more activity than the untreated enzyme.

According to research, the enzymatic reactions in CFS are carried out at the optimum reaction temperature of the enzyme or at lower values, obtaining better results since there is a greater enzyme-substrate affinity.

CONCLUSIONS

Each enzyme has optimal processing parameters, pH and temperature affect the efficiency of the enzymes in reactions since it can modify the structure causing denaturation. In enzymes such as pectinase, cellulase and lipase; pre-sonication helped the enzymatic activity to improve at extreme pH (3 or 7) and non-optimal temperatures. In other enzymes such as xylanase, pectinase, glucoamylase and lipase, they were not affected by the previous sonication treatment at optimum processing conditions, while cellulase increased catalysis.

The pretreatment with ultrasound on the substrate modified the molecular structure and affected the reduction of the activation energy, the Gibbs free energy, the Michaelis-Menten constant and it increased the initial reaction rate. In pretreatment on potato protein, soy sauce, xylan, cellulose, whey protein and starch, the hydrolysate was increased, and the process time was shortened. In the studies they determined the optimal parameters of power and time. The enzymatic activity was lower than in the control for long treatments. This suggests that the effect of ultrasound on the structure of the protein limited the binding with the enzyme in prolonged reaction times. In other substrates such as pectin, sucrose and goat's milk cream, the pretreatment did not have any influence on the reaction, which indicated that the efficiency in pre-sonication on the substrate depends on its structure.

In ultrasound-assisted reactions, reaction time was minimized, energy consumption decreased, reagent amounts decreased, the conversion rate increased, and contaminating by-products were reduced. The increase in activity is because the ultrasounds generated shear forces due to cavitation that prevented the enzymes from aggregating, it altered the configuration and promoted the exposure of active sites in the enzyme.

The reactions in SC-CO2 showed greater activity than the enzymatic reactions without FSC, this may be possible due to the increase in enzymatic activity. Furthermore, the system is more efficient if enzymes are immobilized because of the stability provided by the bed structure. Thanks to high pressures, it allows a better distribution of the substrate around the enzymes, improving productivity.

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