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Influence of microwave irradiation on enzymatic properties: applications in enzyme chemistry

BARBARA REJASSE[1], SYLVAIN LAMARE[1], MARIE-DOMINIQUE LEGOY[1], & THIERRY BESSON[2]

[1]*Laboratoire de Biotechnologies et Chimie Bio-organique, FRE CNRS 2766, UFR Sciences Fondamentales et Sciences pour l'Ingénieur, Bâtiment Marie Curie, Université de la Rochelle, F-17042 La Rochelle cedex 1, France, and* [2]*UMR CNRS 6014, Laboratoire de Chimie Pharmaceutique, U.F.R. Médecine - Pharmacie, Université de Rouen, 22 Boulevard Gambetta, 76183 Rouen Cedex 1, France*

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Abstract

Although microwave-assisted reactions are widely applied in various domains of organic chemistry, their use in the area of enzyme chemistry has been rather limited, due to the high temperatures associated with the microwave heating: Because current technology, allows a good control of reaction parameters, several examples of microwave-assisted enzyme chemistry have been reported, using stable and effective biocatalysts (modified enzymes). The purpose of this review is to highlight the applications and studies on the influence of microwave irradiation on enzymatic properties and their application in enzyme chemistry.

Keywords: *Microwave heating, enzyme chemistry, polar solvents, dry-media reactions*

Introduction

Microwave radiation as an energy source for heating is today widely used in organic chemistry. Indeed, an electromagnetic field of high frequency (2.45 GHz) induces molecular rotation of dipolar species, which is accompanied by intermolecular friction and subsequent dissipation of energy by heating in the core. Reduction in reaction times, enhancement in conversions and sometimes in selectivity have been reported concerning mainly solvent-free reactions conducted under a microwave field. A large number of review articles [1–4] and several books [5–7] provide extensive coverage of the subject.

On the other hand, the use of microwave irradiation in enzymatic synthesis remains still limited. This can be explained by the high temperatures associated with the microwave heating: enzymes are temperature-sensitive macromolecules. Now, with current technology, temperature as low as 40°C can be maintained by precise power input. Furthermore, recent developments to modify enzymes [8, 9] make it possible to obtain stable and effective biocatalyst, for synthetic applications [10, 11].

The aim of this review is to summarize the whole of work dealing with biosynthesis under microwave irradiation, which all was published this last decade. The primary focus is on the microwave influence on the enzymatic properties, such as the enzymatic activity, selectivity and stability. So, the first part of the review will be devoted to the early studies dealing with the irradiation of enzymes, which have been carried out in aqueous media, in view to detect a possible harmful effect of the microwave irradiation to biological processes. Secondly, all the works concerning the use of the microwave energy in biocatalysis field will be summarized, to discuss the future

Correspondence: T. Besson, UMR CNRS 6014, Laboratoire de Chimie Pharmaceutique, U.F.R. Médecine - Pharmacie, Université de Rouen, 22 Boulevard Gambetta, 76183 Rouen Cedex 1, France. Tel: 33 235 148399. Fax: 33 235 148592.
E-mail: thierry.besson@univ-rouen.fr

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potential of the microwave-induced enzyme-catalyzed synthesis.

Studies of the microwave/enzyme interaction in aqueous medium

First studies carried out in quasi-cellular conditions

The first studies concerning the irradiation of enzymes are published in the end of the Seventies. Indeed, the number of devices using microwave irradiation has been increasing the previous years rapidly, and the concern of military, industrial and government organizations with the possible health hazards associated with exposure to microwave irradiation was grown. The heating effect of the microwaves was already well-known but a doubt remained on the existence of a non-thermal effect. Isolated enzymes in aqueous medium were then taken as model to determine if there was an effect of the radiation on the biological processes.

These first studies have been carried out with various microwave apparatuses, directly manufactured in the laboratories. So, the frequencies and powers of irradiation used, from one study to another, were different (Table I). The temperature of the irradiated sample was in all cases maintained constant by means of an external circulating water bath (Figure 1).

Initially, researchers were interested in the influence of a subsequent irradiation on the enzymatic specific activity. Experiments consisted in heating two independent volumes of enzyme solution at the same rate and to the same temperature; one volume was heated by microwave energy and the other by conventional, resistive heating. The two volumes were maintained at that temperature for some length of time, and then assayed, and the results were compared. In most cases, similar enzymatic activity was found [12, 13]. Only one study observed a significant inactivation of the enzyme, the horseradish peroxidase, by applying microwave irradiation at room temperature [14]. However, local heat denaturation could not be ruled out, considering the high irradiation power used (see Table I).

Thereafter, the enzyme activity was measured simultaneously with microwave exposure to observe possible reversible effects [15–18]. No influence of the irradiation was found again.

More recent studies carried out in aqueous medium were interested in the influence of an electromagnetic field of high frequency (≈10 GHz) on the enzymatic stability. Notably, d'Ambrioso and co-workers [19] studied the stability of Acid Phosphatase at 60°C, with irradiation power varying from 160–480 mW/g. A surprising stabilization of the enzyme towards thermal deactivation was found for microwave exposures which exceed 280 mW/g. The authors concluded with a structural modification of the irradiated enzyme. Similar studies were conducted with thermophilic and thermostable enzymes, in view to increase the temperature of the study and consequently the irradiation power [20–22]. An opposite effect was then observed: enzymes are inactivated by the microwave heating, whereas they are stables under conventional heating at the same temperatures. This significant inactivation due to exposure to microwaves was in agreement with conformational changes revealed by fluorescence emission spectra and far ultraviolet CD spectra [20, 21].

Studies resulting from the agroalimentary research

The first application of the microwave energy in biotechnology took place in the agro-alimentary sector. Indeed, in the Nineties, new sterilization processes using microwaves energy were developed. Reduction of process times, energy and water usage may be achieved with microwave heating mode. A higher end product quality can be reached because

Table I. Studies of the microwave/enzyme interaction in aqueous medium.

Enzyme	Frequency (GHz)	Power input (mW/g)	Temperature (°C)	Subsequent/Simultaneous radiation	Effect	Ref
G6P dehydrogenase	2.8	160–300	37–50	Subsequent	No effect	[12]
Trypsin	2.45	100–600	30–95	Subsequent	No effect	[13]
Lysozyme						
Horseradish peroxidase	2.45	62500–375000	25	Subsequent	Inactivation	[14]
Adenylate kinase	2.45	42	25–60	Simltaneous	No effect	[15]
G6P dehydrogenase						
Cytochrome c reductase						
Lactate dehydrogenase	3	30–1800	25–60	Simltaneous	No effect	[16]
Acetylcholinesterase	2.45	1–100	37	Simltaneous	No effect	[17]
Creatine phosphokinase						
Acetylcholinesterase	2.45	2,46–4,29	25	Simltaneous	No effect	[18]
Acid Phosphatase	9.375	160–480	60	Subsequent	Stabilization	[19]
Thermophilic enzymes	10.4	1500–3100	70–90	Subsequent	Inactivation	[20]
Thermophilic enzymes	10.4	800–1700	30–70	Subsequent	Inactivation	[21, 22]

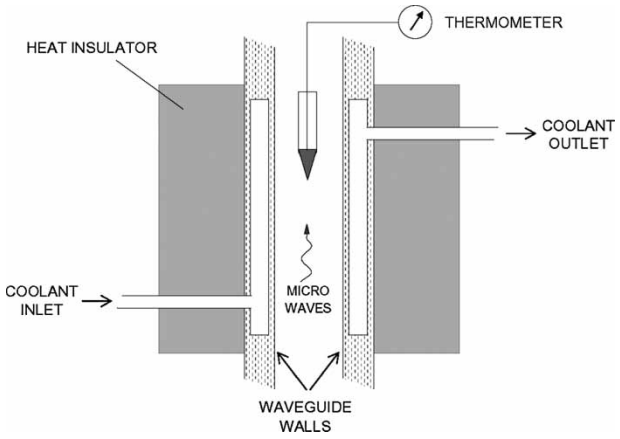


Figure 1. Schematic diagram of the waveguide exposure chamber.

undesirable side reactions and product losses may be minimized, which will result in different colour, flavour and aroma of foods [23].

The development of these processes of sterilization by microwave irradiation allowed the emergence of several studies concerning the microwaves influence on the enzymatic stability. In these studies, which have been conducted in aqueous medium with domestic microwave ovens (2.45 GHz), the enzymatic inactivation appears faster under microwave irradiation than under traditional heating. However, for the majority of this work, either the temperature was not controlled, or the temperature rise rate used was different according to the heating type. Nevertheless, a study carried out with the mushroom polyphenoloxidase (PPO) obtained significant results [24]. The enzyme was incubated at 80°C, either in a water bath or in a microwave oven (22.6 W/g). In both cases, the temperature of the enzymatic solution was continuously measured. PPO inactivation was complete after 20 seconds of microwave irradiation, whereas conventional methods needed more than 6 minutes for thermal inactivation to be complete. That can be explained by the faster heating in the case of microwave treatment. More surprising, residual enzymatic activity obtained according to the temperature reached during the incubation was dependent on the heating mode. Thus, a $T_{1/2}$ (representing the temperature at which residual activity is 50% of the initial activity) of 56.4°C was obtained for the PPO sample treated by microwave, while a $T_{1/2}$ of 63.5°C was calculated for the sample heated in a conventional hot-water bath. Kinetic tests were carried out with enzymatic fractions incubated under microwave irradiation or conventional heating, which have reached the same temperature. Identical limiting velocity V_{max} were obtained for both treatments while the Michaelis constant K_m, opposite to the enzymatic affinity, was higher for the irradiated enzymatic fraction. The authors concluded that conventional and microwave treatments produce different enzyme intermediates with different stability and kinetic properties.

In agro-alimentary sector, enzymes are used to transform the raw materials into food which is more easily assimilated by the organism. Some of these "pre-digestions" were carried out under microwave irradiation. Izquierdo et al [25] studied the enzymatic hydrolysis of bovine β-lactoglobulin at 40°C, in a monomode microwave applicator (Figure 2). The microwave treatment has accelerated the enzymatic reaction, but do not affect the hydrolysis selectivity, compared to the conventional heating treatment.

Roy and co-workers [26] were interested in the chitin enzymatic hydrolysis. The substrate chitin was beforehand incubated at 57°C, in a water bath or in a multi-mode microwave oven. Pre-treated chitin was then hydrolysed by chitinase and kinetical parameters of the enzymatic reaction were determined. Similar V_{max} were obtained with both chitin pre-treatments. On the other hand, the enzymatic affinity ($1/K_m$) of the enzyme for the chitin was 1,5 times higher when chitin was pre-treated with microwaves. Scanning electron micrographs and X-ray diffraction data indicated that this may be due to greater accessibility of the susceptible bonds in the microwave-irradiated chitin.

Studies of the microwave/enzyme interaction in nonaqueous medium

Since the last decade, researchers have attempted to use microwave to improve enzymatic reactions in nonaqueous medium. The first studies dealing with enzymatic synthesis under microwave irradiation appear in the middle of the Nineties and are still very few. Majority of those were carried out with lipases or glycosidases, which are the most frequently used enzymes in biocatalysis. Researchers studied the

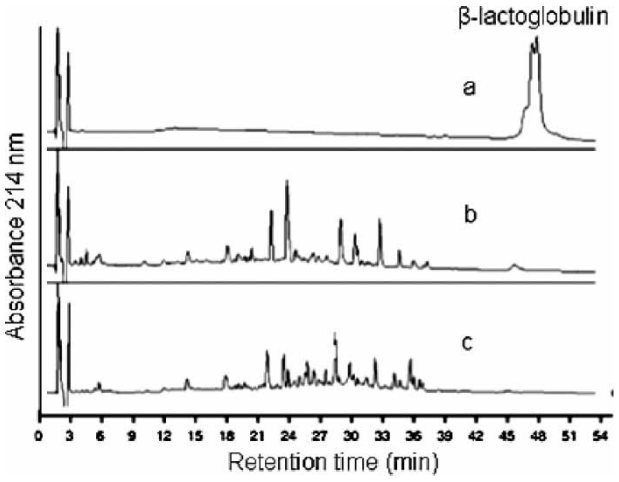


Figure 2. Reverse-phase-HPLC chromatograms from β-lactoglobulin digested by chymotrypsin for 20 minutes under several conditions. (a) undigested protein, (b) conventional heating, at 40°C and (c) microwave irradiation, 15W.

microwave influence on the enzymatic properties which are decisive for an enzymatic synthesis: the activity, the selectivity and the stability of the enzyme. Majority of the studies have been conducted with mono-mode applicators and the temperature of the irradiated samples was controlled.

Enzymatic activity under microwave irradiation

Two particular parameters seem to influence the enzymatic activity under microwave irradiation: the hydration state and the polarity of the reaction medium.

Influence of the hydration state of the reaction medium

Several studies were carried out under dry media conditions [27–30]. Enzymes were immobilized on solid supports which weakly absorb the microwaves. The substrates were then impregnated on the enzyme-loaded support. The enzymatic reactions were performed inside a mono-mode microwave reactor, using an open system, at temperatures close to 100°C. Results are then compared with those obtained under classical heating strictly under the same conditions. In this way, Carillo-Munoz et al [27] tested two lipases for various esterification reactions (Scheme 1). Yields obtained under microwave irradiation were from 2 to 9% higher than those measured under conventional heating.

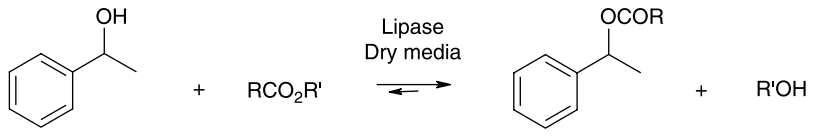
Authors suggested that microwave exposure induce equilibrium shifting by evaporation of light polar molecules (water for esterification reactions, ethanol for transesterification reactions carried out with ethyl esters) which are strongly interacting with the electromagnetic field. This improvement of the water evaporation under microwave heating using an open system was also described by Gelo-Pujic et al [29] which studied a reverse hydrolysis reaction catalysed by almond-β-glucosidase. At 80°C, a dramatic drop in the galactosidase activity was observed in open system under microwave irradiation, whereas intact activity was measured in closed system. The negative effect of microwave in open system could be a consequence of a too fast and irreversible loss of water: galactosidases are enzymes which require a minimum of water to function ideally.

In closed system, enzymatic properties changes under microwave irradiation, depending on the water content of the medium, were also shown. Parker et al [31] studied a transesterification reaction catalysed by a cutinase, in liquid medium, without solvent addition (Scheme 2). Thermodynamic water activities (a_w) of substrates and enzyme powder were equilibrated at a fixed value, before the reaction. For initial a_w of 0.58 and 0.69, microwave irradiation was found to increase the initial rate of the enzymatic reaction by 2–3 fold. On the other hand, when hydration was considerable (a_w of 0.97), significantly lower reaction rates, compared to those observed by classical heating, were obtained.

Huang et al [32] were interested in an esterification reaction (Scheme 3) catalyzed by an immobilized lipase, without solvent addition, for initial water content from 0.5 to 8% (v/v). Microwave heating allowed to improve the initial rate of the enzymatic reaction by 1.5 fold, for all hydration level tested. In organic solvent, similar result was obtained: the activity of two proteases was increased by 2.1–4.7 fold under microwave heating, for water content from 0.05 to 0.5% (v/v) [33].

Influence of the polarity of the reaction medium

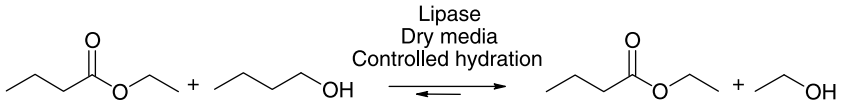
Like the hydration state, the polarity of the reaction medium could influence the enzymatic activity under microwave irradiation. Roy and Gupta [33] carried out subtilisin-catalyzed transesterification



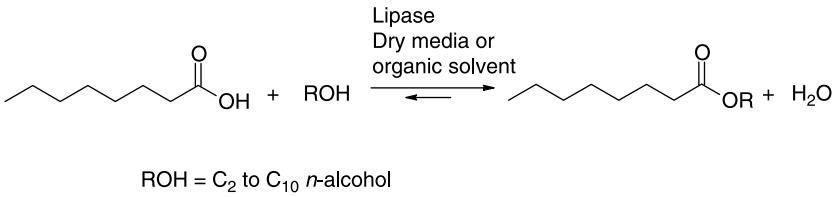
R = CH_3 , C_4H_9 , C_7H_{15}

R' = C_2H_5 , $H_2C{=}CCH_3$

Scheme 1. Microwave esterification of 1-phenylethanol by lipase in dry media conditions.



Scheme 2. Microwave-assisted transesterification reaction catalysed by a cutinase, in liquid medium.



Scheme 3. Microwave esterification of *n*-caprylic acid by an immobilized lipase, in non-aqueous medium.

and α-chymotrypsin-catalyzed esterification in six solvents of differing polarities and at three different temperatures. In all cases, microwave irradiation was found to increase the enzymatic activity. Furthermore, the acceleration of the enzymatic reaction increased with the hydrophobicity of the solvent. With a particular solvent, the increase in initial rates due to microwave irradiation was in a similar range at all the three tested temperatures. Yadav and Lahi [34] studied the transesterification activity of a supported lipase, at 50°C in toluene, and changed the length of the chain of the alcohol substrate. The enzymatic activity was higher under microwave heating in comparison with conventional heating, for all the alcohols. These microwave effect is related to the increase of chain lengths, and consequently, with an increase of the medium hydrophobicity (Scheme 4).

Enzymatic selectivity under microwave irradiation

Some works dealt with the microwaves influence on the enzymatic selectivity. Gelo-Pujic et al [30] carried out transglycosylation reactions catalysed by supported glycosidases with diols possessing a primary and a secondary alcohol function (Scheme 5). The selectivity for primary *versus* secondary function was found to be unchanged (4:1) according to the heating mode. Similarly, the substrate selectivity of various lipases towards triacylglycerides, fatty acids, methyl esters and alcohols, during synthetic and hydrolytic reactions, was identical under microwave irradiation and conventional heating [34, 35].

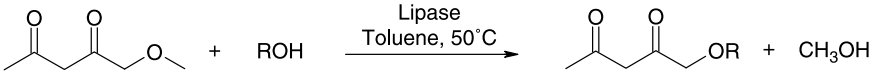
On the other hand, microwave heating seems to have an effect on the enzymatic stereoselectivity. Carillo-Munoz and et al [27] carried out the resolution of racemic 1-Phenylethanol via transesterification reactions catalysed by a supported lipase in dry media (Scheme 6).

The enantiomeric excess (*ee*) of the *S*-1-Phenylethanol was from 5 to 30% higher when microwave irradiation, rather than conventional heating, was used. Lin and Lin [36] obtained similar results with the lipase from porcine pancreas used in toluene.

Enzymatic stability under microwave irradiation

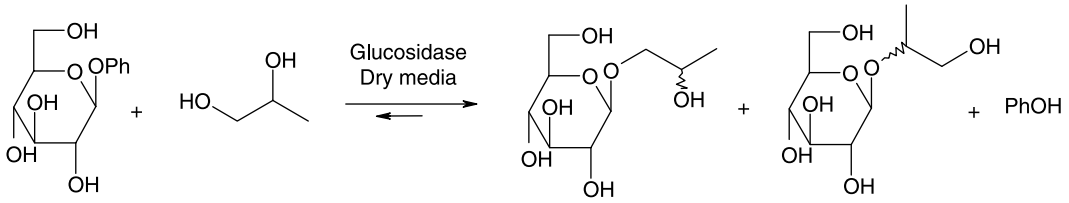
The enzymatic stability under microwave irradiation had been very poorly studied. Carillo-Munoz et al [27] placed supported lipases during 30 minutes under microwave irradiation at the temperature range of 70°–100°C. The enzymatic activity was subsequently tested in a transesterification reaction: the activity was found to be the same as that of freshly prepared, nonheated, supported enzyme. Unfortunately, control with conventional heating was not published. Réjasse et al [37] studied the stability of a supported lipase at 100°C. The microwave irradiation was carried out before or during the enzymatic reaction. In both case, while enzymatic activity was similar under microwave and conventional heating, enzyme stability was higher with the microwave heating. Moreover, this gain of stability appeared to be higher in more polar solvent (Scheme 7).

A further study [38] was made with the free form of the lipase. It showed that only the initial rate of the

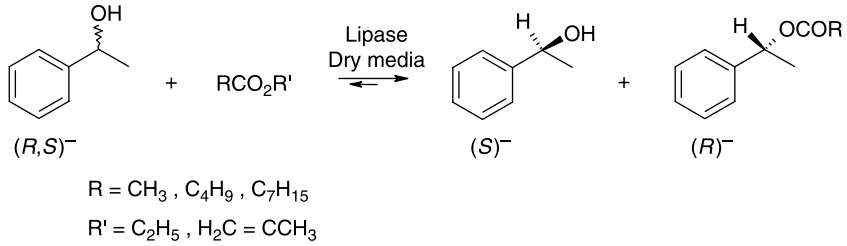


ROH	Conversion (%)	
	Microwave	Conventional
1-Propanol	74	57
1-Butanol	72	53
1-pentanol	66	42
1-Hexanol	61	34
1-Octanol	55	17
1-Decanol	33	9

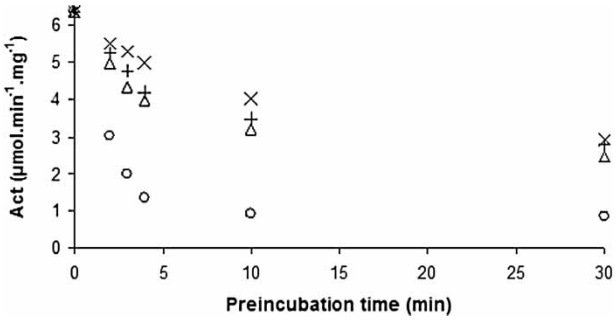
Scheme 4. Conversion rate of methyl acetoacetate transesterification by a supported lipase, at 50°C in toluene.



Scheme 5. Microwaves influence on transglycosylation reactions catalysed by supported glycosidases.



Scheme 6. Resolution of racemic 1-Phenylethanol via transesterification reactions catalysed by a supported lipase in dry media.



Scheme 7. Residual activity of supported *Candida antartica* lipase B after preincubation in organic substrates (○, preincubation in butanol, conventional heating; Δ, preincubation in ethyl butyrate, conventional heating; ×, preincubation in butanol, microwave heating; +, preincubation in ethylbutyrate, microwave heating).

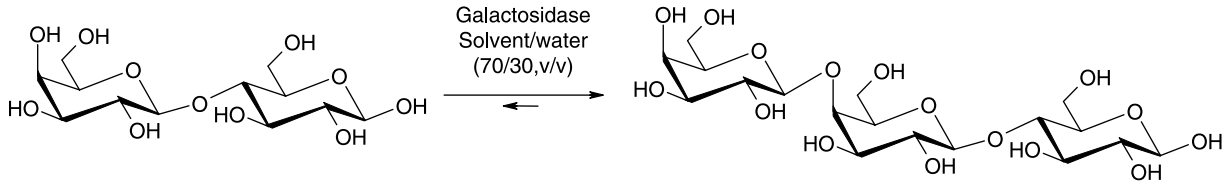
enzymatic deactivation process was affected by the microwave irradiation. Moreover, this microwave effect could be a positive or a negative effect, according to the temperature.

Secondary enzymatic reactions under microwave irradiation

During enzymatic transesterifications and transglycosylations, secondary reactions of enzymatic hydrolysis can limit the yield of synthesis. Some authors were interested in the influence of the microwave heating on these secondary reactions. With an open system, in dry media, although the initial rates of various transglycosylations were increased by the microwave irradiation, the secondary enzymatic hydrolysis of the products was similar under microwave and conventional heating [30]. Conversely, in a biphasic medium (solvent/water, 70/30, v/v) with a supported galactosidase (Scheme 8), the transglycosylation yield of lactose was identical under microwave irradiation, compared to conventional heating, in the first hours of reaction [39]. But when the lactose transformation was complete, galacto-oligosaccharides (GOS) synthesized were hydrolyzed by the galactosidase with conventional heating, while GOS yield remained stable with microwave heating.

Mazumder and co-workers [40] carried out the enzymatic reduction of organic azides. Corresponding amine's yield did not exceed 40% after 4 hours of conventional heating because of the simultaneous decomposition of products, while a reduction yield of 80% was reached in 5 min under microwave irradiation.

All the studies concerning the influence of the microwave irradiation on the enzymatic properties in nonaqueous medium are summarized in Table II.



Scheme 8. Microwave influence on the transglycosylation yield of lactose with a supported galactosidase.

Table II. Studies of the microwave/enzyme interaction in non-aqueous medium.

Enzyme	Reaction medium	Enzymatic reaction	Temperature range (°C)	Tested enzymatic parameter	Microwave effect	Ref
Supported lipases	Dry	(trans)Esterification	70–100	Activity	Higher	[27]
				Selectivity	Higher	
				Stability	No effect	
Supported lipase	Dry	Esterifications	95–110	Activity	Higher	[28]
Supported glucosidase	Dry	Glucosidation	80	Activity	Smaller	[29]
Supported glucosidase	Aqueous	Hydrolysis	60	Activity	Higher	[29]
Supported glucosidase	Dry	(trans)Glucosidations	80–110	Activity	Higher	[30]
				Selectivity	No effect	
Cutinase	Liquid substrates	Transesterification	50–70	Activity	Higher	[31]
Supported lipases	Organic solvent	Esterification	40–70	Activity	Higher	[32]
	Liquid substrates	Esterification	40–70	Activity	Higher	
Chymotrypsin, Subtilisin	Organic solvent	(trans)Esterification	25–60	Activity	Higher	[33]
Supported lipase	Organic solvents	Transesterification	50	Activity	Higher	[34]
				Selectivity	No effect	
Lipases	Micro-emulsion	Hydrolysis	80	Activity	Higher	[35]
				Selectivity	No effect	
Lipases	Organic solvent	Esterification	80	Activity	Higher	[35]
				Selectivity	No effect	
Porcine pancreas lipase	Organic solvent	Transesterification	35, reflux	Activity	Higher	[36]
				Selectivity	Higher	
Supported lipase	Liquid substrate	Transesterification	100	Activity	No effect	[37]
				Stability	Higher	
Lipase	Liquid substrate	Transesterification	40–110	Activity	No effect	[38]
				Stability	Modified	
Supported galactosidase	Solvent/water (70/30, v/v)	Transglycosylation	40	Activity	No effect	[39]
				2nd reactions	Smaller	
Porcine pancreas lipase	Organic solvent	Azide reduction	50–60°C	Activity	Higher	[40]
				2nd reactions	Smaller	
Glycosidases	Solvent/water (9/1 and 4/1, v/v)	(trans)Glycosylations	50–65	Activity	Higher	[41]
Supported lipases	Organic solvent	Esterification	30–70	Activity	Higher	[42]

Conclusion

Microwave technology is emerging as an alternative energy source to accomplish enzymatic reactions. In aqueous medium, the properties of the irradiated enzyme are identical to those obtained under traditional heating. In non aqueous medium, where only the polar species present absorb the microwave energy, the activity, the selectivity and the stability of the enzyme can be improved by microwave heating.

Some authors envisage the existence of a specific effect of the microwave irradiation on the structural and functional properties of enzymes. Concerning the enzyme, a direct energy transfer between the electromagnetic field and the polar protein's domains could induce modification of the flexibility of the enzyme, and consequently change the enzymatic properties [21, 23]. Moreover, a direct absorption of the microwave energy by the polar substrates of the enzyme could lead to a higher reactivity of the functional groups involved in the enzymatic reaction [40].

However, the specific effect of the microwaves could be of thermal origin. The temperature measured during the studies is a macroscopic average parameter of the system. All the enzymatic properties changes which have been observed under microwave irradiation, compared to conventional heating at a fixed macroscopic temperature, could be the consequence of different thermal profiles at the microscopic level.

Whatever the origin of the microwave effect, microwave heating appears to be a promising technology to improve the enzymatic catalysis sector.

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