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Improvements in the extraction of bioactive compounds by enzymes

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A B S T R A C T

Bioactives from plants are always in high demand in nutraceutical, pharmaceutical and functional food sectors due to their health benefits. This intensifies the need of extraction of bioactives by different methods that can improve the yield and purity of the compound. Enzyme helps in the release of bioactives from the plant material under optimized conditions so as to make the extraction process efficient. Though enzymatic extraction of bioactive has been used since long, it needs improvement to further enhance the yield, reduce the process time and to make the process cost competitive. The combination of enzymatic extraction with other green techniques such as ultrasound extraction, supercritical fluid extraction, three phase partitioning, ionic liquid extraction and microwave extraction can boost the advantages of enzymatic extraction. This review focuses on the improvement in the enzymatic extraction techniques for the bioactive in detail.

Keywords

Bioactives
Enzyme-assisted extraction (EAE)
Ultrasound assisted enzyme extraction (UAEE)
Enzyme-assisted supercritical extraction (EASCFE)
Ionic liquids enzyme assisted extraction (ILEAE)
Microwave assisted enzymatic extraction (MAEE)
Enzyme assisted three phase partition (EATPP)
High pressure assisted enzymatic extraction (HPAEE)

Introduction

Bioactive compounds are obtained from natural sources that can modulate metabolic processes leading to better health. The positive effects of bioactive compounds include antioxidant activity, inhibition and/or induction of enzymes, inhibition of receptor activities and inhibition and/or induction of gene expression [1]. These properties make them excellent choice of molecule to be exploited in the area of nutraceutical, pharmaceutical, functional foods and food additives. However they are present in small amount in nature and are mostly in conjugate form which requires laborious extraction and time consuming purification.

Table 1: Recent development in the use of commercial enzyme mixtures in EAE of bioactives

Source	Bioactive(s)	Commercial enzyme	Enzyme concentration	Yield	Reference
Dasylirion wheeleri (wild stool plant)	Fructans	Pectinex ultra SP-L	83.04 U/mL	39.08 g/100 g	[53]
Elettaria cardamomum maton. (Cardamon)	Volatile oil	Viscozyme L	1% (v/w)	7.83%	[18]
Polygonum cuspidatum	resveratrol	Pectinex ®	3950 polygalacturonase units/g	10.38 mg/g	[54]
Medicago sativa	Total phenolics	Kemzyme	2.9% (w/w)	(142.69 ± 5.11 µg/g)	[55]
Black tea leftover	Polyphenols	Kemzyme	2.9% (w/w)	29.15 %	[36]
Pomegranate peels	Total phenolics	Viscozyme L	1.32 ml/100 ml	19.77 mg GAE/g	[56]
Cedrela sinensis	Polysaccharide	Shearzyme plus	1% (v/w)	10.53%	[57]
Okara (soy pulp)	Protein concentrate	Viscozyme L	4.0% (w/w)	56%	[58*]
Cassia fistula pods	Total phenolics	Acid cellulose	3.40% (w/v)	165.63 mg GAE/g	[59]

On the other hand, varied structure, functionalities, site of action, specificity and effectiveness creates difficulties in extraction of bioactive compounds. Hence, there is always a need to find a suitable method to improve production, extraction and purification of these compounds [2*]. Conventional techniques used for the extraction of bioactives from various sources include maceration, soxhlet extraction, hydrodistillation, infusion, etc. These techniques depend on the type of solvent used along with physical operation of a technique. The major disadvantages of conventional extraction methods include, low extraction yield, higher operational time, and hazardous nature of solvents used.

Although conventional extraction techniques are still in operation, the ‘green’ extraction approach by using enzymes is highly preferred, especially because of its higher recovery, quick

operation with improved purity and ease of handling. Therefore, this review focuses on current developments in the enzymatic extraction techniques for bioactives.

Enzyme-assisted extraction of bioactives

Extraction of bioactive compounds is based on molecular diffusion generally using organic solvents which are undesirable especially in food industry because of their harmful effects on human health [3]. Hence, the use of aqueous based extraction systems is desirable. Nevertheless, the use of water as a solvent needs an assistance from various supplementary techniques to obtain high yield in short extraction time.

Therefore, enzyme-assisted extraction is considered to be supplementary and complimentary to aqueous based extraction system. Enzymes can be obtained from microorganisms, plants and mammalian cells that can be used in various industrial processes in free as well as in immobilized form. Some common enzymes used for the extraction of bioactives include cellulase, α-amylase, β-glucosidase, xylanase, β-glucanase, pectinase and other related. Besides, many industrial enzyme preparations such as Viscozyme L (cellulolytic enzyme complex) and Ultrazym (pectinolytic preparation), Pectinex, Lallzyme Beta etc. the recent applications (past two years) of which, are listed in table 1.

Mechanism of enzymatic extraction of bioactives

An effective enzymatic extraction is mainly dependent on the enzyme substrate interaction. Enzyme and substrate cohere to form an enzyme-substrate complex, and this subsequently binds the substrate substrate [4]. Addition of enzyme enhances rate of reaction, until the substrate concentration becomes limiting [4]. The enzyme-substrate interaction is affected by parameters such as size of plant material, concentration of enzyme, reaction time, temperature, pH, and solid to liquid ratio [5, 6] (Fig. 1.). In order for the enzyme to interact in order to facilitate maximum interaction of the enyme with the substrate, these parameters have to be set at their optima. While considering the optimum pH, the isoelectric point should be avoided as proteins are insoluble around this range and affects the extraction of the bioactives [2]. Similarly, while at suboptimal temperatures enzymes show less activity, higher temperatures lead to enzyme degradation, both reducing the efficacy in the extraction of bioactive [2]. Even though higher enzyme concentration would enhance substrate binding and it ultimates break down, the cost of the process must be appropriately considered.

Plant cell wall mainly constitutes of cellulose, hemiclellulose and pectin, which are the main barriers to the extraction of bioactives [5*]. Enzymes degrade these cell wall components and enable the release of compound of interest. The interaction between the enzyme and substrate decides the extent of hydrolysis of these barriers. This ultimately can be directly correlated with the amount of bioactives released. Hence an understanding of the basic structure of cell wall of the source material could assist in formulating optimum enzymatic cocktail [7].

Extraction of bioactives from plants generally entails an enzymatic cocktail. This is because the plant cell walls are comprised mainly of cellulose fibres along with hemicellulose [8] embedded in a matrix made up of pectic substances. For instance, several phenolic hydroxyl groups show hydrogen bonding with the polysaccharides complexes [9] and the use of the aforementioned enzymes could result in an effective release of the phenolics. In certain cases such as extraction of polyphenols from peels, the enzyme-substrate contact can be improved by pre-drying the material to improve the porosity. This leads to an improvement in the yield [2].

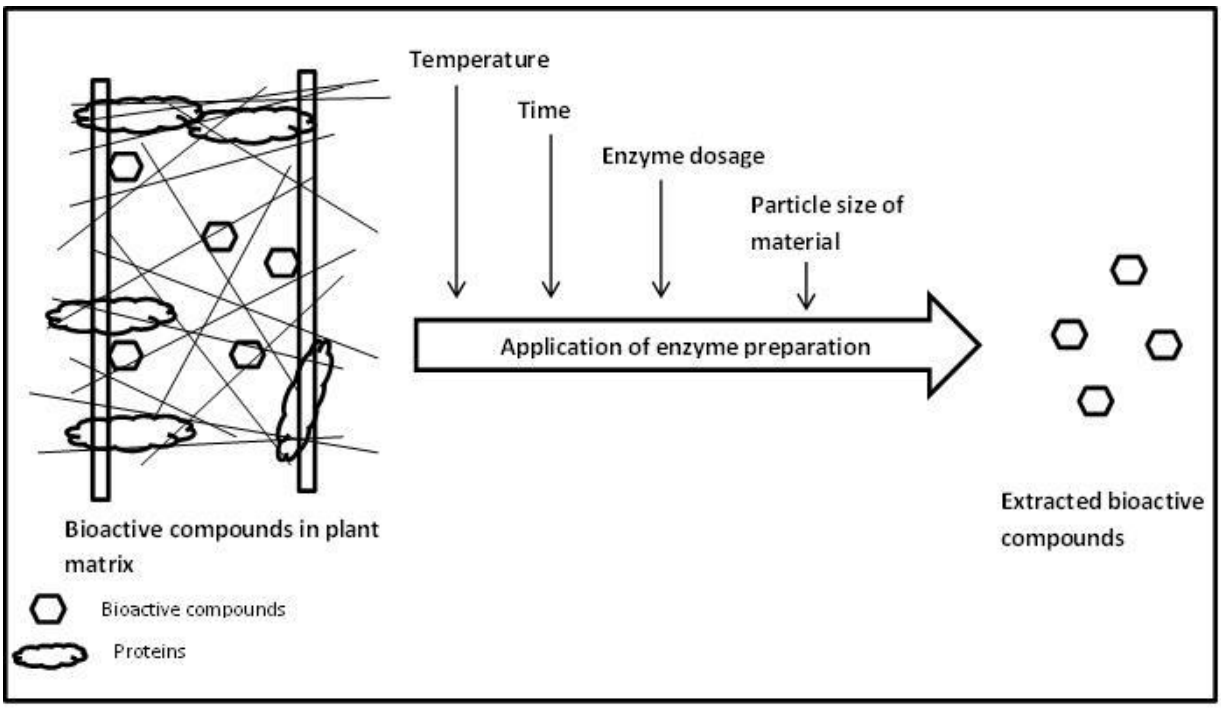

Fig. 1: The mechanism of enzymatic extraction of bioactive compounds from natural sources such as plant

Enzyme assisted extraction of proteins from the algae, *Chondrus crispus* has also been reported, where the algal cell wall was degraded using polysaccharidases like κ-carrageenase, β-agarase, xylanase and cellulase [10]. The authors also reported an increase in protein yield from the algae *Gracilaria verrucosa* on using cellulase and agarase together. This demonstrates the importance of specificity of enzymes towards their substrate in enzyme assisted extraction.

In case of extraction of oils from oilseeds, a similar enzymatic mixture would work in a different fashion. In this case, the carbohydrases would break the cell wall components, leading to the release of oil. At the same time, the oil droplets that are embedded in fibrous protein would be hydrolyzed by the proteases, thus improving the overall yield [11]. It should be noted that while the proteolytic enzymes could facilitate the release of oil, the concurrent breakdown of protein could also lead to an increase in its emulsifying capacity, which could lower the extraction of free oil. Thus, maintaining an optimum concentration of enzymes in the enzyme mixture is important.

Furthermore, the compound of interest may have interaction with other macromolecules that constitute the matrix of the source material. Breaking down these macromolecules by enzymes could further facilitate the release of bioactive compounds leading to higher yield as compared to non-enzymatic processes. Besides increasing the yield of bioactives, enzyme-assisted extraction also reduces the extraction time and quantity of the solvent required [4].

Applications and advantages of enzymatic extraction of bioactives

a) EAE reduces the extraction time

Pre-treating the plant material with enzymes improves the efficiency of extraction, which could be attributed to the reduced cell wall thickness. Based on enzyme assisted extraction (EAE),

hyphenated techniques such as ultrasound assisted enzyme extraction (UAEE), enzyme-assisted supercritical extraction (EASCFE), ionic liquids enzyme assisted extraction (ILEAE), high pressure assisted enzymatic extraction (HPAEE), etc. have been developed and have proved to reduce the extraction time [12*, 13] which could also allow the preservation of thermolabile compounds, thus making EAE more desirable.

b) EAE makes the extract innocuous and pharmacologically more active, thus finding an application in food and pharmaceutical industries

Babbar et al. [14] observed that in comparison to the use of acids and chelators, enzymatic extraction is rapid and the extracts so obtained are greener and safer, which consequently find uses in pharmaceutical industry. Upon enzymatic extraction, increase in various biological activities of bioactives has also been observed. In another study performed by Rostami and Gharibzahedi [15*] an increase in the antitumor, antioxidant and antimicrobial activities of the polysaccharide fractions extracted from *Malva sylvestris* was observed. de Camargo et al. [16] studied the extraction of insoluble-bound phenolics using EAE from winemaking by-products which showed an improvement in the antioxidant as well as alpha-glucosidase and lipase inhibitory activity. Hardouin et al. [17] used EAE for the extraction of bioactives from green seaweed *Ulva armoricana* and observed anti-viral activities against *herpes simplex virus* type-1.

Apart from its use in pharmaceutical applications, EAE also finds its way in food applications [18, 19]. Compounds like pigments and flavors are thermo-sensitive and therefore high-temperature extraction techniques are unfavourable, thus making EAE, a method of choice [20].

c) Improves the extraction yield

The major advantage of EAE is the improved yield of bioactives extracted from various sources. An increase in the yield of bioactives has been observed in various different studies [13, 15*, 21] along with recent findings reporting an increase in the activity of various bioactives [22, 23, 24, 25*].

Improvements in the enzymatic extraction of bioactives

Enzyme-based extractions are the subject of ongoing research and have the perspective to be commercially viable. Enzyme pre-treatment of raw material generally reduces the extraction time as well as volume of solvents, enhances yield, and improves the quality of product. Improvements in the methods of enzymatic extraction by combining other technologies and green procedures like ultrasound assisted enzyme extraction (UAEE), enzyme-assisted supercritical extraction (EASCFE), ionic liquids enzyme assisted extraction (ILEAE), microwave assisted enzymatic extraction (MAEE), enzyme assisted three phase partition (EATPP), high pressure assisted enzymatic extraction (HPAEE) can outplay the limits posed by traditional methods. Improvements in the enzymatic extraction and recent applications have been cited in table 2.

Table 2: Improvements in the enzymatic extraction for extraction of bioactives in recent times

S.N.	Bioactives/s	Source	Enzyme/s/ others	Brief summary	References
Ultra sound assisted enzyme extraction (UAEE)					
1	Lycopene	Tomato peels	Co-immobilized Pectinase and cellulase	Maximum lycopene release was obtained at co-immobilized enzyme conc., 3% (w/w); pH, 5.0; temperature, 50°C, 10 W ultrasound power, 10W; and incubation time, 20 min.	[12*]
2	Resveratrol glycosidases	*Polygonum cuspidatum*	(Pectinex® or Viscozyme®)	Better extraction efficacy was found in the Pectinex®-assisted extraction compared to Viscozyme®-assisted extraction.	[54]
3	Polysaccharide	*Corbicula fluminea*	Papain	Ultrasound power, 300 W; extraction temperature, 62 °C; ratio of water to raw material, 35 mL/g; and extraction time, 32min. yielded higher fractions of polysaacharides.	[60]
4	Phenolics, flavonoids, anthocyanins	Mulberry (*Morus nigra*)	Pectinex uf (puf)	Frequency, 82 kHz; enzyme concentration, 0.010% (v/w); and incubation time, 11.58 min. improved the quality of must and reduced the time during the maceration process of juice or wine.	[61]
5	Polysaccharides	Blackcurrant	Papain and Pectinase (2:1)	Enzyme concentration, 1.575%; pH, 5.3; and ultrasonic time, 25.6 min. resulted in 14.28 % yield of polysaccharide.	[62]
Enzyme assisted supercritical extraction (EASCFE)					
6	Polyphenols	*Medicago sativa*	Kemzyme	Temperature, 68°C; co-solvent (ethanol) 15.5%; at 205 bar gave maximum extraction yield of 142.69 µg/g.	[55]

7	Ployphenols	Black tea leftover	Kemzyme	Polyphenols (521 mg GAE/g of tea extract) were extracted at 55 °C and 300 bar pressure keeping the SC-CO_2 and ethanol flow rates at 2 and 0.2 g/min for 30–120 min.	[36]
8	Valuable components	Buckthorn pomace and seeds	Cellulolytic and xylanolytic enzyme (viscozyme, celustar xl)	Supercritical carbon dioxide, pressurized ethanol and enzyme-assisted extraction yielded 146 and 135 g/kg of lipophilic fraction from pomace and seeds, respectively.	[63*]
Ionic liquid based enzyme extraction (ILEAE)					
9	Genipin	*Eucommia ulmoides*	Cellulase Ionic liquid: 0.5mol/L [C6 mim]Cl	The optimum conditions for genipin extraction was found to be treatment time, 140min; liquid–solid ratio,19.81mL/g; enzyme concentration,.15mg/mL; and pH,5.0.	[64]
10	Alkaloid components camptothecin and 10-hydroxycamptothecin	Samara of *Camptotheca acuminate*	Hemicellulase Ionic liquid: 0.75M [C8MIM]Br	Temperature,40°C; incubation time,12h; pH, 4; liquid-solid ratio,30mL/g; [C8MIM]Br,0.75M; and hemicellulase concentration,0.441mg/mL yielded high amounts of alkaloids.	[65]
11	Chlorogenic acid (CGA)	*Eucommia ulmoides*	Cellulase Ionic liquid: 0.5 M [c6mim]Br	Cellulase in 0.5 M [C6mim]Br aqueous solution was found to provide better performance in extraction.	[39]
12	Curcumin	Turmeric	α-amylase and amyloglucosidase Ionic liquid: *N*,*N*-Dipropyl ammonium *N*′, *N*′-dipropylcarbamate	Ionic liquid yielded 3.58% yield of curcumin without enzymatic treatment which increased to 5.73% after enzymatic pretreatment.	[40]

Microwave assisted enzymatic extraction (MAEE)					
13	Polysaccharides	Fruit of *Schisandra chinensis baill*	Cellulase, papain and pectinase	Microwave irradiation time,10 min; pH, 4.21; temperature, 47.58°C; extraction time, 3h; and enzyme concentration of 1.5% yielded 7.38 % of polysaccharides .	[42]
14	Phlorotannins and antioxidant compound	Seaweed *ecklonia radiata*	Carbohydrases (viscozyme, celluclast, and ultraflo) and Proteases (alcalase, neutrase, and flavourzyme)	Microwave-assisted viscozyme extraction for 5 to 30 min was found to be most effective process with an extraction yield of 52 %.	[66]
15	Seed oil	Pumpkin	Cellulase, pectinase and proteinase	The highest oil recovery of 64.17% was achieved under optimal conditions of enzyme concentration, 1.4%, w/w); temperature, 44 °c; time, 66 min; and irradiation power, 419 W.	[67]
16	Corilagin and geraniin	*Geranium sibiricum li nne*	Cellulase	The extraction yields of corilagin and geraniin achieved were 6.79 and 19.82 mg/g, which increased by 64.01% and 72.95%, respectively, as compared with the control ones.	[68]
	Polysaccharides	*Lentinus edodes*	Papain, pectinase and cellulase	Maximum yiled of 9.83% was obtained at 48°C; pH 5; 440 W microwave power for 10 min.	[69*]
17	Oil	*Isatis indigotica*	Enzyme cocktail (cellulase/protei nase/pectinase)	Concentration, 1.82% (w/w); temperature, 43°C; time, 83 min; and irradiation power, 375 W gave the highest oil recovery of 59.27%.	[70]

Enzyme extraction assisted three phase partition (EATPP)					
18	Oleoresin	Ginger (*zingiber officinale*) rhizome powder	Accellerase	Increase in the yield of [6]-, [8]-, [10]-gingerols and [6]-shogaol by 64.10, 87.8, 62.78 and 32.0% within 4 h was obtained.	[25*]
19	Oleoresin	Turmeric	Enzyme preparation of α-amylase, glucoamylase	The extraction time was lowered as compared to conventional acetone extraction.	[48]
High pressure assisted enzymatic extraction (HPAEE)					
20	Lycopene	Tomato waste	Pectinase	HP extraction at 700 mpa/10 min enabled lower solvent volumes and higher yields.	[71]
21	Oil extraction	*Moringa oleifera* seed kernels		HP pre-treatment at 50 MPa and 60 °C for 35 min resulted in approximately 73% (w/w) free oil recovery.	[13]
22	Taxifolin, quercetin and isorhametin	*Cactus*	Rapidase-Viscozyme mixture	The crude polysaccharides isolated from the extract (51.2% at 1000 μg mL^{-1} for HP extraction at 300 MPa)	[72]

Ultrasound-assisted enzyme extraction (UAEE)

Extraction efficiency can be enhanced by using ultrasound assistance which is mainly attributed to the propagation of ultrasound pressure waves, and resulting cavitation phenomena. It also exerts a mechanical effect that allows greater penetration of solvent into the tissue to increase contact surface area between solid and liquid phase for enhanced diffusion to the solvent phase [26]. However, ultrasound generates extreme heat which may denature heat labile compounds. Therefore combining EAE with ultrasound assisted extraction (UAE) can be used to combat this disadvantage. Acoustic cavitations present hydrophobic surfaces within the extraction liquid, thereby increasing the net hydrophobic nature of extraction medium. Micro-jets impact the surface by pealing, erosion and particle breakdown resulting in the introduction of new surfaces further increasing mass transfer [27]. Thus it is possible to extract polar fractions into otherwise hydrophilic aqueous extraction media, plummeting the need for generally undesirable hydrophobic or strongly polar extraction solvents. Optimization of extraction parameters is crucial for improving the extraction yields.

Ultrasound treatment of waste carrot has yielded β-carotene to obtain a yield of 83.32% under optimized operating conditions (50 min. ultrasound irradiation, 50°C, 100 W, 60% duty cycle, and solid to solvent ratio of 0.3:20) [28]. Polysaccharides from *T. fructus* were obtained using UAE by optimizing several independent variables and their interactions via response surface methodology [29]. Researchers have extracted polyphenols, flavonoids, polysaccharides and anthocyanins from different crop varieties like mulberry, blackcurrant, peanut shells, etc. using the UAEE method (Table 2.). Five types of enzymes; Cellulase, Viscozyme L, Alcalase 2.4L, Protex 6L, and Protex 7L, were evaluated for their effectiveness in releasing oil from ultrasonic pretreated perilla seeds. The highest oil yield of 81.74% was observed in cellulase treated perilla seed samples [30]. UAEE treatment has been found to increase the antioxidant activity in the extract. Guava mash juice treatment by UAE and enzyme (cellulose) treatment was found to increase the antioxidant activity by 19.7% (2,2' -azinobis-3-ethylbenzothiazoline-6-sulfonic acid method) and 11.5% (ferric ion reducing antioxidant power method) compared with control [31].

EAE treatment for extraction of bioactive is usually associated with longer extraction time, which could increase the processing cost. Therefore, UAEE has proved its efficincy with rapid, simple, and inexpensive operation with enhanced yields. UAEE efficiency is influenced by many parameters such as type and concentrations of enzymes, solvent composition, solvent to solid ratio, temperature and reaction time [32]. Enzymes in free form or immobilized state can be used in UAEE. Ladole et al. [12*] reported the extraction of lycopene using UAEE technology from tomato peels using immobilized pectinase and cellulase. UAEE can be successfully employed for extraction of polysaccharides, phenols, glycosides, polyphenols, flavonoids etc.

3.2 Enzyme-assisted supercritical extraction (EASCFE)

Supercritical fluid-based extraction (SCFE) can extract the compounds which are prone to thermal deterioration and oxidation at higher temperature. Due to its mild operational temperature, SCFE prevents thermolabile compounds from deterioration. Enzymatic hydrolysis before supercritical carbon dioxide (SC-CO_2) extraction recompenses the operational cost as it can improve mass transfer, decrease the particle size, increase the contact area and improve solvent distribution [33]. Therefore, EASCFE is considered to be an efficient extraction technique.

The critical temperature and pressure for SC-CO_2 are 31.1°C and 73.8 bar above its critical pressure (P_c) and temperature (T_c). $SCCO_2$ enters into a supercritical state resulting in higher density, high solvation power as like liquid and low viscosity, high diffusivity and low surface tension as like gas [34]. These enhanced properties improve the mass transfer interactions between solute and supercritical fluid and give higher extraction efficiencies. The lower critical temperature enables SC-CO_2 to be used for extraction of heat labile compounds. Moreover, it is inert, non-toxic, non-flammable and readily available in high purity. The limitation of SCFE is the polarity of SC-CO_2, which can extract non-polar bioactives during the process. Therefore, SCFE techniques are considered expensive because of low extraction yield when applied to the extraction of polar components like polyphenols from complex biological matrix. However, this problem has been besieged by incorporating the co-solvents/modifiers/entrainers, such as methanol, ethanol, and water which increases the versatility of this technique to extract diversified phytochemicals.

EASCFE is an excellent alternative to ultrasound [35] extraction technique having wide variety of applications in the area of food technology, therapeutics, neutraceuticals, aroma and flavor industries. Mushtaq et al. [36*] obtained high yields of non-extractable polyphenols from black

tea leftover using Kemzyme and ethanol (entrainer) with SC-CO_2 and ethanol at flow rate of 2 and 0.2 g/min for 30-120 min, respectively.

Ionic liquids enzyme assisted extraction (ILEAE)

In current times, ionic liquids and enzymes have extensively been used in the separation and extraction processes of natural commodities. Use of ionic liquid in aqueous phase enhances the penetrability of solvent over extraction process. Ionic liquids (ILs) along with enzyme, enhances the penetrability of solvent in extraction process and utilizes the characteristics of enzymatic hydrolysis on cell wall to increase efficiency and extraction yield. ILs are composed of bulky organic cations and inorganic or organic anions in the form of are molten salts. Although, there are large numbers of pure ILs which rely on cation-anion combinations, just few of them span a wide range of physical and chemical properties as reactive solvents [37]. ILs can interact with both polar as well as nonpolar molecules and are useful when combined with an array of extraction techniques. The essential parameters gauged during extraction with ILs are chemical structure, concentration, moisture level, enzyme concentration, pH, extraction temperature, and enzyme to substrate ratio [2*]. ILs used in enzymatic extraction are more viscous and denser than organic solvents which in turn increase the stability of enzymes in a medium.

ILEAE is useful to extract various components like genipin, alkaloid components (camptothecin and 10-hydroxycamptothecin), chlorogenic acid, curcumin, forskolin etc (Table 2.). Pectinase in [C6mim] Br aqueous phase has been investigated as an extraction process of chlorogenic acid from *Flos Lonicera japonica* with enhanced yield [38]. Scanning electronic microscopy and circular dichroism spectroscopy of samples demonstrated that both pectinase and IL disposal enabled the extraction process by disintegrating cell wall structure. Liu et al. [39] investigated the solubility of chlorogenic acid and the activity of cellulase in eight 1-alkyl-3-methylimidazolium ionic liquids from *Eucommia ulmoides*. Curcumin extraction from turmeric using a developed carbamate ionic liquid [*N*, *N*-Dipropyl ammonium *N*′, *N*′-dipropylcarbamate (DPCARB) synthesized from dry ice and dipropylamine] was investigated by Sahne et al. [40]. DPCARB was employed for extraction of curcumin from turmeric which resulted in extraction yield of 3.58%, at room temperature (25^0C)within 2h. Enhanced yield of curcumin was obtained with enzymatic pre-treatment (α-amylase and amyloglucosidase) to destruct turmeric cell wall before the extraction process was found to remarkably improved extraction yield to 5.73%.

Microwave-assisted enzymatic extraction (MAEE)

Microwave-assisted enzymatic extraction (MAEE) combining microwave extraction (MAE) and EAE, has gained wide acceptance as a dominant tool for sample preparation of solid matrices. MAEE has been used by various researchers to extract polysaccharides, oil, phlorotannins, etc. from plant materials. Enzymes like cellulase, pectinase and proteinase, etc. individually or in combination (cocktail) can be used in MAEE to aid the extraction of bioactives. MAE utilises effects of microwaves to extract bioactives. Heating of solid materials culminates into the loss of water vapours rapidly thereby resulting in a tremendous increase in pressure. This causes enlargement and rupture of cells, enabling the release of intracellular components into solvent [41].

MAEE yield depends on several factors such as microwave power, time of explosion, sample size, nature of solvent and extraction temperature. The nature of solvent affects the extraction process by affecting solubility (of desired molecule) and its ability to absorb microwave energy [11*]. Higher absorption of microwave energy generates high heat, leading to enhanced extraction.

Various parameters which are known to affect the extraction are: microwave irradiation time, pH, temperature, extraction time and enzyme concentration.

Cheng et al. [42] optimized various parameters of MAEE by response surface methodology (RSM) and orthogonal test design, to improve the extraction of crude polysaccharides (CPS) from *Schisandra chinensis Baill* fruit. Three methods including heat-refluxing extraction (HRE), ultrasonic-assisted extraction (UAE) and enzyme-assisted extraction (EAE) for extracting CPS by RSM were further compared. Results indicated that MAEE method had highest extraction yields of CPS at lower temperature [43]. Polysaccharides from *Rosa roxburghii* were also extracted using MAEE. Specific conditions (microwave power, 575W; microwave time, 18min; liquid-to-material ratio, 13.5:1mL/g; and enzyme dose, 6.5g/mL) generated an experimental yield of 36.21 % [43]. MAEE is a cost-effective method possessing additional advantages, such as shorter operational time, less use of solvent, enhanced extraction yields without any decomposition [44]. Therefore, MAEE has now become one of the prevalent and cost-effective extraction techniques with several improvements in instrumentation and operation.

3.5 Enzyme-assisted three phase partition extraction (EATPP)

Enzyme-assisted three-phase partitioning (EATPP) is an extraction technique which is a combination of EAE and three phase partitioning (TPP). Tertiary butanol (*t*-butanol) is completely miscible with water, but on the addition of sufficient salt such as ammonium sulphate, the solution is separated into two phases as aqueous, and organic phase. Three phases are formed, and a precipitate of protein-rich phase separates top organic layer from bottom aqueous layer. Antichaotropic salt (ammonium sulphate) at high concentration instructs a high dielectric property to water owing to which the t-butanol layer is transformed to hydrophobic layer. This layer gets extracted from water aiding extraction of hydrophobic constituents while polar molecules get concentrated in the lower aqueous phase [45]. Upon mixing with different plant and animal cell sample forms, three layers are formed, separating the proteinaceous layer at the interphase of organic and aqueous layers [46]. Other solvents which can be used for three-phase partitioning (TPP) are *n*-butanol, *iso*-propanol, ethanol, etc. Extraction of bioactives is optimized concerning the concentration of ammonium sulphate loading and the ratio of *t*-butanol to slurry, temperature conditions and enzymatic optimization [47].

Pre-treatment of slurries with commercial enzyme preparation (α-amylase and/or glucoamylase) followed by three phase partitioning is important. Enzyme pretreatment before TPP has been reported to increase the yield of compound by breaking complex cellulosic structure [48]. EATPP is advantageous over conventional extraction methods as it involves mild operational conditions and intact structural stability. EATPP is an economical process which uses inexpensive chemicals such as *t*-butanol and ammonium sulfate, therefore it can be successfully employed in large scale operation [49].

EATPP can be employed for the extraction of oleoresins and oil from different plant resources [25*, 48]. Varakumar et al. [25*] reported an increase in the yield of gingerols and shoagaol by EATPP using acellarse enzyme from ginger (*Zingiber officinale*) rhizome powder. Kurmudle et al. [48] extracted turmeric oleoresin using enzymatic preparations of α-amylase and glucoamylase.

High- pressure assisted enzymatic extraction (HPAEE)

HPAEE is enzyme assisted extraction method leading to higher yields of biomolecules by implying ambient pressure condition. EAE methods are gaining more attention, because hydrolytic

enzymes break down the structural integrity of cell walls and rendering intracellular materials to be more exposed to extraction. Combinational use of EAE and high pressure processing (HPP) cause structural changes, such as cell deformation or cell membrane disintegration that increases the cell permeability as well as secondary metabolite diffusion and consequently mass transfer rates. High pressure treatment affects the stability of molecule which could upset its catalytic activity, substrate affinity, enzyme interactions with activators, inhibitors, cofactors etc. Correspondingly the stability of biomolecules such as enzyme substrates and their secondary products could also be affected under pressure, leading to changes in the susceptibility of substrates towards enzymatic reaction, ion changes, substrate degradation etc. [50]. Parameters for optimization in HPAEE are pressure range, time and enzyme selection, and enzyme activity.

HPAEE has been reported to improve the extraction yield, hydrolysis and functional properties of the desired molecule. The HPAEE treatment increased the yield of polysaccharides up to 8.55% from longan (*Dimocarpus longan Lour.*) using cellulase with high pressure conditions of 407 Mpa for 6 min. [51]. The extraction yield of peanut protein isolate was 39.86% at 80 Mpa. Reasearchers have also investigated HPAEE to extract tricin from rice hull [52]. Enzymatic hydrolysis performed with Celluclast (0.5%, w/w) before HPP (500 MPa) treatment yielded maximum tricin content (32.9 mg/kg rice hull). The efficacy of rice hull extract obtained by HPAEE was significantly higher than that of the extract prepared by traditional solvent extraction [52].

Conclusion and future perspective

Application of enzymes for bioactive extraction is gaining much attention and thus requires intense research inputs to establish its commercial operation. EAE along with some modifications has been practiced by researchers in recent years. Moreover, the positive improvements in this technique mitigate several challenges posed by conventional extraction processes. Interestingly, use of enzymes reduces extraction time and gives better product yield together with minimal solvents usage. However, foremost limitation of this method is cost of enzymes and could be overcome by tailor-made enzyme preparations.

Determination of enzyme interactions and stability during processing is a significant area of research. Additionally, in-depth understanding about polysaccharide structure of plant substrate would be beneficial to reach the active site quicker. More efforts in the field of biodiversity and protein engineering are needed to improve available extraction techniques. Besides, new enzymes with added functionalities can be designed through modern biotechnology and process engineering approaches. There is a wide scope for further research in the line of customized enzymes for extraction of value added products which is not exploited so far. Furthermore, enzyme driven flavour and colour extraction appear to have a high potential in near future.

Enzyme immobilization is another active area of research which could help in the context of repeated use of enzymes. State-of-the-art strategies are needed to design immobilization supports so as to make overall process economically competitive. Future investigations are needed to enlarge the currently available enzymatic processes, specifically to enhance the yields of bioactive compounds. In case of heat sensitive bioactives, enzymatic extraction is a good choice and hence advances in enzyme designing are vital and must be explored. On the other hand, expressing enzymes in plants would also be a suitable way out to minimize extensive processing. Indeed, enzymes could be produced on-site at bio-refineries so as to eliminate surplus charges.

More importantly, enzymes do not kill pathogens; instead they simply remove the protecting shelter of bacteria. Therefore, harmonious combinations of several bactericides with enzymes are now becoming a method of interest and thorough understanding is needed in near future. In addition, knowledge of bioinformatics, proteomics, and gene shuffling could be helpful in developing EAE as future technology. Usually, conventional extraction processes can be performed in batch as well as continuous mode. Continuous extraction is most suitable from industrial point of view. Hence extensive research efforts are required to make EAE systems to be used at commercial scale.

Conflict of interest

None declared

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as: * of special interest

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